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Homeostasis: Humidity and water relations in honeybee colonies *(Apis mellifera)*

by

Michael B. Ellis

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DECLARATION

I declare that the thesis entitled **Homeostasis: Humidity and water relations in honeybee colonies *(Apis mellifera),*** which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work (unless otherwise indicated) and has not previously been submitted by me for a degree at another institution.

Michael Ellis November 2008

ABSTRACT

One of the benefits of colonial living in insect societies is the ability to build a nest which enables the maintenance of a homeostatic microenvironment. The detrimental and uncertain effects of fluctuating ambient conditions are thus avoided. An extensive amount of work has documented the regulation of respiratory gases and temperature by honeybee *(Apis mellifera)* colonies but relatively little is known of their water relations. Nest humidity influences the fitness of the honeybee colony by affecting adult and brood mortality, microbial and parasitic growth, nectar concentration and thermoregulation. This study aims at determining whether honeybee colonies are able to actively regulate humidity within their nest or whether humidity is stabilised merely as consequence of other socially regulated parameters. As a first step in understanding water relations in a hive, the daily, seasonal and two-dimensional humidity patterns are described in diverse contexts: various subspecies, nest architectures, ambient climates and colony conditions. The humidity in the brood nest of a healthy honeybee colony does not show a daily pattern: mean hourly RH remains between 50 and 60 % and high vapour pressure deficit results in a large evaporative capacity. Two-dimensional humidity patterns show that a vapour pressure gradient exists from the central brood area to the periphery of a hive. This finding suggests possible active regulation by workers and to test this idea we determined the behavioural response of a group of workers to a humidity gradient. Young honeybee workers in the absence of brood exhibit a weak hygropreference for approximately 75% RH. When brood is present the expression of this preference is further weakened, suggesting that workers tend to the brood by distributing evenly in the gradient. In addition, fanning behaviour is shown to be triggered by increasing humidity adding to our understanding of this behaviour. Although these results suggest that humidity in honeybee colonies is actively controlled by workers, passive mechanisms are also involved in the observed patterns. Cocoons that are spun by the larvae accumulate in cells and these hygroscopic cocoons contribute to passive stabilisation of humidity. Old comb containing cocoons absorb 11 % of its own mass in water when placed in high humidity and this water can readily evaporate into the atmosphere when humidity decreases. This buffering effect may increase brood survivorship by maintaining a high and stable humidity in the brood cells. This study contributes to our understanding of the complex mechanisms that govern microclimatic regulation in social insect nests and specifically the active and passive mechanisms that ensure homeostasis of honeybee nest humidity.

Photographs: V Dietemann & MB Ellis

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PUBLICATIONS AND CONFERENCE PROCEEDINGS

A list of publications and conference proceedings arising for this research:

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Ellis MB (2007) Hive humidity & its regulation by honeybees. BeeCon

CHAPTER ONE

General Introduction

"Water ...is the one substance from which the earth can conceal nothing. It sucks out its innermost secrets and brings them to our very lips."

- Jean Giraudoux, 1943

Water is essential for the sustainability of life and one can even state that water preceded life on earth. Water relations of living organisms are interesting in that there is a need to maintain a homeostatic balance of their internal fluids in a varying external environment. The smaller the organism, the more susceptible it is to environmental fluctuations, which makes the water relations of arthropods particularly fascinating. The surface area of an organism shows a proportional decrease relative to an increase in that organism's volume. An arthropod's small body size makes it susceptible to evaporation of water via the cuticle and this is a major avenue of water loss (Edney, 1977; Hadley 1994). Although the cuticle can be divided into multiple layers, the epicultricle, by virtue of its position and chemical composition, provides the greatest contribution to integumental waterproofing (Hadley 1994). The degree of waterproofing differs between species and it also comes at a cost; respiratory gaseous exchange via the cuticle is inhibited. In order to deal with this problem, arthropods have evolved a series of internal air-filled tubes that open to the outside through spiracular pores in the cuticle, which thus constitute another avenue of water loss. Respiratory water loss differs significantly between species, but is generally less, unusually much less, than 20 % of total water loss (Chown, 2002).

Although the small body size of arthropods makes them prone to desiccation, it also enables them to locate favourable microclimates. Many arthropods do this in order to evade harsh ambient conditions. The microclimate created by some leaves has a much higher humidity near the transpiring surface and particularly the underside of the leaf; a fact that has clear implications for the arthropod occupants. The burrow of a sand-dwelling wasp provides a very constant hydrothermal environment just 10 cm below the soil surface (Willmer, 1982) and

some flowers such as the sacred lotus *(Nelumbo nucifera)* actively create suitable microclimates and can maintain temperatures between 16 and 20 °C above ambient through thermogenesis (Seymour and Schultze-Motel, 1996; Lamprecht et al., 2002). These flowers act as a thermal refuge for various arthropods; a mutually beneficial relationship in which pollination of the plant is improved and the behaviour of the arthropod is profoundly influenced.

Apart from being able to access suitable microclimates, some arthropods occur in conspecific aggregations and can themselves create and maintain suitable local environments (Chown and Nicolson, 2004). First instar cockroach nymphs *(Blattella germanica)* do not aggregate when placed in high humidity, but at extremely low humidity (<2% RH) they exhibit a nearest neighbour distance of less than their anntenal length (Dambach and Goehlen, 1999). Each individual is surrounded by a water vapour envelope caused by respiratory and cuticular transpiration and when aggregation occurs these envelopes overlap, thus reducing water loss. Klok and Chown (1999) showed similar physiological benefits for aggregations of the caterpillars of the emperor moth, *Imbrasia belina*, which they compared to solitary caterpillars with significantly lower body temperature and higher rates of water loss. Aggregations of arthropods certainly affect microclimate and this is taken to another level when arthropods evolve into social colonies which are able to maintain a homeostatic nest environment.

1.1 Homeostasis of a superorganism

A superorganism by definition consists of numerous individual organisms which can be differentiated into sterile and reproductive non-uniform individuals that serve distinctly different functions (Moritz and Southwick, 1992). These authors assign various traits to a superorganism: they are usually sessile, are either well armed or highly cryptic, have a large number of colony members that function as a cooperative unit and are able to maintain intraorganismic homeostasis.

The word homeostasis was coined by Walter Cannon (1926) whose work grew from that of Claude Bernard who first recognised the importance of maintaining stability in the *milieu intérieur*, or internal environment. Cannon expounded on this concept in his book, "The Wisdom of the Body" (Cannon, 1932), in which he stated the following:

> *"The constant conditions which are maintained in the body might be termed equilibria. That word, however, has come to have fairly exact meaning as applied to relatively simple physico-chemical states, in closed systems, where known forces are balanced. The coordinated physiological processes which maintain most of the steady states in the organism are so complex and so peculiar to living beings - involving, as they may, the brain and nerves, the heart, lungs, kidneys and spleen, all working cooperatively - that I have suggested a special designation for these states, homeostasis. The word does not imply something set and immobile, a stagnation. It means a condition - a condition which may vary, but which is relatively constant."*

In the same manner in which Cannon described the homeostasis of a body, this concept can be applied to various states within a superorganism. Just as the amount of carbon dioxide is regulated in the blood of a mammal, the concentration of carbon dioxide in the internal environment of a social insect nest is maintained at homeostasis. An example of such a homeostatic balance is that of respiratory gases in many termite nests. The architecture of these nests enables the survival of large colonies by ensuring the homeostasis of respiratory gases; the existence of these large colonies would otherwise be impossible in this subterranean habitat. The levels of the respiratory gases in these nests are indeed, as Cannon suggested, not a stagnation but they do fluctuate both diurnally and seasonally (Lüscher, 1961; Korb and Linsenmair, 2000). A superorganism can benefit in various ways from nest homeostasis (Schmickl and Crailsheim, 2004). Certainly the most obvious is that by maintaining a constant internal environment, the colony is able to avoid the detrimental and uncertain effects of a fluctuating ambient environment.

It is not merely the "steadiness" of the internal nest environment that implies homeostasis. "Steadiness" is the *outcome;* however the *process* on which this "steadiness" is based, is equally important in defining homeostasis (Turner, 2000). Due to its thermal inertia, a rock lying in the sun has a much steadier temperature than the surrounding ambient air and could thus be defined as homeostatic if this definition was based on an *outcome*. Homeostasis in the honeybee nest is both based on a *process* and an *outcome:* it is not merely the cumulative effect of the aggregation of thousands of individual honeybees within the nest. The process that determines homeostasis in the nest is dependent on the partitioning of specific tasks to different individuals. The division of labour within the colony is based on the age and past

experience of each worker and on the demography and current demand within the colony (Ribbands, 1953; Lindauer, 1952; Johnson, 2008).

An example of such a homeostatic *process* in honeybee colonies is the regulation of water collection that is carried out by a specialised group of workers with overrepresentation from some patrilines (Robinson et al., 1984; Kryger et al., 2000). Water, unlike nectar, is not readily stored in the nest although there are reports of such storage in hot, dry climates (Park, 1923). Water collection is determined by a variable hive demand for water whereas nectar collection is determined by a variable field supply of nectar (Seeley, 1995). When the colony's need for water is high, water collectors are rapidly unloaded by water receivers and the number of failed unloading attempts is reduced (Lindauer, 1954; Kühnholz & Seeley, 1997). Water collection is indirectly affected by the concentration of nectar being transported to the nest and the ambient temperature, which affects the amount of water being used for evaporative cooling in the nest. If water collection is interrupted by bad weather the workers will exit the hive *en masse* at the first available opportunity in order to return the amount of water in the colony to a homeostatic state (Lindauer, 1954).

Homeostatic mechanisms may consist of numerous feed-back and feed-forward loops (Emerson, 1954). A process that determines one aspect of nest homeostasis can be affected by another homeostatic process. A nest parameter may, therefore, be regulated at suboptimal levels due to the regulation of another nest parameter: a situation which is termed a trade-off (Kleineidam and Roces, 2000). *Macrotermes bellicosus* colonies adjust their nest architecture in different, albeit adjacent, habitats; in cooler gallery forests their mounds are dome-shaped and have thick walls and in open savanna habitats the mounds are taller and cathedral shaped with thin walls. A trade-off is evident due to the necessity of insulating the nest in the cooler environment causing a decrease in the exchange of respiratory gases, thus elevating the CO2 concentration in the nest (Korb and Linsenmair, 1999). Interrelationships of nest parameters have also been shown in wood ant *(Formica polyctena)* nests: higher moisture content increases the nest temperature due to higher microbial heat production, which in turn elevates nest CO_2 concentrations (Frouz, 2000).

In order for a superorganism to actively maintain nest homeostasis it is essential for the individual workers to detect fluctuations in the relevant nest parameters. Workers are able to detect temperature fluctuations due to the excitation of thermoreceptors on their antennae

(Lacher, 1964). This information is then used to maintain the brood in a honeybee nest between 33 – 36 °C (Hess, 1926; Lindauer, 1954; Kronenberg and Heller, 1982). These temperatures ensure normal development and deviations can cause malformations and mortality of brood (Himmer, 1927; Weiss, 1962), as well as altered adult behavioural performance (Tautz et al., 2003). At low temperatures workers produce heat by shivering the flight muscles in their thorax without moving their wings; these muscles efficiently convert chemical energy to mechanical power, and due to biochemical inefficiencies heat is produced (Southwick and Heldmaier, 1987; Heinrich, 1993). Heat producing workers either sit on the surface of the capped brood (Bujok et al., 2002) or in empty brood cells where their T_{th} can reach 40.6 °C (Kleinhenz et al., 2003). At high nest temperatures workers reallocate themselves from other labour in order to cool the nest (Johnson, 2002). Cooling can be due to workers spreading droplets of water onto the surface of the comb or droplet extruding behaviour (otherwise known as tongue lashing), during which water is regurgitated and forms a droplet on the proboscis (Lindauer, 1954). Workers also respond to high nest temperatures by fanning; a behaviour that improves ventilation and exchanges the nest air with ambient air (Hazelhoff, 1954).

1.2 Sensing water vapour

Honeybees can sense fluctuations in the amount of water vapour in the air due to the electrochemical phasic-tonic excitation of the moist, dry and thermo receptor cells within the coelocapitular sensilla (Fig. 1.1) situated on eight of the ten antennal annuli (Yokohari et al., 1982; Yokohari, 1983). In fact the first records of an impulse from a hygroreceptor were made on the antennae of a honeybee by Lacher (1964). These were the impulses from a moist receptor and it was Waldow (1970) who made the first recoding from a dry receptor on the antennae of *Locusta migratoria.*

The mechanism by which insect hygrosensory cells detect humidity is unclear, although, they are thought to function similarly to mechnoreceptors in spite of being morphologically distinct. Yokohari (1978) showed that the antennal hygroreceptors of *Periplaneta* will increase their firing rate when antagonistically stimulated by movement of an electrode. This idea is supported by the discovery that the antennal neurons responsible for hygrosensation in *Drosophila* (i.e. those that contain transient receptor potential channels encoded by the *nanchung* and *water witch* loci) send their axonal processes into the mechanosensory region of the brain (Lui et al., 2007).

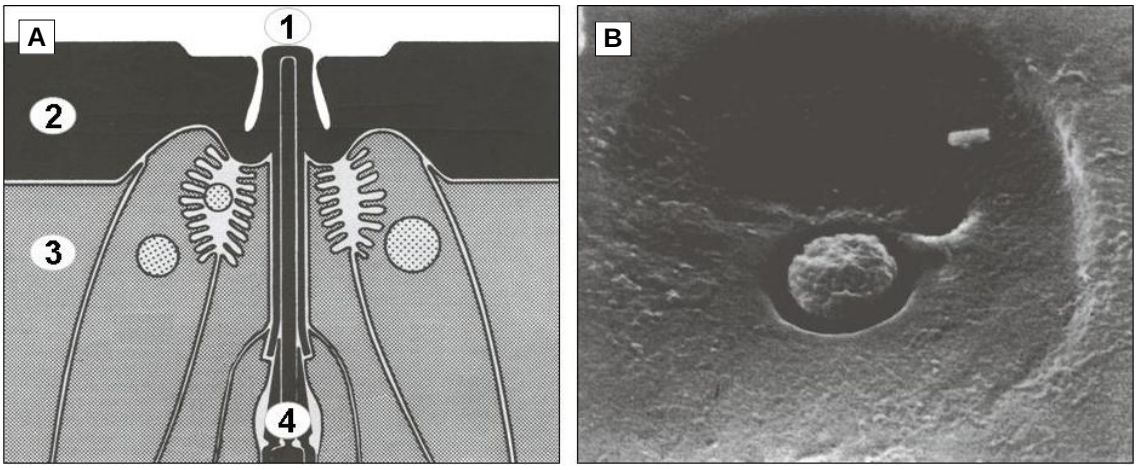

Fig. 1.1 A) Schematic drawing of the coelocapitular sensillium showing *1* cuticular apparatus, *2* cuticular wall, *3* epidermal cell, *4* sensory cilium **B)** scanning electron micrograph of the external structure of a honeybee coelocapitular sensillium (x 12000), showing a circular shallow depression and mushroom-shaped protrusion (Yokohari, 1983; Reprinted with permission from F. Yokohari)

Humidity induced mechanical stimulation of the hygroreceptors is possibly due to the hygroscopic swelling and shrinking of the cuticlar wall (Altner and Loftus, 1985). In male *Anopheles* mosquitoes, the rigidity of the antennal hairs is altered by changing the hydration state of the cuticular annulus at their base (Nijhout and Sheffield, 1979). In a similar way, volumetric changes in the coelocapitular sensilla of the honeybee antennae could cause mechanical deformation of the dendritic membranes and thus affect their polarisation (Altner et al., 1981). There are also other models which explain how hygrorecptors may be stimulated and they emphasise the varied ideas that exist. One idea is that they rely on a temperature differential between a moist and a dry surface on the receptor and thus function much like a psychrometer (Tichy and Loftus, 1996). In the wandering spider, *Cupiennius salei*, it is more probable that the concentration of electrolytes in a thin layer of lymph surrounding the dentrites is responsible for humidity detection (Ehn and Tichy, 1994).

The detection of humidity by the hygroreceptive coeloconic sensilla of *Periplaneta* is not determined by absolute but rather relative humidity; these sensillae have a similar morphology to those of the honeybee (Yokohari and Tateda, 1976). Altering the stimulus flux (i.e. velocity of air stream), which determines the number of water molecules per unit time which contact these sensillae does not change the response of the moist or dry receptors. The functioning of hygroreceptors is therefore very different from olfactory reception. The response curve to humidity is more closely related to relative humidity (RH) or vapour pressure deficit (VPD) than absolute humidity. This curve is affected by the temperature of the organism irrespective

of RH or VPD but does not appear to be affected by the temperature of the air. Tichy (2003) showed that the response curve is not only dependent on the instantaneous humidity but also on the rate of change of humidity. In this study the response curve of *Periplaneta* was altered by rates of change as low as 1% RH per second.

1.3 Humidity in the honeybee nest

Despite the research conducted on honeybee hygroreceptors, relatively little is known of how the information gained from these receptors is utilised within the social context of the colony. Humidity based decision-making has been shown in numerous ant species, such as *Atta sexdens* (Roces and Kleineidam, 2000) which relocate their fungal gardens to relative humidities above 90%. Similar choice tests have been carried out on four species of fire ants (Potts *et al.,* 1984), the meat ant *Iridomyrmex* sp. and the Argentine ant, *Linepithema humile* (Walters & Mackay, 2003) and thus provide evidence that some social insects do exhibit a colonial response to nest humidity. Lindauer (1954) tested whether honeybees alter their droplet extruding behaviour based on humidity but found no response, although this experiment was carried out with small groups of bees in an artificial environment.

Nest humidity is a parameter that influences the fitness of the honeybee colony for numerous reasons. Doull (1976) showed that the survival of honeybee eggs is dependent on RH with no eggs hatching below S0% RH. Humidity affects adult survival (Woodrow, 1935) and also influences microbial activity within the hive (Wohlgemuth, 1957; Büdel, 1948). The percentage of brood mummification caused by chalkbrood *(Ascosphaera apis)* was shown to increase by 8 % when RH was increased from 68 % to 87% (Flores *et al.,* 1996; Liang et al., 2000). The parasitic mite *Varroa jacobsoni,* which reproduces in the brood cells, shows lower reproductive success at higher humidity (Kraus and Velthuis, 1997). Humidity is an important factor for nectar concentrating and thermoregulation because the efficiency of these behaviours depends on the evaporation rate (Reinhardt, 1939; Ribbands, 1953). For instance, in order to down-regulate nest temperature workers utilise the droplet extruding behaviour to form a water droplet between their mouth parts thus increasing the surface for evaporation (Lindauer, 1954; Lensky, 1964). Evaporative cooling is, however, impossible if the air is saturated.

Honeybee nest humidity has been studied by various authors but these studies are far fewer

than the number conducted on nest temperature (Büdel, 1948; Oertel, 1949; Wohlgemuth, 1957; Kiechle, 1961; Human et al., 2006). The reason for this has been the technical difficulties associated with recording humidity. Johansson and Johansson (1979) stated that the "recent availability of electronic sensors should fill this information gap in the near future." It was a further twenty seven years before the first study using electronic sensors recorded humidity in honeybee nests (Human et al., 2006).

Humidity in the honeybee nest exists within a certain range but fluctuates temporally. Büdel (1948) recorded a mean relative humidity of 40 % in the brood nest where it was infrequently found to be above 50 % or below 30 %. In this study, Büdel stated as the first rule of nest humidity that vapour pressure of the air is equal in any part of the hive at a given time but that it varies through time. Wolgemuth's (1957) records agreed with the equality of humidity between the brood nest and the rest of the hive but he noted a large vapour pressure gradient between the hive and ambient air. In contrast to the earlier studies Human et al. (2006), using smaller and more effective recording devices, showed that vapour pressure does in fact vary between the brood and nectar stores and suggested an optimum relative humidity in the brood nest of 40 % RH.

Honeybee nest humidity is determined by the various avenues of uptake and loss of water from the nest cavity (Fig. 1.2). The cavity which has a volume of approximately 40 litres, is lined with propolis that is impermeable to water (Seeley and Morse, 1976; Schneider and Blyther, 1988). Any water leaving or entering the nest must do so either via the air in the entrance channel or be transported directly by the bees. Both the brood and adults in the nest produce metabolic water, some of which they lose via cuticular and respiratory evaporation (Louw and Hadley, 1985) and via excretion, which takes place during cleansing flights (Marshall, 1986; Woyke et al., 2004).

Foragers collect both pure water and water-rich nectar, which is then returned to the nest. Nectar is sometimes concentrated by foragers during transport (Nicolson and Human, 2008), however, it is usually concentrated within the nest. Foragers are unloaded by house-bees via trophallaxis and the nectar is then concentrated by droplet extruding behaviour and stored in a cell (Park, 1925). Droplet extrusion usually takes place in an uncrowned nest region, away from the brood (personal observation; Park, 1925; Ribbands, 1953). Nectar stores can serve as a source or sink for water, depending on the sugar concentration (Nicolson, 2009). Pure water

brought to the hive by foragers is either spread into the hexagonal depressions between the capped brood cells, placed as a hanging droplet onto the upper surface of a cell, especially those containing eggs or larvae, or used during droplet extruding behaviour (Lindauer, 1954). The use of pure water in the nest has only been directly attributed to thermoregulation.

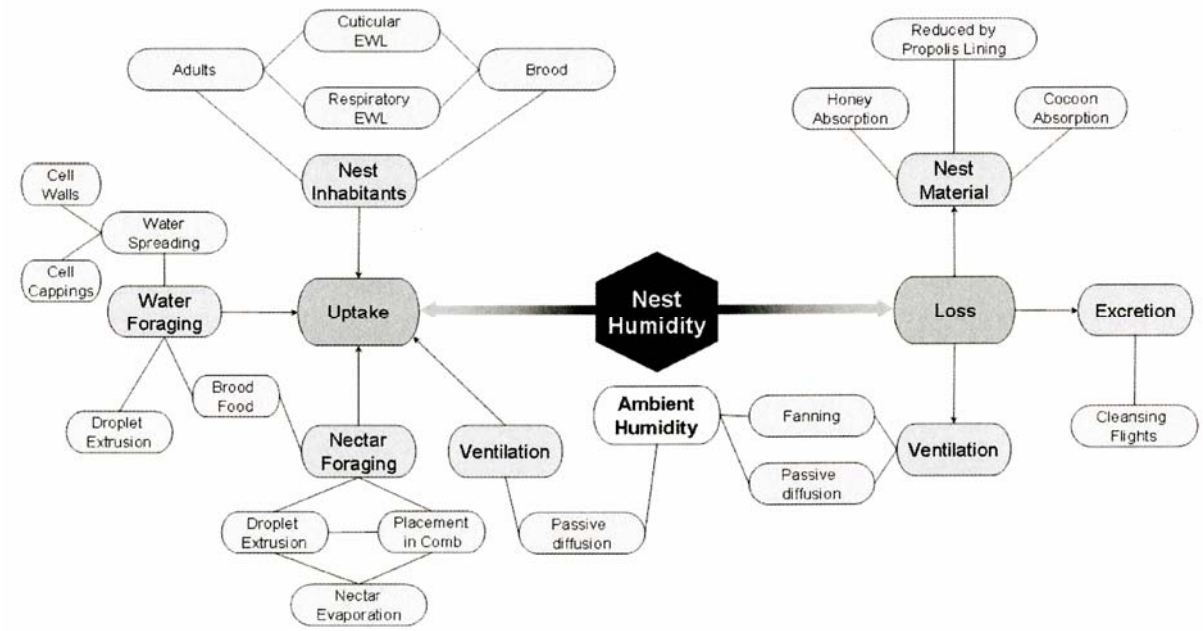

Fig. 1.2 Nest humidity and colony water relations depend on various avenues of water uptake and loss, an overview of which is provided in this diagram.

Workers actively influence humidity through fanning behaviour which ventilates the nest. Ventilation takes place in a cyclic breathing manner consisting of inspiration and expiration phases which remove excess carbon dioxide, heat and water vapour (Reinhart, 1939; Hazelhoff, 1954; Seeley, 1974; Southwick and Moritz, 1987). Similar cycles have been found in the colonies of two species of stingless bees, *Trigona denoiti* and *Trigona gribodoi* (Mortiz and Crewe, 1988). The expiration phase is depended on the fanning workers orientating themselves with their heads toward the nest thereby forcing air out of the nest cavity (Hazelhoff, 1954). Inspiration is due to the passive movement of air back into the nest, however, as Turner (2000) suggests, this exchange cycle may be different in natural nests where there is more than one entrance. Although numerous authors have suggested that fanning behaviour is an active regulatory response to nest humidity, there is no experimental evidence to support this (Reinhart, 1939; Ribbands, 1953; Hazelhoff, 1954).

1.4 Humidity calculations

There are numerous ways to describe the amount of moisture in the air and each of these needs to be interpreted in a specific way. In the study of water balance of arthropods several misconceptions have arisen from the misinterpretation of these concepts (Edney, 1982).

Vapour pressure (P_w)

This is the partial pressure of water vapour present in the air mass which is often given in millibars. Dalton's law of partial pressures states that the total air pressure (P_{tot}) is the sum of all the partial pressures of its components and water vapour pressure (P_w) is one of these partial pressures:

$$\mathbf{P_{tot} = P_w + P_{nitrogen} + P_{oxygen} + P_{other}}$$

Absolute humidity (AH)

Instead of presenting the actual amount of water vapour in the air as a pressure, it can also be expressed as a mass of water per volume of air, which is termed absolute humidity (AH) and can be given as g/m^3. It is defined by the following equation:

$$\mathbf{AH = m_v / V}$$

where m_v is the mass of water vapour and V is the volume of air.

Saturation vapour pressure (E_s)

Saturation vapour pressure is a function of temperature and indicates the total amount of water vapour that can be held in the air at a certain temperature. It can be defined as the vapour pressure at which two phases of water are at equilibrium in a body of air at a certain temperature.

All calculations of saturation vapour pressure in this study are derived from the Magnus Tetens formula as expressed by Murray (1967):

$$\mathbf{E_S = 6.1078*10\text{^}(u*T/(T+v))}$$

where E_s is saturation vapour pressure (mb), T is temperature (°C), u = 7.5 and v = 237.3.

The Tetens equation is widely used both for its simplicity and because it is accurate over a temperature range of -10 to 40 °C. If more accuracy is required at lower temperatures then equations such as the Goff-Gratch formulation can be used (Goff and Gratch, 1946). Such equations are typically used for calculation of humidity in the upper troposphere. Computational speed is important when analysing large data sets. Instead of using the exponential Tetens formulation for calculation of E_s the polynomial formulation by Lowe (1976) can be used to increase computation speed.

Relative humidity (RH)

Relative humidity (RH) is the amount of water in the air relative to the maximum amount of water that can be held in the air at a certain temperature. It is described by the following equation:

$$\mathbf{RH = P_w / E_s *100}$$

where P_w is the vapour pressure and E_s is the saturation vapour pressure.

Vapour pressure deficit (VPD)

This is the difference between the saturation vapour pressure (E_s) and vapour pressure (P_w). It indicates the deficit of water that can still evaporate into a body of air and is sometimes termed saturation deficit (SD). It was first used in insect physiology by Bacot and Martin (1924) and can be useful in such studies because it combines the effects of temperature and humidity (Edney, 1982). For example, in the carpenter bee, *Xylocopa capitata*, VPD is highly correlated to evaporative water loss (Nicolson and Louw, 1982). VPD can be given in millibars and is defined by the following equation:

$$\mathbf{VPD = E_s - P_w}$$

where E_s is the saturation vapour pressure and P_w is the vapour pressure .

Evaporation Rate

Relative humidity gives very little, if any, indication of evaporation rate and vapour pressure deficit is a better predictor (Anderson, 1936). It must be noted that vapour pressure deficit is not the only influencing factor of evaporation and variables such as wind velocity also play a role. The effect of wind can be substantial and increasing the air velocity from 0 to 3 m/s can cause a 3-fold increase in evaporation rate from an atmometer (Kucera, 1954). Evaporation

rate is also dependent on the surface area exposed for evaporation: a large water droplet will certainly have a higher evaporation rate than a smaller one. Evaporation from a social insect nest is therefore affected by the ventilation of the nest and the surface area available for evaporation within the nest.

1.5 Scope of this study

This study aims at determining whether honeybee *(Apis mellifera)* colonies are able to actively regulate the level of humidity within their nest or whether humidity is stabilised merely as consequence of other socially regulated parameters. Chapter two documents the spatial and temporal fluctuations of temperature and humidity in honeybee nests and provides descriptions of humidity in both artificial and natural nests and in different subspecies of honeybees. It also describes the daily, seasonal and two-dimensional patterns of humidity in the nest, as well as in an absconding colony and a winter cluster. Humidity in a winter cluster has previously only been dealt with theoretically (Omholt, 1987). Chapter three investigates the active behavioural response of workers to a humidity gradient, in order to determine their hygropreference and whether humidity acts as a behavioural impetus. Chapter four investigates the passive stabilisation of humidity by documenting the hygroscopic effect of the cocoons which accumulate in cells with successive generations of workers.

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CHAPTER TWO

Patterns of humidity in honeybee (*Apis mellifera*) nests

Abstract

One of the benefits of colonial living in insect societies is the ability to build a nest which maintains a homeostatic microenvironment. Although the regulation of the nest microclimate can be due to the passive effect of the nest architecture, active regulation by workers can also be substantial. An extensive amount of work has been conducted on the regulation of respiratory gases and temperature in honeybee nests but relatively little is know of humidity regulation. In order to better understand the regulation of humidity in honeybee nests this study aims at describing seasonal and daily humidity patterns, comparing nests in diverse contexts (i.e. different nests sites, subspecies, climates & seasons) and determining the association of nest humidity with ambient climate and colony condition. The humidity (mean hourly) in the brood nest of a healthy honeybee colony is relatively constant throughout the day: RH remains between 50 and 60 % and vapour pressure deficit (V PD) is high resulting in a large evaporative capacity. Although, desiccation sensitive larvae and eggs are located in the brood nest, the high VPD will prevent moulding of the comb and higher humidity is probably maintained within the brood cells. VPD in the nectar store is more variable and lower than in the brood nest but higher than ambient VPD throughout the day. It is thus more energy efficient for workers to evaporate nectar in the nest rather than in the ambient air. Two dimensional humidity patterns show that a vapour pressure gradient exists from the central brood area to the periphery of a hive; a fact that is at varience with Büdel's (1948) idea of constant vapour pressure throughout the nest. Brood nest microclimate of a healthy colony is not highly correlated to ambient temperature, humidity, wind, rain or solar radiation but this is not the scenario in a weak colony. The amount of uncapped brood in the nest is correlated with nest humidity, highlighting the importance of humidity patterns in the honeybee colony.

Keywords: humidity, vapour pressure deficit, homeostasis, honeybee, *Apis mellifera*

1. Introduction

Colonial living in insects is a strategy that maximises the organism's fitness while minimising energetic costs. Many social insects build nests and these range from the small simple nests of orchid bees (Apidae: Euglossini) to the large complex nests built by termites such as *Macrotermes* (Augusto and Garofalo, 2004; Lüscher, 1961). All nests provide some benefit to their occupants, whether it is merely a protective function or an intricately regulated microcli mate.

Microclimatic parameters can be regulated passively by the nest architecture but they are not independent of each other. The mounds built by *Macrotermes* depend on environmental conditions: a thin-walled cathedral shape is built in the warmer savannas and thick-walled dome shape in the cooler gallery forests (Lüscher, 1956; Korb and Lisenmair, 1999). The nests that are built in cooler environments have better insulating properties, however, this causes a trade-off with the exchange of respiratory gases and leads to elevated CO_2 levels in the nest. It is thought that the ventilation in *Macrotermes* mounds is driven mainly by external factors such as wind and solar energy (Korb and Linsenmair, 2000), and not by the internal thermosiphon mechanism as proposed by Lüscher (1961). Although the ventilation system of these termite nests is efficient, it does not negatively affect nest humidity (Korb and Linsenmair, 1998). The air is maintained near saturation despite the evaporation rate being as high as 8.S litres/day in some *Macrotermes subhalinus* nests (Weir, 1973). The saturated air ensures the survival of the desiccation sensitive brood and workers.

Although the passive effects of nest architecture, orientation and site selection contribute to homeostatic nest environments, active regulation by workers can be substantial (Jones and Oldroyd, 2007). Colonial thermoregulation is a well studied aspect of active microclimatic regulation (e.g. Kronenberg and Heller, 1982; Worswick, 1987). In honeybee colonies a small discrepancy in brood rearing temperature (i.e. 34-36°C) can lead to developmental abnormalities (Himmer, 1927; Weiss, 1962) and can even affect adult behaviour later in life (Tautz et al., 2003). In order to maintain the nest temperature above ambient the workers will actively raise their body temperature through shivering thermogenesis (Harrison, 1987; Stabentheiner et al., 2003). These bees then either position themselves in empty comb cells (Kleinhenz et al., 2003) or press their thorax onto the brood comb surface (Bujok et al., 2002).

During heat stress workers reallocate themselves from other labour within the nest in order to cool the nest by droplet extruding and fanning (Johnson, 2002). The thermoregulatory ability of a colony is depended on the degree of genetic diversity (Jones et al., 2004) and polyandry ensures numerous patrilines exist in the colony. Each patriline having a different response threshold and thus, thermoregulation occurs in a series of graded responses to temperature fluctuations.

Some social insects are known to actively regulate the humidity of the air in their nests. The leaf cutting ant *Acromymex ambiguus* will plug tunnels that carry dry air into the nest; they locate these tunnels by determining the direction of airflow (Bollazzi and Roces, 2007). Workers of *Atta sexdens* relocate their fungal gardens to areas in the nest with the highest humidity (Roces and Kleineidam, 2000) and dispose of waste in drier nest regions which in turn arrests fungal growth (Ribeiro and Navas, 2006). Nest humidity in the leaf-cutting ant *Atta vollenweideri* is never below 90% RH and even high ventilation rates during summer do not compromise this (Kleineidam and Roces, 2000). High humidity in these nests is important to maintain growth of the symbiotic fungus on which the brood feed. It is also interesting to note that the larger mature nests were found to have more variable humidity, due to greater volumes of dry air flowing through the tunnels.

Humidity is an important microclimatic variable for honeybees (Woodrow, 1935; Reinhardt, 1939; Doull, 1976; Flores et al, 1996; Bruce et al, 1997; Kraus and Velthuis, 1997; Liang et al, 2000). For instance, if isolated at high vapour pressure deficit, a honeybee worker can lose 19 $mg.g^{-1}$ body mass of water per hour though evaporation while only producing 2 $mg.g^{-1}$ metabolically (Louw and Hadley, 198S). Evaporative heat loss is minimised in the homeostatic microclimate of a honeybee nest (Simpson, 1961; Chown and Nicolson, 2004); however, studies on humidity patterns and water economy in honeybee colonies have been limited. Büdel (1948) could not detect any difference in water vapour pressure within the different regions of the nest and stated that by subsampling at any position in the hive one could derive relative humidity from the temperature at any other position. He did, however, state that there was probably a steep water vapour gradient in a winter cluster of honeybees. In a classic study, Oertel (1949) needed to remove five frames from a hive in order to replace them with a thermohygrograph. Measurements were then made in the brood nest and honey store for six months and although these provided an indication of the fluctuations of nest humidity they lacked precision. Human et al. (2006) studied hive humidity using smaller and

more accurate recording devices and could detect differences in vapour pressure in different nest regions. They present evidence that workers influence humidity in the hive but do so at suboptimal levels: 40 % RH in the brood nest. There is still a need to adequately describe patterns of nest humidity on a seasonal timescale with a short sampling interval.

The aim of this study is to describe the seasonal fluctuations of humidity occurring within honeybee nests, to determine whether a daily pattern of humidity exists and if this differs between different nest regions. I also compare humidity regimes in different subspecies of *Apis mellifera* and between hives and natural honeybee nests. I determine the association between nest microclimate, ambient climatic conditions and colony dynamics (e.g. brood rearing). By describing the pattern of nest humidity and comparing this to ambient conditions we determine whether humidity is actively regulated or whether regulation is a passive consequence of the homeostasis of other nest microclimatic parameters.

2. Methods

2.1 Daily nest climatic patterns

In order to determine whether there is a daily nest humidity pattern, three honeybee *(Apis mellifera scutellata)* colonies (ExpH1,2&3) were selected from the University of Pretoria apiary (25°45'11"S, 28°15'29"E) and positioned with four meters between each of the Langstroth hives. Each colony was housed in a brood box containing nine frames, of which at least four contained brood, and one shallow super containing ten frames of drawn comb partially filled (50-70%) with capped honey. The hives were positioned with all their entrances facing north (down-slope) and were situated in a partially shaded, semi-urban apiary. Each hive was placed on a 20 cm high metal stand with greased legs to prevent ant raids.

The three hives were set-up on 5 August 2006 and temperature and humidity were recorded in the hives from 1 September 2006 to 31 August 2007. A Hygrochron iButton data logger (DS 1923, Dallas Semiconductor, USA) was embedded in the centre of the 5th frame in each of the three brood boxes and it recorded the temperature and humidity between frames 5 and 6 (Fig. 2.1a), an area where brood is usually present. A HOBO H8 data logger (Onset Computer Corporation, Pocasset, MA, USA) was imbedded in the centre of the super; it recorded the microclimatic conditions in the nectar store of each hive (Fig. 2.1b). The iButton and HOBO loggers were covered with metallic mesh to prevent the workers from damaging the sensors with propolis. The loggers were set to record at an interval of 12 min and the data was manually downloaded every 15 days. All data from 12:00 on the day prior to downloading up until 19:00 on the subsequent day was excluded from analysis due to the disturbance caused by opening the hives. The data set was divided into four seasons for analysis: spring (Sept / Oct / Nov 2006), summer (Dec 2006 / Jan / Feb 2007), autumn (Mar / Apr / May 2007) and winter (Jun / Jul / Aug 2007). Data from the three hives were aligned to ensure that comparisons between hives had an accuracy of ±6 min. The daily pattern of temperature, RH and vapour pressure deficit (VPD) was determined for each hive separately by calculating the mean ± SD for each hour of the day; this was done for each season.

2.2 Abnormal nest microclimate: absconding and winter clustering

Two specific characteristics of a colony's life history, absconding and winter clustering, were identified and studied in detail. One of the three *A. m. scutellata* colonies in the University of Pretoria apiary was observed to be present on 15 May 2007 and had absconded by 5 June 2007; data analysis revealed that this event had taken place on 1 June 2007. Winter cluster formation was evident from observations in all three of the colonies but the cluster of one colony (ExpH2) was offset in such a way that the iButton became located relatively further from the cluster centre on each consecutive day. Winter clustering in this colony took place over approximately two and a half weeks, and microclimatic variables were recorded during this period. Swarming was also evident and took place at least twice in each colony; it was identified by observation of queen cells and a reduction in the amount of uncapped brood. There was no clearly observable pattern in nest microclimate preceding or subsequent to swarming, however future analysis may reveal changes at a finer scale.

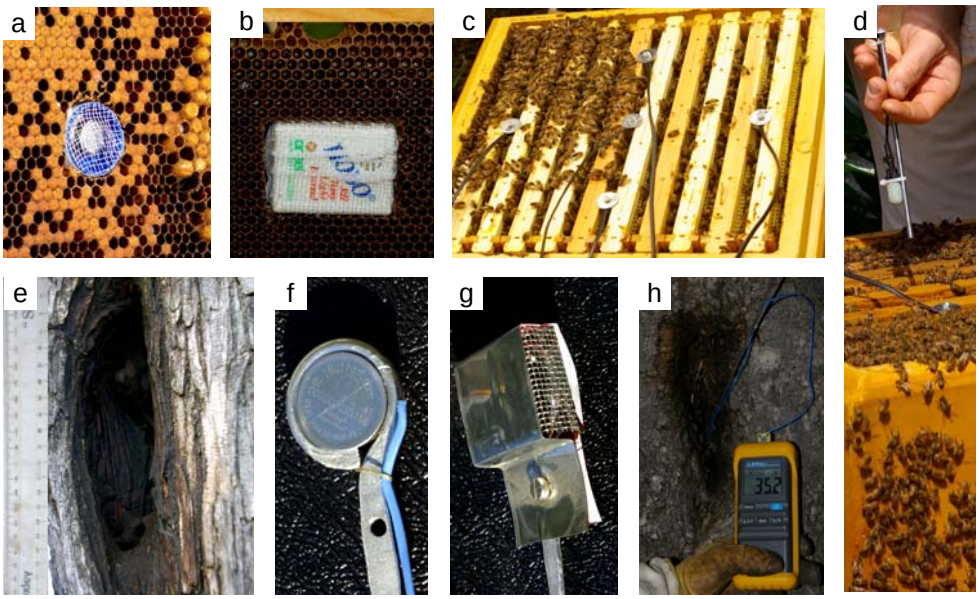

Fig. 2.1 Photographs showing: a) an embedded Hygrochron iButton and an embedded b) HOBO in brood and nectar comb respectively, c) the position of five Sensirion RH sensors athwart the length and breadth of an *Apis mellifera* (buckfast) colony (FlaH1), d) a mounted Sensirion RH sensor in its protective filter cap which is about to be inserted into a hive, e) a natural nest entrance (CYB34H) in Kruger National Park, f) a mounted iButton with attached theromocouple wire, g) protective casing for insertion in natural nest and h) thermally locating the brood rearing portion of a natural nest.

2.3 Correlation between nest and ambient climate

A weather station was established within 6 m radius of the hives. Wind velocity and direction were recorded with a wind monitor (05103, Young, Michigan, USA, ±3° & 0.3mls) that was orientated with a magnetic compass (0° = north). Rainfall was measured with a tipping bucket (TR-525i, Texas instruments, Dallas, 0.2mm) and solar radiation with a pyranometer (Li-200, Li-cor, USA). A CR10 data logger (Campbell Scientific, Logan, Utah) was programmed to record all the parameters at an interval of 12 min. Ambient temperature and humidity were measured with a Hygrochron iButton which was mounted in a solar shield. All data was manually downloaded every 15 days. All ambient data were aligned with the nest data from the three colonies to ensure an accuracy of ±6 min was maintained.

Data for wind direction were converted from a 360° scale to 180° by transforming all values greater than 180° and less than or equal to 360° to the appropriate values between 0 and 180°. This process regarded the direction of wind moving perpendicular to the hive entrance (i.e. east or west) as irrelevant. A value of 0° therefore indicates that air is blowing toward the hive entrance and 180° indicates movement away from the entrance.

All climatic parameters were found to be non-normally distributed and therefore nonparametric statistics were used for analysis. Spearman rank order correlations were used to determine the relationship between a colony's microclimate and ambient climatic parameters, without making any assumptions of the variables' frequency distribution. Each hive was assessed separately and analysis was grouped according to season.

2.4 Correlation between nest microclimate and colony condition

Observations were made of each frame in the brood box of the three colonies every 15 days at the time of data downloading. Frames were removed individually and the percentage of the comb area utilised for brood rearing and pollen and nectar storage was recorded for each side of the frame. Categories of comb utilisation were defined as: capped brood, uncapped brood, pollen, capped honey and uncapped nectar.

To determine the accuracy of the above mentioned observations, 10 frames were selected at random and assessed by the observer. Photographs of these frames were then analysed using

Adobe Photoshop to determine the total number of pixels per frame and the number of pixels for each comb utilisation category. The observational error which is the differences between observed comb utilisation (% of comb area) and calculated comb utilisation (% of comb area) was 5.1% and this was considered to be within an acceptable range.

Spearman rank order correlations were used to determine the association between each of the observed comb utilisation categories and the nest microclimate. Data from the three colonies were pooled for analysis as the differences between colonies were not significant (Kruskall-Wallis ANOVA: $H<3.8$, $n=3$, NS).

2.5 Two dimensional hive humidity patterns

Distinct humidity patterns may occur in different nest regions and this was tested by recording nest humidity in two dimensions (in one colony in South Africa and one in Denmark). Thirteen capacitance-type RH sensors (SHT75, Sensirion, Zürich, Switzerland, ±1.8% RH) with a range of 0-100 % RH were placed in a single Langstroth hive containing 10 deep frames. The *A. m. scutellata* colony (BomaH4) contained a marked laying queen and the total comb area consisted of 17 % brood (3 frames), 3 % pollen, 58 % capped honey and 8 % uncapped nectar. The RH sensors were placed in filter caps to prevent them from being damaged by bees, dust or other contaminants and were then mounted on a steel rod (length: 15 cm) to standardise the depth to which they were inserted between the frames in the hive (Fig. 2.1d). The sensors were positioned in an equidistant cross athwart the length (i.e. between the two centre frames) and breadth (i.e. between each consecutive frame) of the hive. An evaluation kit EK-H3 (V2.3, Sensirion, Zürich, Switzerland) was used to record temperature and humidity from the sensors every 15 s. Recording took place at the isolated boma (i.e. livestock enclosure) from 19 to 22 June 2007 on University of Pretoria experimental farm during a relatively cold and dry South African winter (ambient temperature: 12.1 ± 12.9 °C, ambient RH: 36.8 ± 4.9 %, recording interval: 15 s).

In order to compare climatic variations within honeybee nests under different climatic conditions, the experiment was repeated in Denmark at the Flakkebjerg Research Centre of the University of Aarhus during a humid summer (ambient temperature: 16.3 3.2 °C, ambient RH: 81.9 ± 12.5 %, recording interval: 1 h) from the 14 to 17 July 2007. Five RH sensors were placed in an equidistant cross athwart the length and breadth of an *Apis mellifera*

(Buckfast) colony (FlaH1) of mixed European origin housed in a single polyurethane hive (Fig. 2.1c). Photographs were taken of each frame before and after the recording period and a scale continually recorded the colony mass (±10 g).

These data were interpolated using the software package STATISTICA version 7.1 (Statsoft Inc., 1996) and presented as temperature and humidity profiles over time for the length and breath of the hive separately.

2.6 Patterns of nest humidity in diverse contexts

In order to determine the variability of microclimatic parameters in the brood nest of honeybee colonies when in different contexts, recordings were made in three different *Apis mellifera* subspecies, natural nests and under different ambient conditions. Five Sensirion RH sensors were placed in each of two *Apis mellifera* (buckfast) colonies (FlaH2&3) situated at the Flakkebjerg Research Centre, Denmark and housed in polyurethane hives (4 stacked deep brood boxes). Three sensors were placed in the brood rearing portion of the nest and two in the nectar store. Recording took place from 14 to 17 July 2007. The entrance hole, orientated 200° South, was the entire breath of the hive and the bottom board contained a ventilation mesh. Humidity and temperature was also recorded in two *Apis mellifera mellifera* colonies (LaesoH7&H12) housed in wooden Langstroth hive on Laeso Island, off the north eastern coast of Denmark. A Hygrochron iButton was placed in the brood and HOBO H8 data logger in the nectar store of each colony from 7 to 14 July 2007.

Humidity and temperature were measured in five natural nests in the Kruger National Park, South Africa. Hygrochron iButton sensors were mounted on a 1 m aluminium rod (Fig. 2.1f) and then shielded with a protective casing (Fig. 2.1 g) with open mesh ends which prevented workers from damaging the sensor. A thermocouple wire attached to the aluminium rod enabled the brood nest to be thermally located with an Appa 51 Thermometer (AppaA Technology Corporation, Taiwan, 0.01 °C, Fig. 2.1 h) thereby ensuring the standardised placement of each iButton. It was not always possible to locate 35 °C in the nest and on one occasion the thermocouple was presumably heat balled by workers, causing the temperature to rise to 40 °C. A HOBO logger was placed no further than 2 m from each nest entrance in order to record ambient temperature and humidity.

Other parameters recorded for each natural nest included volume, orientation and entrance size. The nest volume was measured by inserting an aluminium rod into the cavity to determine the length and breadth. Volume was then crudely calculated by assuming a cylindrical nest: all measured nests were situated in hollow trees. It was not possible to calculate nest volumes for natural nests situated in baobabs (*Adansonia digitata)* in the northern part of the Kruger National Park; these are presumably much larger cavities. Orientation of the nest entrance was determined using a magnetic compass. A photograph was taken of each nest entrance (e.g. Fig. 2.1e) with a ruler alongside. The size of the entrance and the amount of propolis used to decrease its size was determined with Adobe Photoshop; the ruler was used to calculate a conversion factor by determining number of pixels in 4 cm^2.

3. Results

3.1 Daily nest climatic patterns

Daily humidity and temperature patterns were determined by recording these parameters at 12 min intervals for a year in *A. m. scutellata* colonies and presented per season. The brood nest of colony 1 (ExpHl) showed a constant daily VPD during spring but this became more variable throughout the year (Fig. 2.2). The mean summer VPD in the nest varied by 8 mb throughout the day; from 17 mb at 8:00 am to 25 mb at 15:00 pm. During winter VPD varied by 27 mb with a maximum of 33 mb at 15:00 pm. Colony 2 (ExpH2) was a strong colony (Appendix C) and showed little change in the daily pattern of VPD, in the brood nest, throughout the year. The difference between minimum and maximum VPD was not more than 6 mb during any season of the year. A maximum mean of 29 mb was recorded (at 4:00 am) during spring and maximum of 24 mb during winter. The daily pattern of VPD was slightly less variable in summer (SD: 4 mb) than winter (SD: 6 mb). The fluctuation of VPD in the nectar store was larger than that of the brood nest, both during summer (20 mb to 32 mb) and winter (10 mb to 31 mb, Appendix A). Ambient daily VPD (Fig. 2.2) fluctuated in summer by 28 mb from 6:00 am to 13:00 pm when it peaked at 32 mb. During winter it fluctuated by 18 mb with a peak of 22 mb.

Temperature in colony 1 was effectively maintained in the brood nest at 35 °C during spring and summer (Fig. 2.3) however during autumn it was only held constant between 14:00 pm and 18:00 pm. Autumn temperature varied most at 7:00 am with an SD of 6 °C. In colony 2, temperature was a constant 35 °C throughout the day in every season, however in autumn the variability was greater than other seasons with a SD of 5 °C. Ambient daily temperature fluctuated by 15 °C in summer from 17 °C at 5:00 am to 32 °C at 14:00 pm. In winter it fluctuated by 17 °C from 6 °C at 6:00 am and 23 °C at 14:00 pm.

RH in colony 1 stayed in a narrow range between 55 and 68 % during spring and summer (Fig. 2.4). In autumn the daily RH showed greater fluctuations with a maximum RH of 59 % at 9:00 am. In colony 2, RH (mean daily) fluctuated within a 10 % range in all seasons. The maximum mean summer RH was 61 % and winter was 66 %. Ambient RH fluctuated by 47 % in summer from 33 % (at 14:00 pm) to 80% (at 6:00 am) and by 22 % in winter.

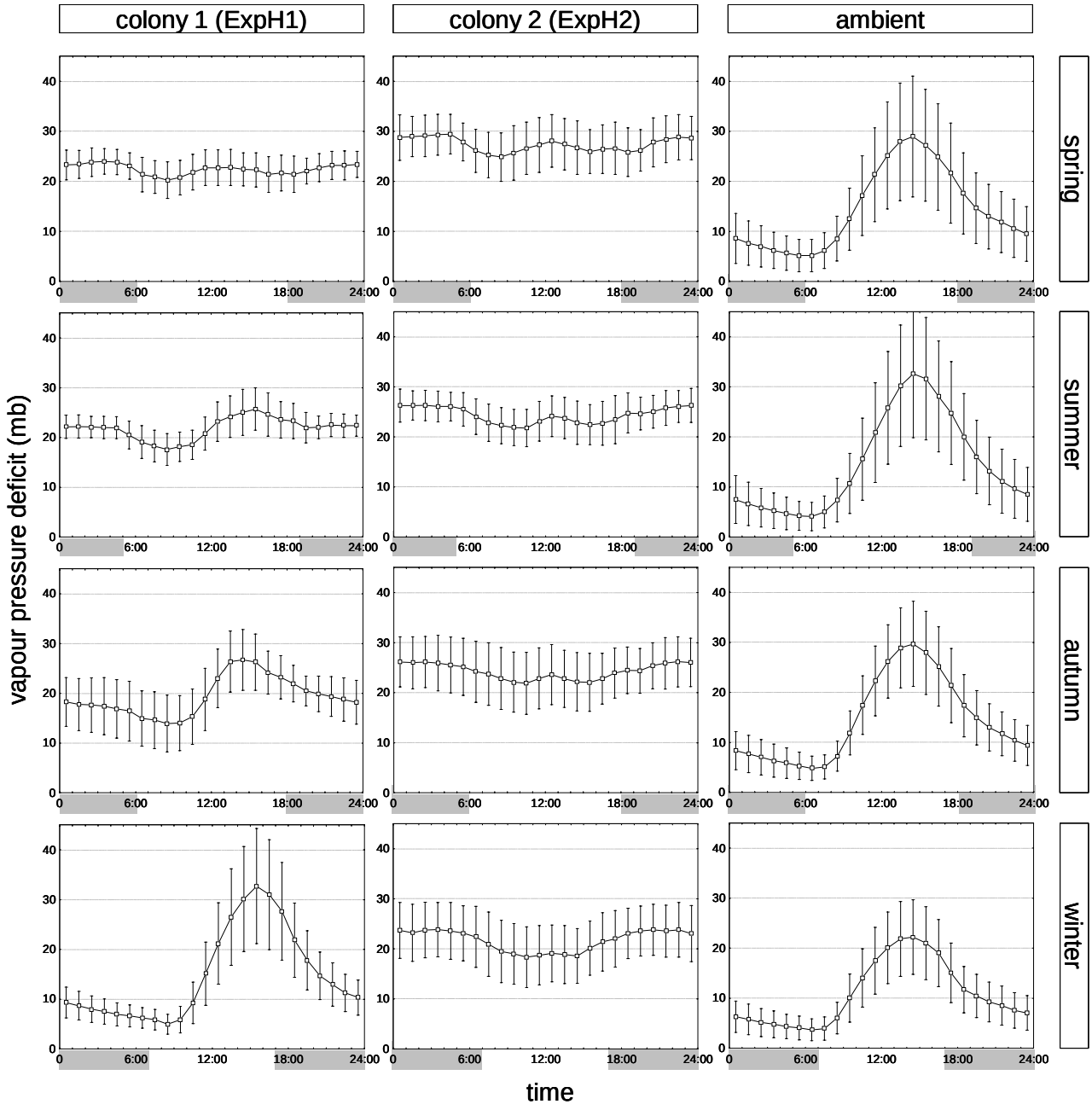

Fig. 2.2 Daily vapour pressure deficit in the brood nest of two honeybee colonies (ExpH1&2) and the associated ambient conditions. Each point represents an hourly mean ± SD of measurements taken at 12 min intervals over a three month seasonal period. Grey bars indicate night time.

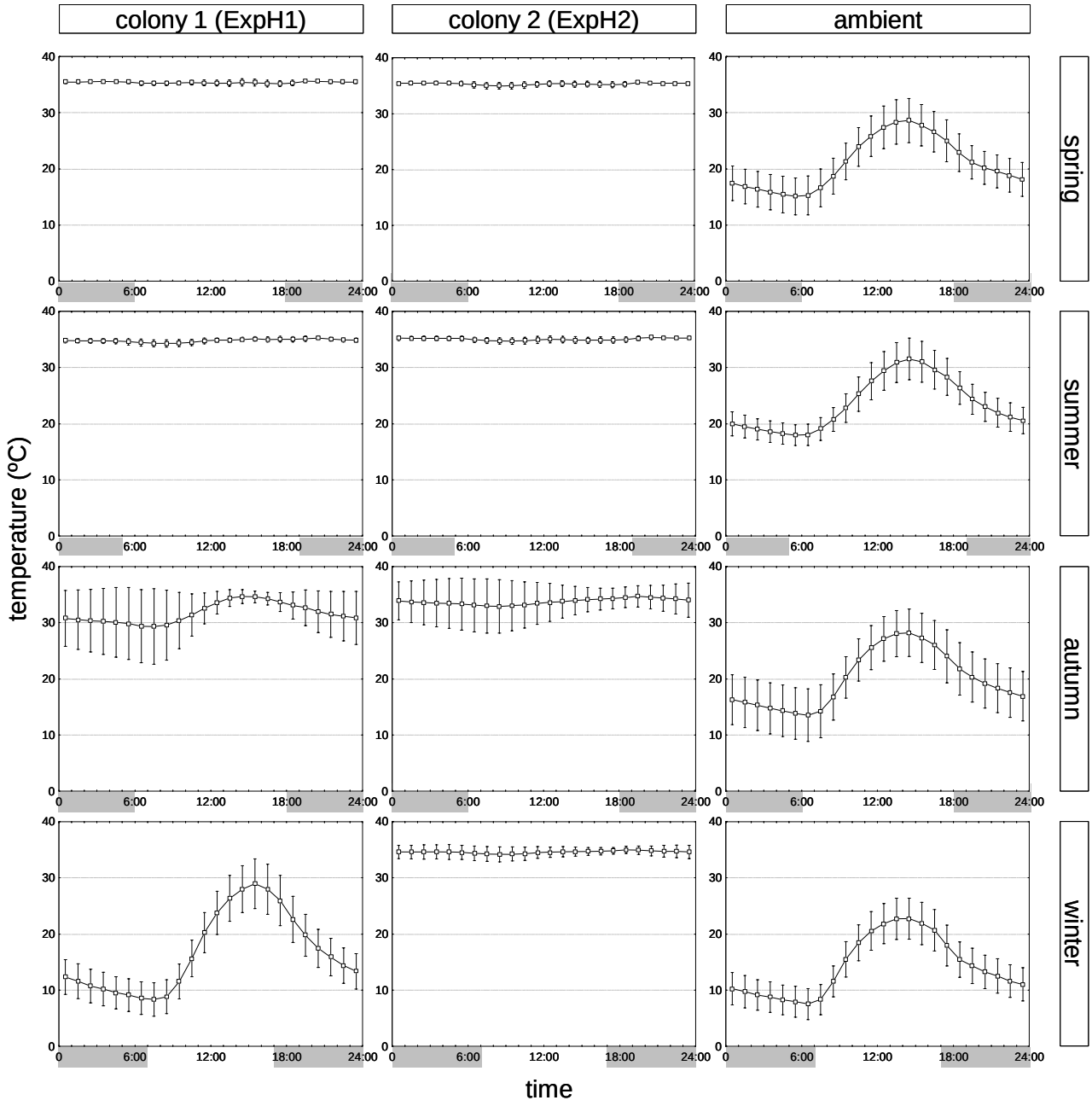

Fig. 2.3 Daily temperature in the brood nest of two honeybee colonies (ExpHl &2) and the associated ambient conditions. Each point represents an hourly mean ± SD of measurements taken at 12 min intervals over a three month seasonal period. Grey bars indicate night time.

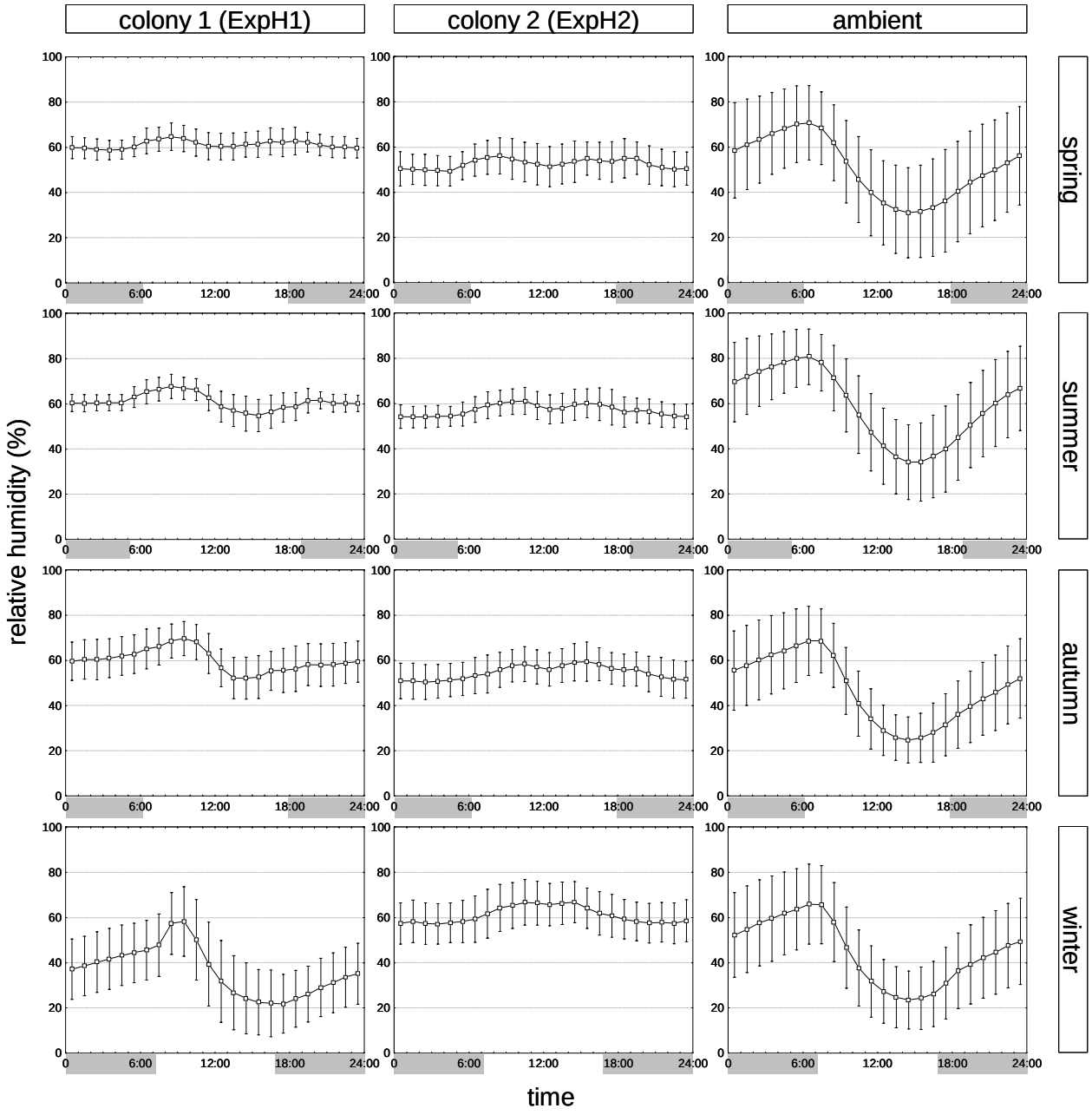

Fig. 2.4 Daily relative humidity in the brood nest of two honeybee colonies (ExpH1&2) and the associated ambient conditions. Each point represents an hourly mean ± SD of measurements taken at 12 min intervals over a three month seasonal period. Grey bars indicate night time.

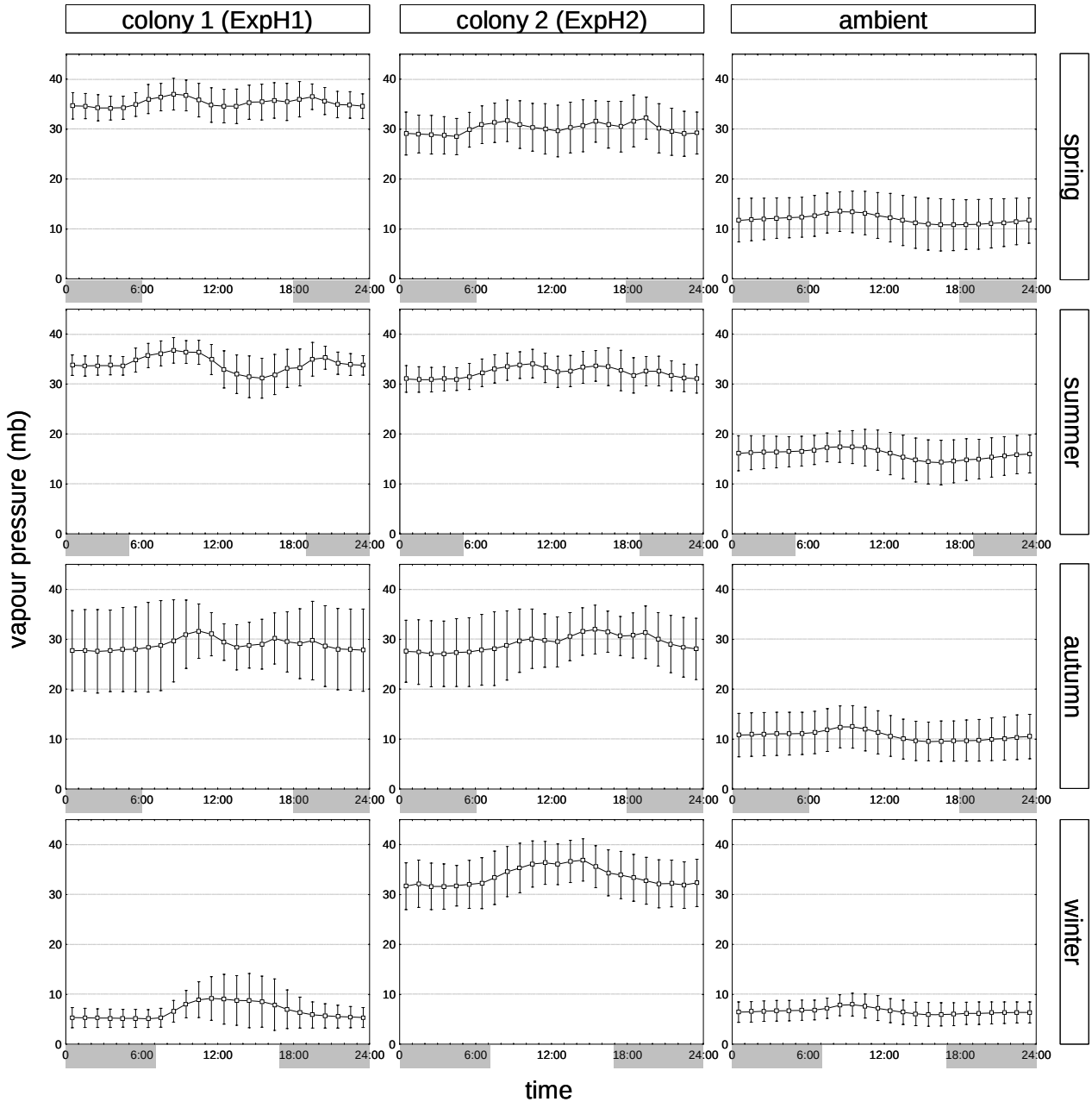

Fig. 2.5 Daily vapour pressure (mb) in the brood nest of two honeybee colonies (ExpH I &2) and the associated ambient conditions. Each point represents an hourly mean ± SD of measurements taken at 12 min intervals over a three month seasonal period. Grey bars indicate night time.

Vapour pressure (P_w in colony 1 remained around 30 mb from spring to autumn but it dropped to 5 mb in winter (Fig. 2.5). In colony 2, P_w remained around 30 mb throughout the year; with the highest variability (as indicated by the SD) during autumn. Ambient Pw remained constant throughout the day; being highest during summer (15 mb) and lowest during winter (5 mb).

3.2 Abnormal nest microclimate: absconding and winter clustering

Nest homeostasis under abnormal colony conditions were described by selecting data for records of temperature and humidity in brood nest of two A. *in. scutellata* colonies in the University of Pretoria apiary. One of the experimental colonies (ExpH 1) absconded on 1 June 2007; distinct humidity and temperature patterns were evident for at least a month prior to this event. Two months prior to absconding, the VPD in the brood nest of the colony did not fluctuate with ambient and only peaked for a short period at 14:00 pm each day (Fig. 2.6b1). VPD showed micro-fluctuations of approximately 10 mb throughout the day. Temperature remained constant at 35 °C in the brood during this period (Fig. 2.7b1) and was maintained 5 °C above the midday ambient peak of 30 °C and 20 °C above the midnight minimum of 15 °C. One month prior to absconding, both VPD and temperature began to fluctuate with ambient conditions (Fig. 2.6b2 & 2.7b2). Micro-fluctuations were still evident in the VPD cycle and temperature was maintained 10 °C above ambient but fluctuated between 28 °C and 35 °C. Subsequent to absconding (Fig. 2.6b3 & 2.7b3), in the vacant hive, the nest VPD and temperature fluctuate with ambient; VPD shows an 8 mb greater fluctuation than ambient and temperature peaks 5 °C above the midday ambient temperature peak.

Honeybee colonies cluster in winter to conserve energy but studies on the water relations of winter cluster have been largely theoretical (Omholt, 1987b). In order to compliment the theoretical studies, data was analysed from a winter cluster that was present in one of experimental colonies (ExpH2) at the University of Pretoria apiary between 24 April and 10 May (Fig. 2.8). April 29 was one of the coldest days in the year with an average temperature of 12.6 °C (min: 4.6 °C). Prior to cluster contraction the temperature in the brood nest was maintained constant at 35 °C. VPD and RH remained fairly constant around 25 mb and 60 %, both showing micro-fluctuations throughout the day. Cluster contraction was abrupt and on 24 April a temperature fluctuation of 5 °C was suddenly evident. The cluster contracted from 24 to 29 April, during which time the temperature fluctuations became larger and closely approximated ambient. Temperature fluctuations were offset from ambient and the minimum

nest temperature occurred approximately 1.5 h after the 7:00 am ambient minimum. Maximum nest temperature during this period occurred 4 h subsequent to the 15:00 pm ambient maximum. VPD and RH showed similar offset values. RH and VPD micro-fluctuations are not evident after the evening minimum on 28 April and they reappear after the morning maximum on 1 May. The winter cluster began to expand on 20 April and took 11 days to regain a constant 35 °C at the point in the nest where the sensor was situated.

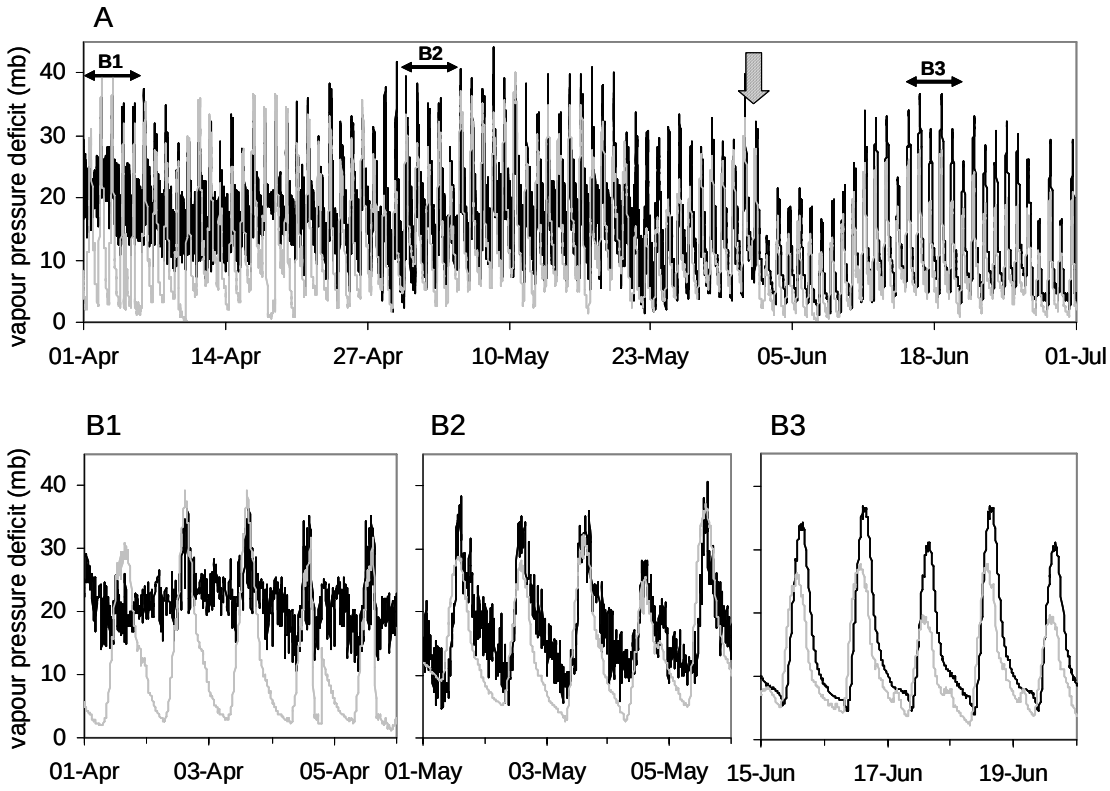

Fig. 2.6 Absconding**: a)** Vapour pressure deficit in an *Apis mellifera scutellata* colony (ExpHl) two months before and one month after absconding (depicted by hashed arrow). Grey line indicates ambient and black line, brood nest fluctuations. Six day time periods are given for **b1)** two months before **b2)** one month before and **b3)** 15 days after absconding.

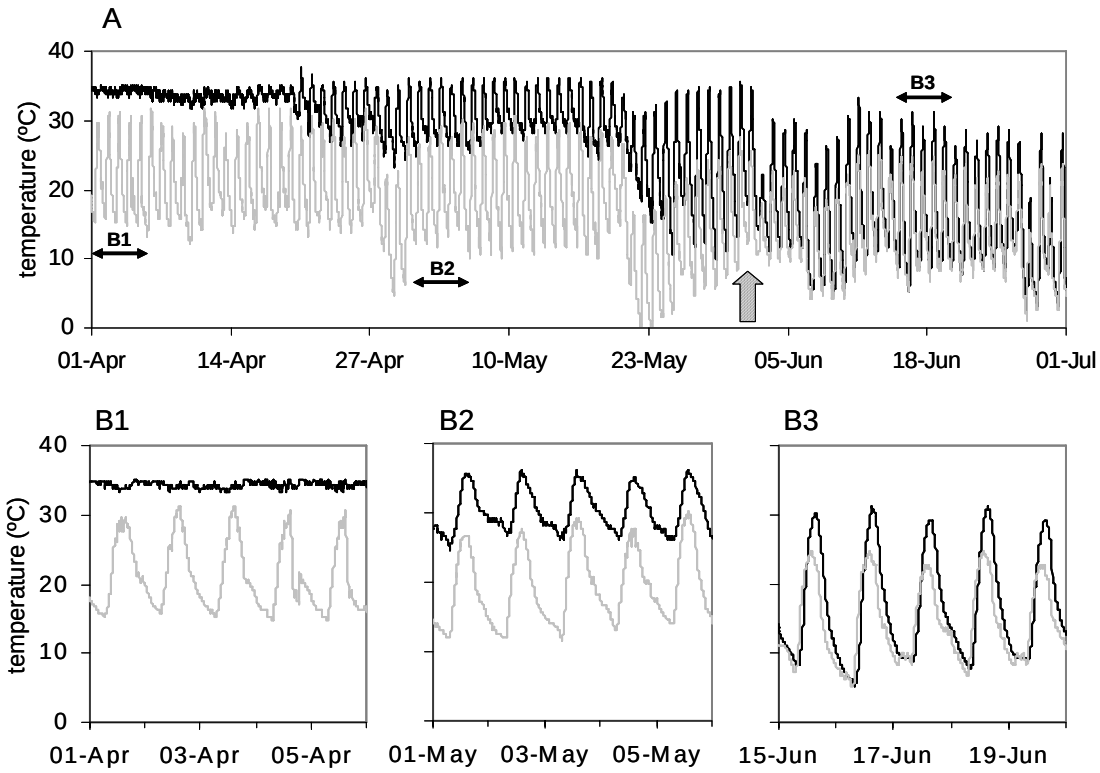

Fig. 2.7 Absconding: a) temperature in an *Apis mellifera scutellata* colony (ExpHl) two month before and one month after absconding (depicted by hashed arrow). Grey line indicates ambient and black line, brood nest fluctuations. Six day time periods are given for **b1)** two months before, b2) one month before and b3) 15 days after absconding.

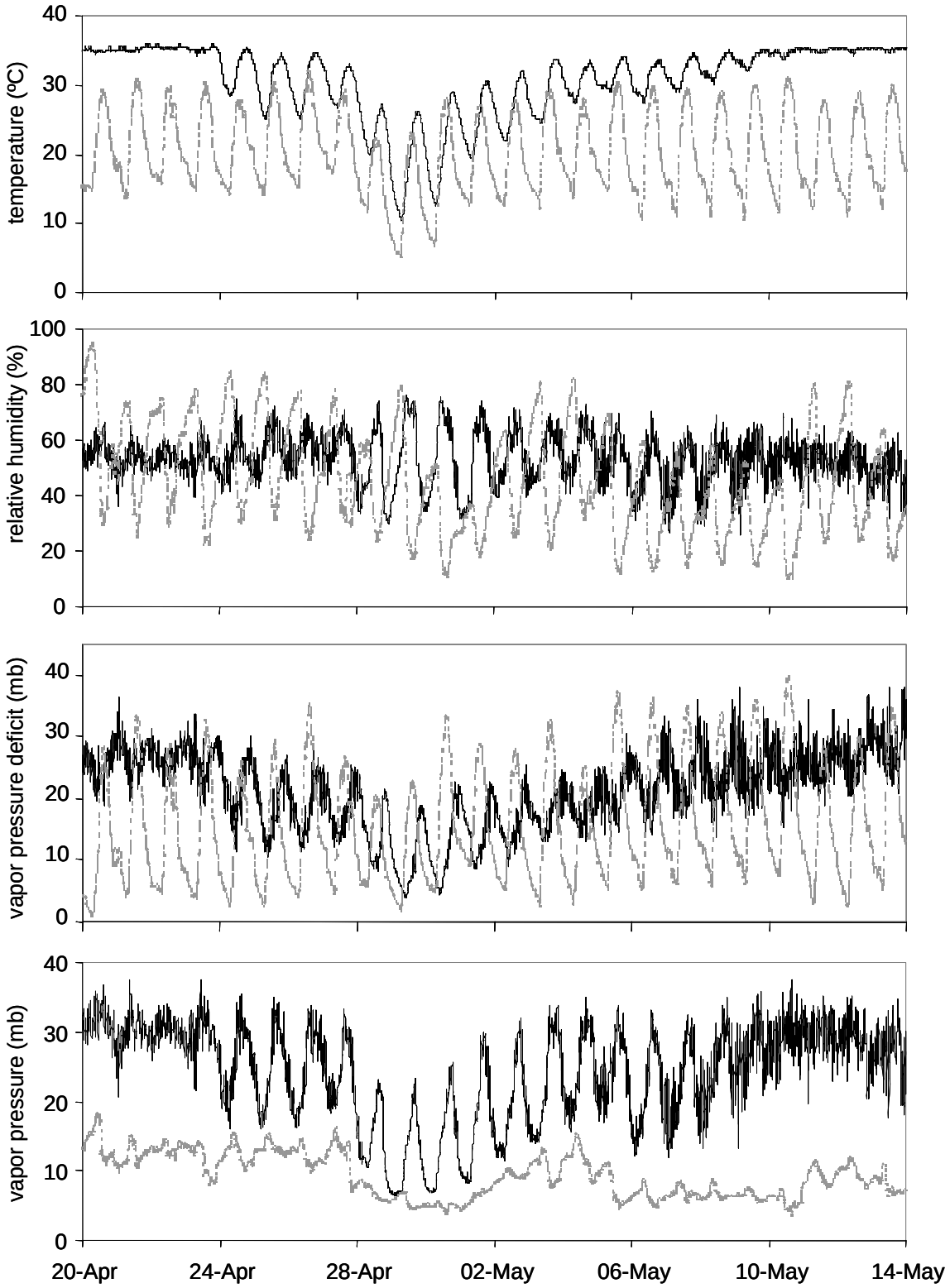

Fig. 2.8 Winter clustering: temperature, relative humidity, vapour pressure deficit and vapour pressure in an *Apis mellifera scutellata* colony (ExpH2) as the winter cluster contracts (from 24 April), leaving the sensor outside the cluster and then expands (from 2 May) to its original size (from 10 May). Grey line indicates ambient and black line brood nest fluctuations.

3.3 Correlation between nest and ambient climate

The relationship between brood nest microclimate and ambient parameter was determined from data obtained from a weather station situated in close proximity to the three experimental *A. m. scutellata* colonies. The brood nest temperature of colony 1 and 3 (ExpH 1 & H3) showed a progressively stronger correlation with ambient temperature throughout the year (Table 2.1); colony 1 and 3 with an autumn r value of >0.3 and winter of >0.9, colony 2 with seasonal r value <0.07. Brood temperature in these colonies during winter was weakly correlated with both wind speed (0.3, $p<0.01$) and direction (-0.3, $p<0.01$). Brood temperature of colony 3 was not strongly correlated with any other variables throughout the year.

RH in the brood nest of colony 1 and 3 was positively correlated with RH in both the nectar store and ambient environment during summer, autumn and winter. Wind direction was only weakly correlated with RH (-0.47, $p<0.01$) in the brood nest of colony 3 during winter but not during any other season in colony 1 and 2. Wind speed was only weakly correlated with temperature (>0.3, $p<0.01$) in colony 1 and 3 during winter but not during any season in colony 2. RH in the brood nest of colony 2 was not correlated with ambient RH except during winter when it was negatively correlated (-0.37, $p<0.01$). RH in this nest was correlated with that in the nectar store during spring, summer and autumn but not in winter (-0.04, $p<0.01$)

Brood nest VPD in colony 1 was progressively more correlated with ambient VPD throughout the year. Brood VPD in colony 3 was only correlated with ambient VPD during winter (0.60, $p<0.01$). Brood VPD in colony 2 was negatively correlated with ambient temperature (-0.25, $p<0.01$) and VPD (-0.36, $p<0.01$) in winter but not correlated in the other three seasons.

Table 2.1 Spearman rank order correlation showing the association between the microclimate in the brood nest of three *Apis mellifera scutellata* colonies (ExpH1,2&3) and those in the nectar store and ambient environment. Data is presented per season and shaded values depict an r value of >|0.3| (light), >|0.5| (medium) and >|0.7| (dark). Values marked with ***italics*** are **not** significant, p>0.01.

	SPRING	**Colony 1 (ExpH1)**			**Colony 2 (ExpH2)**			**Colony 3 (ExpH3)**		
	Brood	**T(ºC)**	**RH (%)**	**VPD (mb)**	**T(ºC)**	**RH (%)**	**VPD (mb)**	**T(ºC)**	**RH (%)**	**VPD (mb)**
Nectar	T (ºC)	-0.15	0.08	-0.10	0.12	-0.25	0.26	-0.06	-0.22	0.19
	RH (%)	0.15	0.18	-0.13	0.09	0.57	-0.54	***0.02***	0.25	-0.22
	VPD (mb)	-0.17	-0.05	***0.01***	0.03	-0.46	0.45	-0.04	-0.26	0.23
Ambient	T (ºC)	-0.12	-0.05	***0.00***	-0.06	-0.13	0.11	-0.14	-0.17	0.14
	RH (%)	0.22	-0.07	0.11	***-0.02***	-0.05	0.05	0.19	0.03	***0.00***
	VPD (mb)	-0.21	0.03	-0.08	-0.03	-0.03	***0.02***	-0.20	-0.08	0.05
	Wind (m/s)	-0.09	-0.06	0.04	0.04	***-0.03***	0.04	-0.07	-0.06	0.05
	Wind Dir (º)	0.15	-0.06	0.10	0.12	-0.05	0.07	0.21	-0.10	0.12
	Rain (mm)	0.06	-0.05	0.05	0.00*	***0.00***	***0.01***	0.06	***-0.02***	0.03
	Solar (w/m2)	-0.21	0.12	-0.17	-0.20	0.10	-0.13	-0.31	0.11	-0.14
	SUMMER									
Nectar	T (ºC)	0.37	-0.40	0.44	0.27	-0.18	0.21	0.09	-0.16	0.16
	RH (%)	-0.15	0.49	-0.48	-0.25	0.45	-0.45	-0.07	0.37	-0.36
	VPD (mb)	0.29	-0.47	0.49	0.28	-0.34	0.36	0.09	-0.28	0.27
Ambient	T (ºC)	0.23	-0.30	0.32	-0.04	0.09	-0.08	***0.01***	-0.08	0.08
	RH (%)	-0.19	0.37	-0.38	-0.04	0.08	-0.08	0.07	0.18	-0.16
	VPD (mb)	0.21	-0.37	0.38	0.03	-0.03	0.03	-0.04	-0.15	0.13
	Wind (m/s)	0.11	-0.17	0.17	***-0.01***	***0.01***	***-0.01***	0.07	-0.08	0.08
	Wind Dir (º)	0.06	0.03	***-0.02***	0.18	-0.19	0.20	***0.00***	-0.16	0.15
	Rain (mm)	0.04	0.03	***-0.02***	***-0.01***	***0.02***	***-0.02***	0.05	***-0.01***	***0.01***
	Solar (w/m2)	-0.05	***0.01***	***-0.01***	-0.26	0.29	-0.30	-0.13	0.26	-0.26
	AUTUMN									
Nectar	T (ºC)	0.69	-0.25	0.67	0.12	0.39	-0.14	0.34	0.31	-0.22
	RH (%)	-0.16	0.45	-0.42	***0.01***	0.31	-0.13	0.23	0.33	-0.27
	VPD (mb)	0.61	-0.31	0.66	0.11	0.20	-0.04	0.11	0.03	***0.02***
Ambient	T (ºC)	0.69	-0.15	0.58	-0.05	0.47	-0.27	0.35	0.39	-0.30
	RH (%)	-0.07	0.42	-0.31	-0.03	0.06	-0.08	0.26	0.34	-0.29
	VPD (mb)	0.41	-0.32	0.50	-0.04	0.22	-0.11	0.05	***0.02***	***0.01***
	Wind (m/s)	0.12	-0.12	0.19	-0.05	0.10	-0.05	***0.03***	-0.04	0.06
	Wind Dir (º)	-0.23	***0.00***	-0.19	0.10	-0.29	0.20	-0.10	-0.18	0.18
	Rain (mm)	0.03	***0.01***	***0.02***	***0.02***	0.03	***-0.01***	0.05	0.04	-0.04
	Solar (w/m2)	0.28	0.04	0.22	-0.17	0.34	-0.25	0.08	0.21	-0.20
	WINTER									
Nectar	T (ºC)	0.96	-0.53	0.85	0.14	0.31	-0.25	0.91	0.26	0.65
	RH (%)	-0.27	0.54	-0.46	-0.14	-0.04	0.03	-0.65	***-0.01***	-0.52
	VPD (mb)	0.94	-0.61	0.88	0.16	0.28	-0.23	0.84	0.15	0.63
Ambient	T (ºC)	0.90	-0.48	0.79	0.07	0.39	-0.33	0.89	0.34	0.59
	RH (%)	-0.73	0.82	-0.84	-0.09	-0.37	0.32	-0.69	***0.00***	-0.58
	VPD (mb)	0.85	-0.70	0.87	0.06	0.41	-0.36	0.82	0.17	0.60
	Wind (m/s)	0.33	-0.18	0.29	***0.01***	0.16	-0.13	0.30	0.11	0.18
	Wind Dir (º)	-0.28	-0.10	-0.12	0.06	-0.21	0.18	-0.30	-0.47	***0.01***
	Rain (mm)	***-0.02***	0.04	-0.03	***-0.02***	-0.04	0.04	***-0.02***	***0.01***	***-0.02***
	Solar (w/m2)	0.33	0.00	0.20	-0.11	0.34	-0.32	0.36	0.61	-0.03

3.4 Correlation between nest microclimate and colony condition

The condition of three colonies (ExpH1, H2 & H3) in the University of Pretoria apiary was determined by assessing the amount of capped and uncapped brood, pollen, capped honey and uncapped nectar (Appendix C). These nest parameters were then correlated with the brood, nectar and ambient microclimate (Table 2.2). The amount of capped brood was positively correlated with brood temperature (0.39, $p<0.01$) but not with any other variables. The amount of uncapped brood was correlated positively with RH (0.53, $p<0.01$) and negatively with VPD (-0.52, $p<0.01$). The amount of uncapped brood was not significantly correlated (<|0.33|, N.S.) with brood temperature or nectar temperature, RH or VPD. The amount of pollen, uncapped or capped nectar were not correlated (<|0.33|, N.S.) with any other variables.

Table 2.2 Spearman rank order correlation showing the association between the condition (i.e. amount of brood, pollen, honey and nectar) of 3 honeybee colonies (ExpH1,2&3) and the climatic conditions in the brood nest, nectar store and ambient environment. Shaded values depict an r value of >|0.3| (light) and >|0.5| (dark). Marked (*) values are significant, $p<0.01$

		capped brood	uncapped brood	pollen	capped honey	uncapped nectar
brood	T(ºC)	**0.39***	-0.30	-0.08	0.20	-0.01
	RH (%)	-0.13	**0.53***	0.13	-0.15	-0.21
	VPD (mb)	0.19	**-0.52***	-0.17	0.17	0.18
nectar	T(ºC)	0.06	-0.30	0.25	0.28	0.21
	RH (%)	0.14	0.33	-0.30	-0.26	-0.30
	VPD (mb)	-0.05	-0.34	0.33	0.25	0.25
ambient	T(ºC)	0.17	-0.19	0.13	0.26	-0.06
	RH (%)	-0.07	0.11	-0.16	0.33	0.12
	VPD (mb)	0.13	-0.20	0.22	-0.17	-0.07
	Wind (m/s)	-0.02	-0.04	0.17	0.25	0.11
	Rain (mm)	0.12	0.08	-0.15	0.02	-0.29
	Solar (w/m2)	0.00	-0.11	0.27	-0.18	0.05

3.5 Two dimensional hive humidity patterns

An *A. m. scutellata* colony (BomaH4) housed in a single Langstroth hive during the South African winter revealed interesting two dimensional thermal (Fig. 2.9) and hygric (Fig. 2.10) profiles. Temperature was constant at 35 °C between the centre three frames and for two thirds of the frames' length. Temperature showed a gradient toward the outer walls of the hive with 15 °C being reached at the front and back of the hive and as low as 10 °C on the western side of the hive. The minimum temperatures were reached at approximately 6:00 am. Low VPD (5 mb) was evident on the peripheries of the hive where it showed steep fluctuations. Higher VPD was measured in the central region (25 mb) of the nest. VPD fluctuated more than the temperature in the centre region.

Two dimensional thermal (Fig. 2.11) and hyrgic (Fig. 2.12) profiles were calculated for an *Apis mellifera* (buckfast) colony (FlaH 1) housed in a polyurethane hive during a Danish summer. Temperature fluctuated within extremely narrow limits (31-36 °C) within the entire nest and showed no daily pattern. VPD in the central nest region fluctuated around 25 mb and in the nest peripheries from 10 to 30 mb.

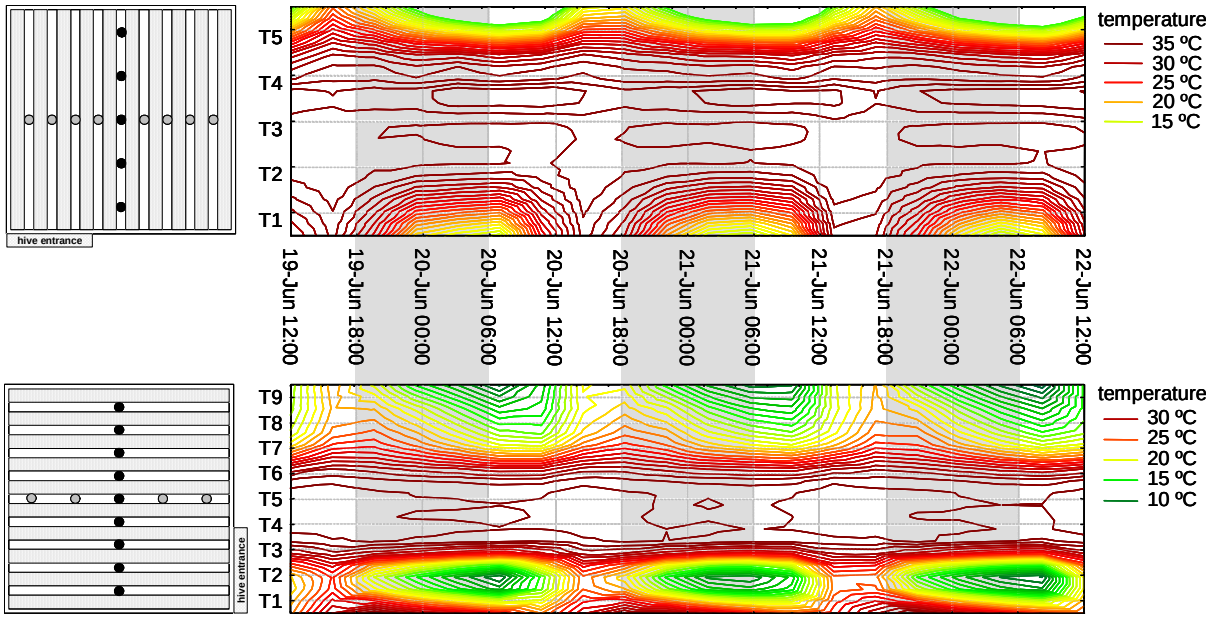

Fig. 2.9 A thermal profile of an *Apis mellifera scutellata* colony (BomaH4) over a 4 day period seen in two dimensions through the hive: front to back (top) indicated by probes T1 to T5 and side to side (bottom) indicated by probes T1 to T9. Shaded areas indicate night and the coloured lines link areas of equal temperature.

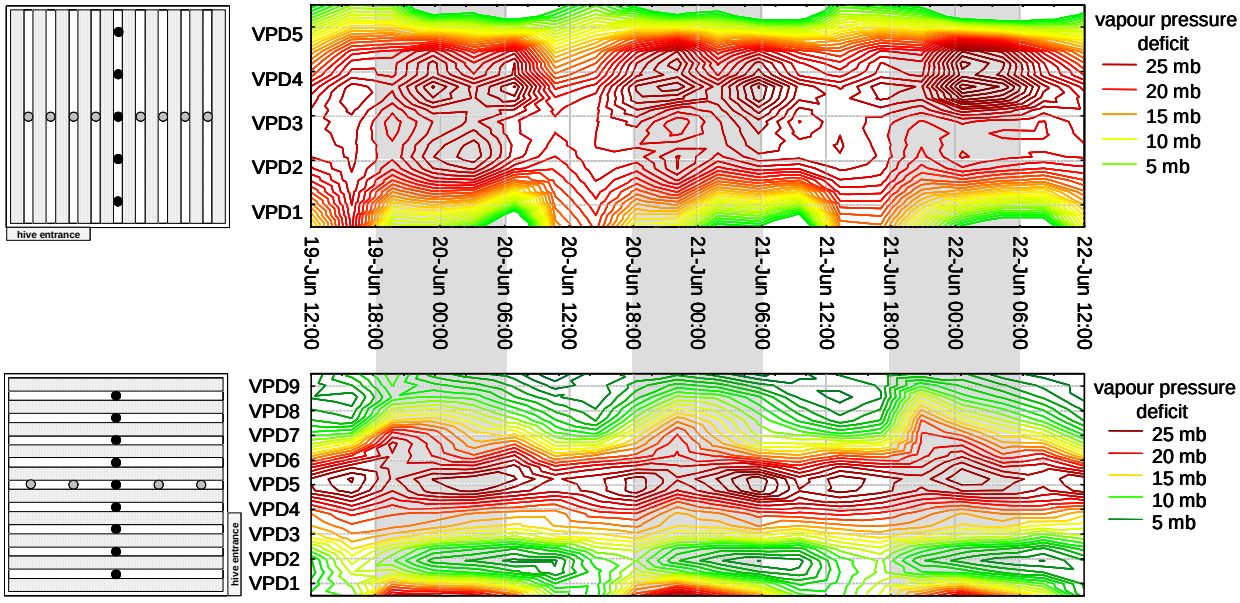

Fig. 2.10 A hygric profile depicting vapour pressure deficit of an *Apis mellifera scutellata* colony (BomaH4) over a 4 day period seen in two dimensions through the hive: front to back (top) indicated by probes VPD1 to VPD5 and side to side (bottom) indicated by probes VPD1 to VPD9. Shaded areas indicate night and the coloured lines link areas of equal vapour pressure deficit.

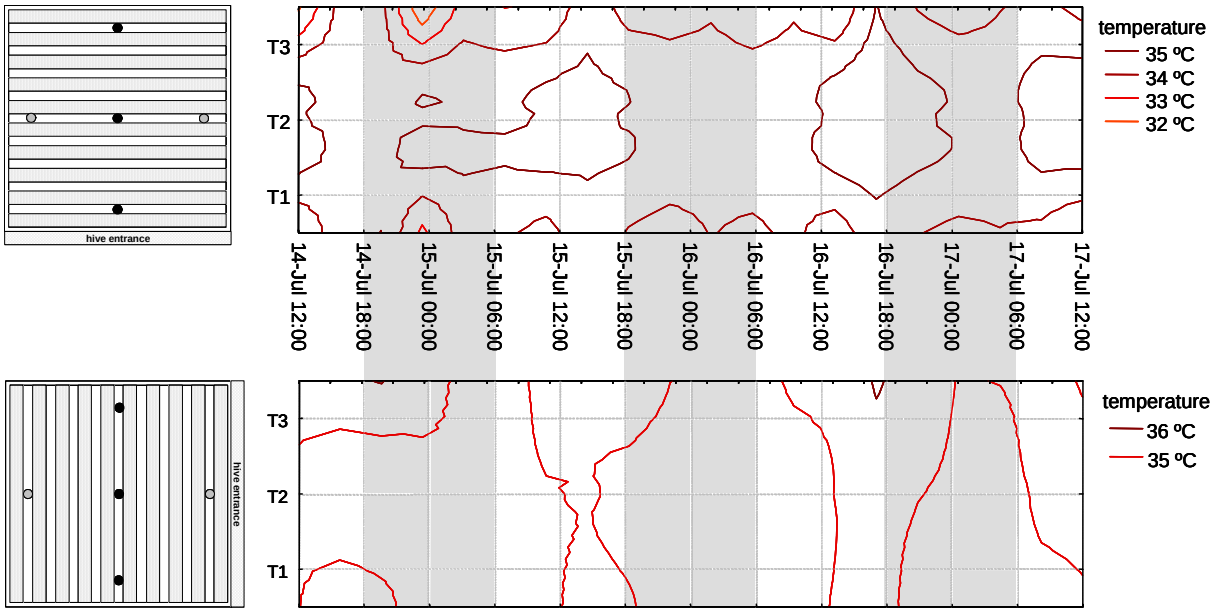

Fig. 2.11 A thermal profile of an *Apis mellifera* (buckfast) colony (FlaH1) over a 4 day period seen in two dimensions through the hive: front to back (top) and side to side (bottom). Shaded areas indicate night and the coloured lines link areas of equal temperature, as recorded by probes T1 to T3.

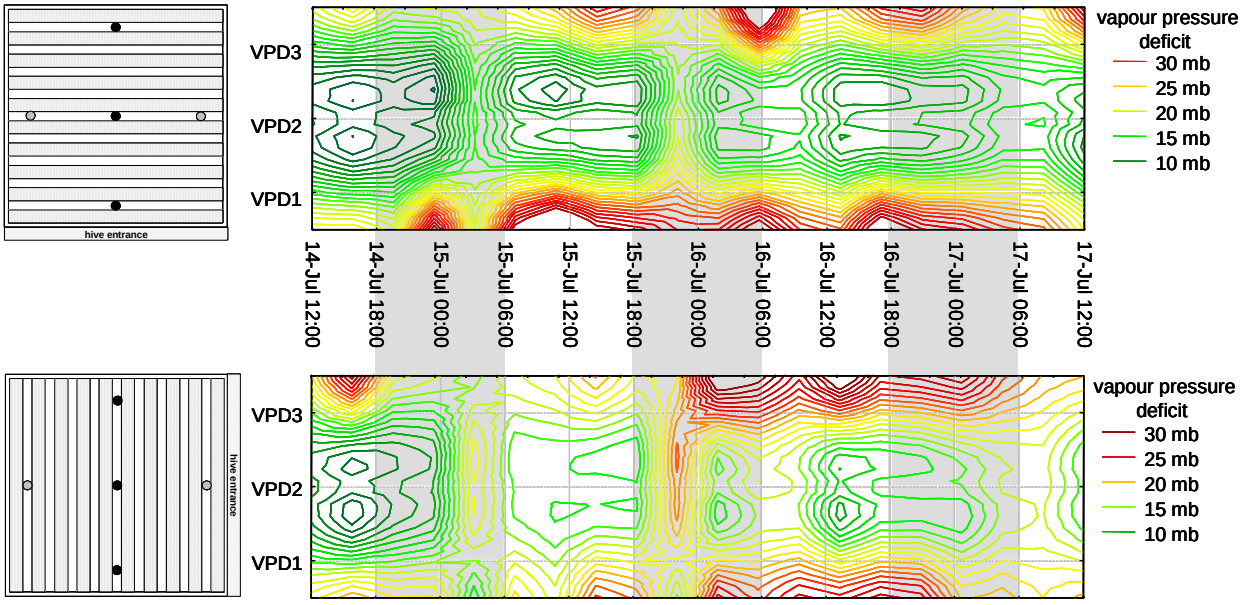

Fig. 2.12 A hygric profile depicting vapour pressure deficit of an *Apis mellifera* (buckfast) colony (FlaH1) over a 4 day period seen in two dimensions through the hive: front to back (top) and side to side (bottom). Shaded areas indicate night and the coloured lines link areas of equal vapour pressure, as recorded by VPD1 to VPD3.

3.6 Patterns of nest humidity in diverse contexts

Recordings were made in three different *Apis mellifera* subspecies, in natural nests and under different ambient conditions in order to determine the variability of brood nest microclimatic parameters in different contexts. The RH median in the brood nest of the three honeybee subspecies in different contexts is always found between 50 and 60% RH and the VPD median ranges from 20 mb to 30 mb (Fig. 2.13). Temperature in the brood nests is constant at 35 °C. The nectar store of the three A. *m. scutellata* colonies (ExpH1, H2 & H3) show larger fluctuations (indicated by the 25-75% quartiles) in temperature, RH and VPD than the *Apis mellifera* (buckfast) colonies. The *A. m. scutellata* (BomaH4) with a similar sampling interval (Appendix D) to the *Apis mellifera* (buckfast) colonies did not show such large fluctuations. The nectar stores of the two A. *m. mellifera* colonies show large fluctuations in temperature, RH and VPD.

Natural nests of *A. m. scutellata* in the Kruger National Park showed median RH values of between 60 and 70%. VPD of these nests was between 20 and 30 mb. The median temperature ranged between 25 and 30 °C giving evidence that the sensors were not always placed in the brood rearing portion of these nests. The range and quartiles of RH in the natural nests are proportional to the fluctuations seen in the nectar store of colonies housed in Langstroth hives.

The mean nest volume was calculated as 50 litres (Appendix D) which is similar to the mean of 44 litres calculated by Schneider and Blyther (1988) for *A.m. scutellata* nests in the the Okavango Delta, Botswana. The average entrance size was 5 cm^2 with a propolis plug of 73 cm^2. One baobab nest contained a propolis plug of 519 cm^2 which reduced the entrance to 11 cm^2.

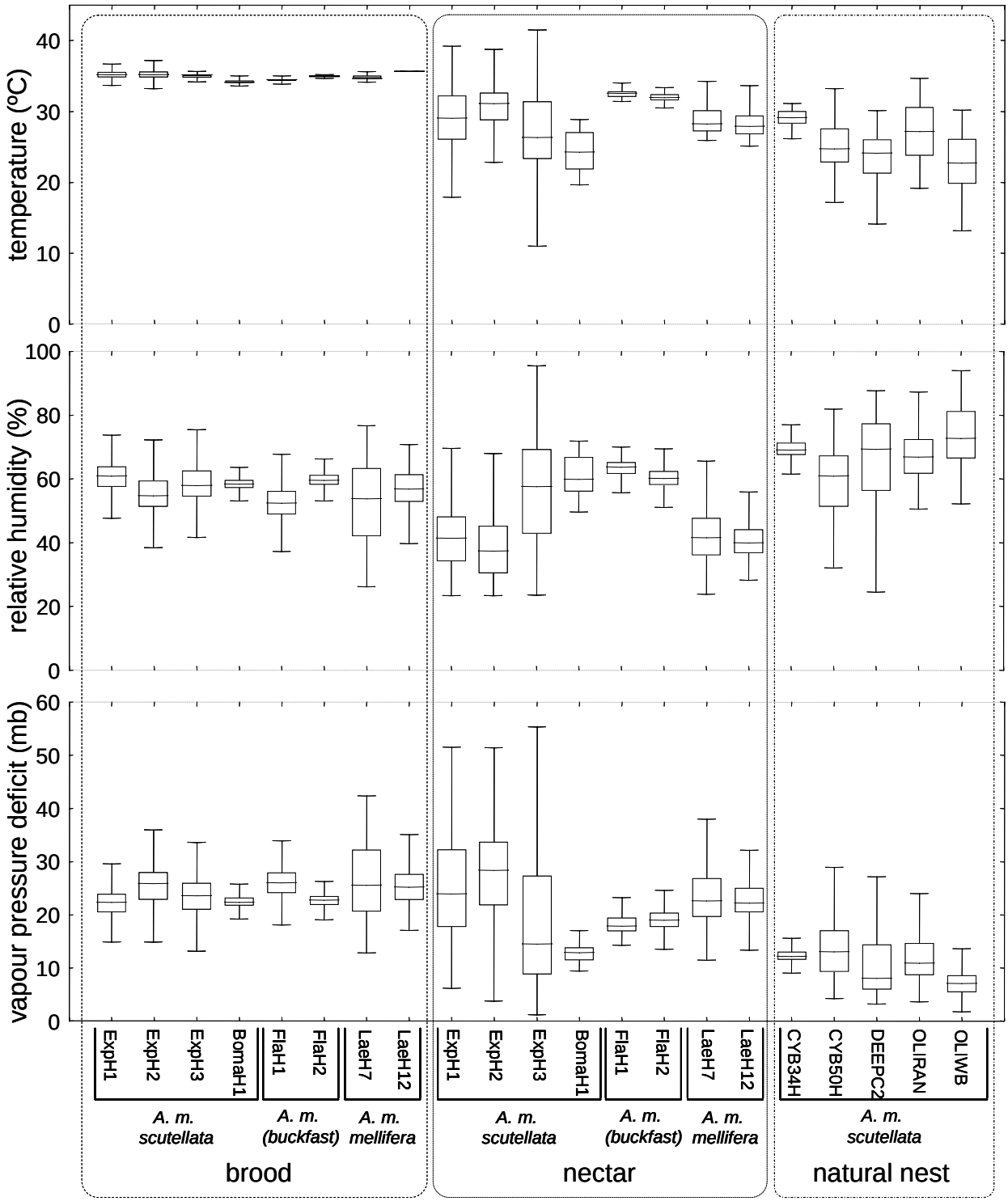

Fig. 2.13 The median, quartiles (25-75%) and non outlier range of temperature, relative humidity and vapour pressure deficit in the brood nest and nectar store of three different honeybee subspecies and in natural nests of *A.m. scutellata.*

4. Discussion

4.1 Daily nest climatic patterns

The (mean hourly) humidity in the brood nest of a healthy established honeybee colony is relatively constant throughout the day. RH remains at a level of 60 %; however VPD is high and therefore the air in the brood nest has a large evaporative capacity. It may seem surprising that low humidity is evident in the brood nest where the desiccation sensitive larvae and eggs are located. This may prevent the moulding of the comb (Wohlgemuth, 1957) and higher humidity is probably maintained within the brood cells. Park (1925) and Lindauer (1954) described a behaviour by which workers spread water onto the surface of the brood or hang droplets in the comb cells. These droplets expose a maximum surface for evaporation and could also be abosorbed by the accumulated cocoons (Ellis et al., submitted).

There is not an observable daily pattern to humidity in the brood nest of a healthy colony, but this is evident when a colony becomes weaker. The range of VPD was not more than 6 mb in a strong colony with RH occurring between 50 and 60 %. These data for a strong colony are in contrast with Büdel (1948) who observed a daily humidity pattern in a colony and RH fluctuated between 40 and 50 %. He described a distinct peak at 13:00 pm which he attributed to the activity of the workers. This may be an artefact of him observing a weak colony or a limitations imposed by his recording devices. It is interesting to note that this daily pattern will be influenced by water vapour moving down a gradient and out of the hive. Vapour pressure in the hive is always higher than ambient and water vapour will move down this gradient as described by Fick's law.

There is a clear distinction between the humidity patterns in the brood nest compared to that of the nectar store. The mean hourly P_w, in the nectar store is always below 20 mb whereas in the brood nest it is above 20 mb. VPD in the nectar store shows greater fluctuation with a peak between 12:00 pm and 16:00 pm. The fluctuations in the nectar store are due to changes in temperature and are not driven by vapour pressure, as this remains fairly constant throughout the day. It would be most efficient for workers to evaporate nectar during the VPD peak, however VPD in the nectar store remains higher than ambient at all times during the day. This explains why honeybees remain inside the hive to evaporate nectar rather than doing this outside the colony. An exception to this was shown by Nicolson and Human (2008)

where forgers concentrated the nectar between the forage site and the nest. This is presumable done by droplet extrusion in dry ambient air while the workers are foraging and on the flight back to the nest.

4.2 Abnormal nest microclimate: absconding and winter clustering

Absconding is a behavioural trait in the honeybees of Africa (Hepburn and Radloff, 1998), but nothing is known of nest microclimate conditions leading up to absconding. It is evident from this study that there are alterations in the patterns of nest microclimate prior to absconding, although this breakdown in nest homeostasis may not have a causal effect on absconding. It is known that there are numerous factors that can induce absconding (Schneider and McNally, 1992); parasites (eg. wax moth and small hive beetles), predators (eg. bee wolves and honey badgers), reduction in field resources and restricted cavity size. *Apis mellifera scutellata* is also known to abscond more over the wetter parts of its range (Hepburn and Radloff, 1998). This could be due to migrating swarms having a lower probability of surviving in drier regions and colonies are thus more hesitant to abscond or that nest homeostasis is more difficult to maintain in wetter regions and colonies, therefore, abscond more frequently.

This study shows clearly distinct patterns in the nest microclimate one and two months prior to absconding. Temperature and humidity levels begin to fluctuate with ambient conditions one month prior to absconding; however temperature is still 10 °C higher than ambient. There is very little brood in the nest during this period due to preparation before absconding (Hepburn & Radloff 1998) which makes it unnecessary for the workers to expend energy on nest homeostasis.

An interesting characteristic evident in the nest prior to absconding is that VPD shows distinct micro-fluctuations. These micro-fluctuations are a characteristic of inhabited nests but are not evident once the bees have left the nest. They are caused by changes in vapour pressure, as they are not evident in the temperature cycles. The reason that vapour pressure fluctuates on such a small scale is uncertain but is probably due to both the respiration of individual bees and the active ventilation of the colony. Honeybee nest ventilation exchanges large volumes of ambient air with that in the nest and follows distinct cycles with colonies taking approximately three breaths per minute (Southwick and Moritz, 1987). Similar cycles are evident in smaller cavity dwelling species of stingless bees (Moritz and Crewe, 1988).

Once a colony has absconded, the temperature and VPD in the nest surprisingly show greater fluctuations than ambient, with temperature peaking 5 °C above ambient. Solar radiation which heats the exterior walls could cause a heating effect inside the nest. This is certainly due to both the design of a Langstroth hive and lack of midday shade in the apiary.

Winter clustering constitutes another abnormal period of nest microclimatic conditions. Clustering takes place when ambient temperature falls to 19-14 °C (Johansson and Johansson, 1979); however, African honeybees do not cluster as tightly as their European counterparts (Southwick et al., 1990). Differences in thermorgegulatory ability have also been shown in African subspecies: *A. m. scutellata* maintains a significantly large brood area at higher core temperature than *A. m. capensis* (Worswick, 1987). By forming a spherical cluster the workers in essence reduce the surface to volume ratio of the superorganism, thus minimising heat loss and energy expenditure. Both theoretical (Omholt, 1987a; Lemke and Lamprecht, 1990) and practical (Worswick, 1987; Stabentheiner et al, 2003) studies have been conducted on the thermoregulation of winter clusters, but little is known of their water economy. This study supports Omholt's (1987b) theoretical study on the water economy of winter clusters. Cluster vapour pressure does in fact decrease with increasing radius from the cluster. Temporally, vapour pressure on the periphery of a cluster fluctuates substantially but does not exhibit any micro-fluctuations as in a normally regulated colony. Temperature and humidity in the winter cluster is slightly offset from ambient conditions by a couple of hours. This offset is due to the thermal and hygric inertia of the hive.

4.3 Correlation between nest and ambient climate

Ambient temperature, humidity, wind, rain and solar radiation are not highly correlated with brood nest microclimate in a healthy colony. It is well known that brood nest temperature can be maintained constant irrespective of ambient conditions, but it is interesting that humidity is also not correlated with ambient. The brood nest humidity in a strong colony (eg. colony ExpH3) is not highly correlated with ambient. Nest microclimate was, therefore, independent of ambient conditions and more important factors within the nest must be responsible for the observed variation. RH and VPD in this colony were however found to be negatively correlated with corresponding ambient conditions during winter. This may be due to the diurnal pattern of nest humidity, but the underlying mechanism producing this pattern is not known.

There are cases in which correlations between nest microclimate and ambient conditions are apparent: when a colony is weak the nest microclimate becomes highly correlated with ambient conditions. This can occur as winter approaches (eg. colony ExpHl & H2) and as brood production stops, since it becomes costly for the workers to maintain these parameters at optimal levels when they are not required to ensure brood development. Another case in which this correlation can appear is before absconding RH and VPD (in colony ExpH1) were correlated with ambient conditions as early as summer. This was probably an early indication of the breakdown in regulation of nest homeostasis before swarm departure.

Wind direction and velocity had surprisingly little association with nest microclimate: they were not strongly correlated to any nest parameter besides during winter when they correlated to temperature and RH in two weak colonies. Some social insects are dependent on wind-induced ventilation, such leaf-cutting ant *Atta vollenweideri* (Kleineidam et al., 2001) that occurs in large subterranean nests. This study confirms that honeybees are not dependent on wind to ventilate their nests. If honeybee colonies were primarily dependent on wind for ventilation then the air movements would be slow and erratic due to the relatively large nest volume (42 litres) and small entrance size (5 cm^2). Nest homeostasis would presumably be affected by a constant high velocity wind but this upper limit may not have been reached in our apiary.

4.4 Correlation between nest microclimate and colony condition

The amount of uncapped brood in the nest is associated with the humidity in the brood nest, but not with any other climatic parameters. Uncapped brood is sensitive to desiccation due to the permeability of the larval cuticle and the egg chorion. Doull (1976) showed that hatching success of eggs is indeed dependent on relative humidity. Although a correlation does not imply a causal relationship it seems possible that workers regulate humidity to improve brood development.

Brood nest humidity is not correlated with the amount of capped brood in the nest. The cappings on the cells of brood are permeable to water because they are constructed from a combination of wax and silk (Hepburn, 1986). They in fact become more permeable with age because workers gradually remove material from the capping and 60% has been removed

prior to adult emergence (Meyer & Ulrich, 1952). It is surprising that the amount of capped brood is not associated with nest humidity but this is presumably because the pupae are more resistant to desiccation and that they are surrounded by hygroscopic cocoons in a relatively isolated microenvironment. Temperature is the only microclimatic parameter that is significantly correlated with the amount of capped brood in the nest. This is expected because amount of capped brood is the prime determinant of thermoregulatory patterns in the colony (Kronenberg and Heller, 1982).

4.5 Two dimensional hive humidity patterns

In order to fully understand nest homeostasis and water economy of honeybee colonies, there is a need to study nest microclimate on a spatial and temporal scale concurrently. I did this in two honeybee colonies and discovered that indeed there are interesting thermal and hygric patterns within the nest. A steep humidity and temperature gradient existed from the central brood area to the periphery of a hive during a South African winter. Büdel (1948) suggested that although vapour pressure fluctuates over time, it is constant throughout the hive at any given time. He proposed that only RH in different nest regions fluctuates and that this is due to fluctuations in temperature. The data of this study contradicts this idea and supports the study of Human et al. (2006) by providing evidence of humidity following distinctly different patterns in different parts of the nest independently of temperature.

Whereas temperature in the central brood area remains constant, vapour pressure fluctuates within certain limits. These fluctuations do not show a distinct daily cycle and could be related to the ventilation of the colony. The periphery of the hive showed low VPD with associated low temperatures. In the *Apis mellifera* (Buckfast) VPD fluctuated more on the periphery (10-30 mb) than in the central nest region (20-25 mb).

A comparison of the thermal and hygric profiles of an *A. m. scutellata* colony in South Africa and an *Apis mellifera* (buckfast) colony in Denmark show distinctly different patterns (fig. 2.9 – 2.12). There are numerous possible reasons for the observed differences. Firstly, the hives were constructed from different materials; with the Danish hive made from polyurethane and the South African hive made from wood. Secondly, recordings were made under different climatic conditions. Thirdly, differences exist between the regulatory abilities of the subspecies. European honeybees are known to tolerate lower temperatures and have more

dense clusters than African subspecies (Southwick and Heldmaier, 1987; Southwick et al., 1990). Heinrich (1993) states that, for these reasons, European bees are able to thermoregulate better at lower energy cost. As seen with their thermoregulatory ability, it is likely that different subspecies would have different water regulation abilities. African colonies could be adapted to more arid climates (i.e. higher VPD) and regulate humidity levels in the colony more effectively. In order to quantify these differences, colonies of different subspecies would have to be exposed to the same ambient conditions. Such a study could be conduced in the America's, as in the abovementioned temperature studies, were the range of Africanized and European honeybee overlap.

4.6 Patterns of nest humidity in diverse contexts

Nest humidity was recorded in honeybee colonies in diverse contexts: in different subspecies, different ambient climates, at different times of year, in different hemispheres and in different nests structure (i.e. polyurethane and wood hives and natural nests). Humidity does indeed vary in the nests of honeybee colonies in different contexts, however, the median RH in the brood nest of all the colonies occurred between 50 and 60 % RH and VPD between 20 and 26 mb. These results, obtained using accurate humidity sensors and high sampling intensities, show higher brood nest humidity than other records (Büdel, 1948; Oertel, 1949; Human et al., 2006). There is inter- and intra-colonial variation in humidity. Inter-colonial variation is possibly driven by a combination of external factors such as water and nectar supply, ambient climate and possibly internal factors such as the regulatory ability of different subspecies and colony status. Intra-colonial variation could be driven by changes in the amount of brood, nest ventilation and evaporation from the workers.

The recorded VPD and temperature in natural nests is both lower and more variable than in hived colonies. The RH was found to be between 60 – 70 %; higher than that in hived colonies. This is preliminary data and these differences may be due to the difficulty of locating the brood area in the natural nests. The loggers may have accidentally been placed on the periphery of the nest where VPD is lower.

Concluding remarks

This study has contributed to our understanding of water relations in honeybee colonies. The mean hourly brood nest humidity in a healthy honeybee colony does not exhibit a daily pattern and remains relatively constant throughout the day. A weak colony or one preparing to abscond exhibit nest microclimatic fluctuations that are similar to the ambient climate. This study also improves our knowledge of water relations in winter clusters: it supports the theoretical study of Omholt's (1987b) and shows that vapour pressure on the periphery of a cluster fluctuates substantially but does not exhibit the micro-fluctuations that are evident in a normally regulated colony. Although ambient temperature, humidity, wind, rain or solar radiation is not highly correlated with brood nest microclimate in a healthy colony, the amount of uncapped brood is associated with the nest humidity.

We are beginning to describe the patterns of humidity within a honeybee colony, however, little is known of the influence that moisture has on the thermodynamics of a nest. Thermal loss is influenced by the moisture in the nests of *Formica polytena* wood ants (Frouz, 2000) and vapour pressure gradients induce circadian adsorption and desorption cycles which stabilise temperature in the paper nests of *Vespa crabro* (Klinger et al., 2005). It would therefore be very interesting to understand the effect that moisture has on thermoregulation, especially in the context of an actively ventilating and metabolising superorganism such as a honeybee colony.

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CHAPTER THREE

Hygropreference and brood care in the honeybee (*Apis mellifera*)

Abstract

Terrestrial organisms need to limit evaporation from their bodies in order to maintain a homeostatic water balance. Owing to a large surface to volume ratio, arthropods are particularly susceptible to desiccation and have evolved behavioural and physiological mechanisms to conserve water. In social insects, water balance is also affected by the interactions between nestmates and by the architecture of the nest. For honeybees, humidity is particularly important for the brood because it affects the hatching success of eggs and because, unlike ants, honeybees cannot relocate their brood to parts of the nest with more favourable humidity. To advance the understanding of the water economy in honeybee nests, we investigated whether workers exhibit a hygropreference when exposed to a gradient of 24% to 90% relative humidity (RH) and whether the expression of this preference and their behaviour is affected by the presence of brood. The results show that young honeybee workers in the absence of brood exhibit a weak hygropreference for approximately 75% RH. When brood is present the expression of this preference is further weakened, suggesting that workers tend to the brood by distributing evenly in the gradient. In addition, fanning behaviour is shown to be triggered by an increase in humidity above the preferred level but not by a decrease. Our results suggest that humidity in honeybee colonies is actively controlled by workers.

Keywords: honeybee, *Apis mellifera scutellata*, hygropreference, relative humidity, brood, nest homeostasis

1. Introduction

The large surface to volume ratio of arthropods accounts for their susceptibility to desiccation through cuticular and respiratory transpiration (Hadley, 1994). However, several aspects of arthropod physiology and behaviour serve to counteract this consequence of their small body size. For example, some tick species are able to absorb water vapour from unsaturated ambient air (Gaede and Knülle, 1997) and individuals of some Collembola species are able to locate microenvironments with low vapour pressure deficits and hence reduce evaporative water loss (Hayward et al., 2000). In social arthropods, water balance is not only dependent on the physiology and behaviour of individuals, but is also affected by the interactions between colony members and by their nest environment. For instance, the nest architecture of some social insect species ensures that suitable microclimatic conditions occur in the nest (Scherba, 1959; Frouz, 2000; Kleineidam and Roces, 2000) thus making it possible for the workers to select certain areas of the nest for certain activities. Humidity based decision-making has been shown in leaf-cutting ants of the genus *Atta* (Roces and Kleineidam, 2000; Ribeiro and Navas, 2006), four species of fire ants from the genus *Solenopsis* (Potts et al., 1984), the wood ant *Formica rufa* (North, 1991), the meat ant *Iridomyrmex* sp. and the Argentine ant, *Linepithema humile* (Walters and Mackay, 2003). These studies have shown that ants prefer humidities of greater than 90% RH, and *Atta sexdens* and *Solenopsis* sp. relocate their fungus garden or brood to locations where the growing conditions are optimal.

Humidity is also an important microclimatic variable for honeybees *(Apis mellifera* L.) since their eggs require a relative humidity (RH) of above 55% to hatch successfully, with the highest survival between 90 – 95% RH (Doull, 1976). High humidity would also benefit brood development indirectly since the reproductive success of *Varroa* parasitic mites decreases with increasing humidity (Kraus and Velthuis, 1997). However, adult honeybee survival has been shown to decrease with increasing humidity (Woodrow, 1935) and the percentage of brood mortality caused by chalkbrood *(Ascosphaera apis)* was shown to increase by 7% when RH was increased from 68 % to 87% (Flores et al., 1996). Unlike ants, honeybees are unable to relocate their brood to the part of the nest most suitable for development. Indeed, eggs remain in the cell in which the queen laid them and develop in this same cell until emergence of the adult. Honeybee workers would therefore need to regulate humidity to optimal levels in the brood nest. There are a number of behaviours in the repertoire of honeybee workers that may be used to alter nest humidity. Ventilation of the hive

through fanning behaviour has been implicated in thermoregulation (Hazelhoff, 1954; Lindauer, 1961; Lensky, 1964) and carbon dioxide regulation (Hazelhoff, 1941; Seeley, 1974; Southwick and Moritz, 1987), but is also expected to influence nest humidity. Furthermore, nectar dehydration (Reinhardt, 1939) and water collection and spreading in the nest (e.g. Lindauer, 1954, Kühnholtz & Seeley, 1997) could be used to increase relative humidity.

Electrophysiological studies have demonstrated that coelocapitular sensilla located on the antennae of honeybees are stimulated by changes in humidity (Lacher, 1964; Yokohari et al., 1982). This shows that honeybees can detect fluctuations in humidity, but it is not known whether they alter their behaviour according to such stimuli or change the intensity or frequency of their behaviour. This study investigates whether honeybee workers exhibit a hygropreference when exposed to a humidity gradient of 24% to 90% RH, and whether the expression of this preference is dependent on the presence of brood. We also determined how different humidities affect fanning and general activity levels. We hypothesised that in the absence of brood honeybee workers would detect differences in humidity in different chambers and relocate to decrease their evaporative water loss. In contrast, in the presence of brood, we expected them to respond to suboptimal RH by fanning or by altering their activity levels in an attempt to improve developmental conditions.

2. Methods

2.1 Experimental animals and rearing conditions

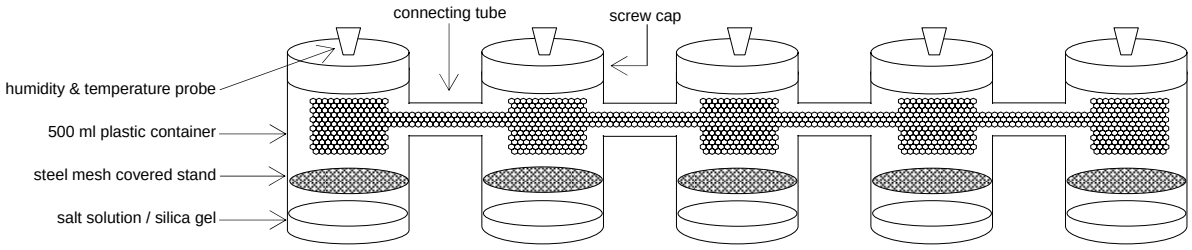
We used honeybee *(Apis mellifera scutellata)* workers from eight different colonies housed in the University of Pretoria apiary. A frame of brood was removed from each colony and placed into a Perspex box in an incubator at 60% RH and 35°C, which is the optimal temperature for brood development. Within 24 h of emergence, the workers were collected, placed in hoarding cages (dimensions: 90 x 100 x 70 cm) with *ad libitum* food (a sucrose, honey and pollen mixture) and water and returned to the incubator. Due to the age polyethism that is partially responsible for differentiation of tasks within a colony, hygropreference of social insects could vary according to age. Workers of 3 and 6 days old are normally involved in cell cleaning and brood tending respectively (Lindauer, 1952) and these age groups may respond differently to a humidity gradient. Freshly emerged workers from a single colony were therefore maintained in hoarding cages for 3 or 6 days and then tested for hygropreference

(n = 8 colonies tested per age) to determine whether age influences worker behaviour in a humidity gradient.

2.2 Hygropreference of workers without brood

Experimental trials were conducted in a dark climate-controlled room which was heated by two heater fans (Tempadait, Fan Heater, Johannesburg, SA) regulated by a thermistor (A419, Johnson Controls, Milwaukee, USA). Although insects are known to alter their hygropreference based on temperature (Haywood et al., 2001, Prange and Hamilton, 1992), we tested hygropreference of honeybee workers at a single temperature of 34.5 ± 0.5 °C since this is the temperature at which brood is reared and the temperature at which the experimental workers would be found within the nest.

Gradients of RH (as in Roces and Kleineidam, 2000; Walters and Mackay, 2003) were established in a set of five linearly arranged 500 ml plastic screw cap jars, interconnected with transparent tubing (length 5 cm, diameter 2 cm) and containing mesh covered stands to prevent workers from contacting the salt solution or silica gel (Fig. 3.1). Pieces of freshly drawn comb (10 by 20 cells) were placed in each container and connected by a strip of wax (length 9 cm, height 2 cm) placed in each connecting tube. This created a continuous vertical substrate for movement of bees from one chamber to another. The RH gradient was generated using silica gel (24% RH) and the following set of saturated salt solutions: 33% RH, $MgCl_2$; 51% RH, $Mg(NO_3)_2$; 76% RH, NaCI; 97% RH, $K_2Cr_2O_7$ (Winston and Bates, 1960). Although no volatiles are expected from the salts, they were substituted by the following combination after completion of half the trials in order to prevent bias caused by preference for a particular salt: 34% RH, NaI; 51% RH, $Na_2Cr_2O_7$; 71% RH, $NaNO_3$; 96% RH, K_2SO_4. Since the chambers were interconnected, gas exchange might occur between them and alter the expected humidity. To account for this effect, humidity was recorded in each chamber with a probe (SHT75, Sensirion, Zürich, Switzeland, ±1.8% RH, set to record every 2 s): no overlap of RH between chambers was recorded. Based on the measured values, chambers were termed the 24%, 40%, 55%, 75% and 90% RH chambers respectively (Fig. 3.1).



Chamber	24%	40%	55%	75%	90%
RH Gradient	Silica Gel 24 ± 3.4%	$MgCl_2$ 40 ± 6.0%	$Mg(NO_3)_2$ 57 ± 4.8%	NaCl 76 ± 3.8%	$K_2Cr_2O_7$ 90 ± 2.0%
	Silica Gel 24 ± 8.8%	NaI 39 ± 1.4%	$Na_2Cr_2O_7$ 54 ± 1.5%	$NaNO_3$ 72 ± 1.8%	K_2SO_4 89 ± 2.1%

Chamber	55%
RH Constant	$Mg(NO_3)_2$ 53 ± 3.4

Fig. 3.1 Five linearly arranged humidity chambers used to maintain two different sets of humidities; one setup with a gradient of 24 – 90% RH and another with 55% RH in all chambers. Two sets of salts were used in the 24 - 90% RH chambers to prevent bias caused by a particular salt. Values indicated are the measured % RH (±SD) in each chamber.

Before each trial, 100 workers were placed in a refrigerator and cooled down to facilitate handling. Twenty individuals were then introduced into each chamber and for each consecutive trial the sequence of introduction was alternated between the ends of the RH gradient. Workers were allowed to acclimate for the first hour which also ensured that the RH level stabilised after the disturbance created by opening the chambers. Observations were carried out every 30 min for the subsequent 3.5 h after which the distribution of workers stabilised. In order to determine the hygropreference of honeybees the number of workers in each chamber was recorded. If a worker was located in the tube between chambers, the direction of its head was used to indicate its preference.

The distribution of workers in linearly arranged chambers can be influenced by uncontrolled factors with individuals aggregating non-randomly in chambers at either end of the array. The occurrence of this bias can be excluded if workers distribute themselves randomly between chambers with identical humidity. We therefore measured the distribution of workers in a setup where a humidity of 55% RH (which frequently occurs in honeybee nests, Human et al., 2006) was maintained in all chambers using a $Mg(NO_3)_2$ solution (n = 5 colonies).

2.3 Hygropreference of workers in the presence of brood

It is possible that, like fire ants which fail to show a clear hydrokinetic response in the absence of brood (Potts et al., 1984), the behaviour of honeybees in a humidity gradient could be altered by the availability of brood. Since 6-day-old workers are more likely to perform tasks related to brood care (Lindauer, 1952), we did not test the hygropreference of 3-day-old workers in the presence of brood. We monitored the behaviour of 6-day-old workers exposed to eggs and larvae. Differences in behaviour between experiments in which workers were exposed to eggs or larvae was expected if workers respond to different desiccation rates of these brood types or if they display preference for one of these brood types based on age polyethism (Ribbands, 1953, p. 301). Trials (n = 4 colonies) were conducted in which ten eggs were grafted into the comb within each chamber and another set of trials (n = 4 colonies) using ten 1st to 3rd instar larvae. Grafting enabled selection of brood from the relevant colony and standardization of the amount and developmental stage of the brood that was placed into the comb in each chamber. After grafting, workers were introduced into the chambers and after one hour of acclimation the distribution of workers was recorded as described above. At the end of each trial the brood was removed, and pieces of wax were changed every three to four trials.

2.4 The effect of humidity on fanning behaviour and worker mobility

We monitored the number of fanning workers per chamber and the number of actively mobile workers per chamber. Since we transferred workers into an artificial setup where few tasks can be performed some behaviours might not be expressed. We therefore monitored workers' mobility as a proxy for general activity level. Mobility was determined by counting the number of workers that were moving for longer than 2 s around the chamber or across the surface of the comb. Observations were made using a low power headlamp. Fanning and mobility observations were recorded as a percentage of the total number of workers in a particular chamber.

2.5 Statistical analysis

The mean percentages of live workers, fanning workers and mobile workers per chamber were calculated for all observations during the 3.5 h experimental period and these values were used for analysis. The mean mortality (±SD) was 1.3 ± 2.86% at the end of all replicates

and all replicates with a mortality exceeding 16% were excluded from analysis (n=4). Some colonies were tested more than once for a particular age and the data were averaged for each chamber and constituted one replicate. The effect of humidity on the distribution and behaviour of workers in the five chambers was determined using a Friedman ANOVA. Pairwise comparisons between chambers were calculated using a Wilcoxon Matched Pairs test (with Bonferroni correction). In order to determine whether age affects hygropreference, the mean number of workers per humidity (i.e. chamber) was calculated across the eight replicates for 3-day-old individuals and likewise for 6-day-old individuals. These mean distributions were compared using a Mann-Whitney U test in order to determine the effect of age on hygropreference. The same test was used to compare the mean distribution of workers in the presence and absence of brood to determine the effect of the availability of brood on hygropreference. The software STATISTICA version 7.1 (Statsoft Inc., 1996) was used for statistical analysis.

3. Results

3.1 Hygropreference of workers without brood

Data from the linear array of humidity chambers (24 to 90% RH) showed that the mean number of workers in each chamber did not differ significantly between ages 3 and 6 days (Mann-Whitney test: U = 9.0, N = 5, N.S.). The data for 3 and 6 days were therefore pooled and showed a non-random distribution of workers in the chambers with different humidities (Friedman ANOVA x^2 = 28.4, d.f. = 4, $p<0.01$; Fig. 3.2). In the absence of brood, the number of workers in the 75% RH chamber was significantly higher than in all other chambers (Wilcoxon matched pair test: $Z<15.1$, N = 16, $p<0.05$) and the number in the 90% RH chamber was significantly lower than all others (Wilcoxon matched pair test: $Z<16$, N = 16, $p<0.05$) except for the 55% RH chamber (Wilcoxon matched pair test: Z = 27, N = 16, N.S., Fig. 3.2).

The preference of the workers was not dependent on the position of the chamber in the linear setup since the distribution of workers between the five 55% RH chambers was not significantly different from random (Friedman ANOVA x^2= 8.16, d.f. = 4, N.S.).

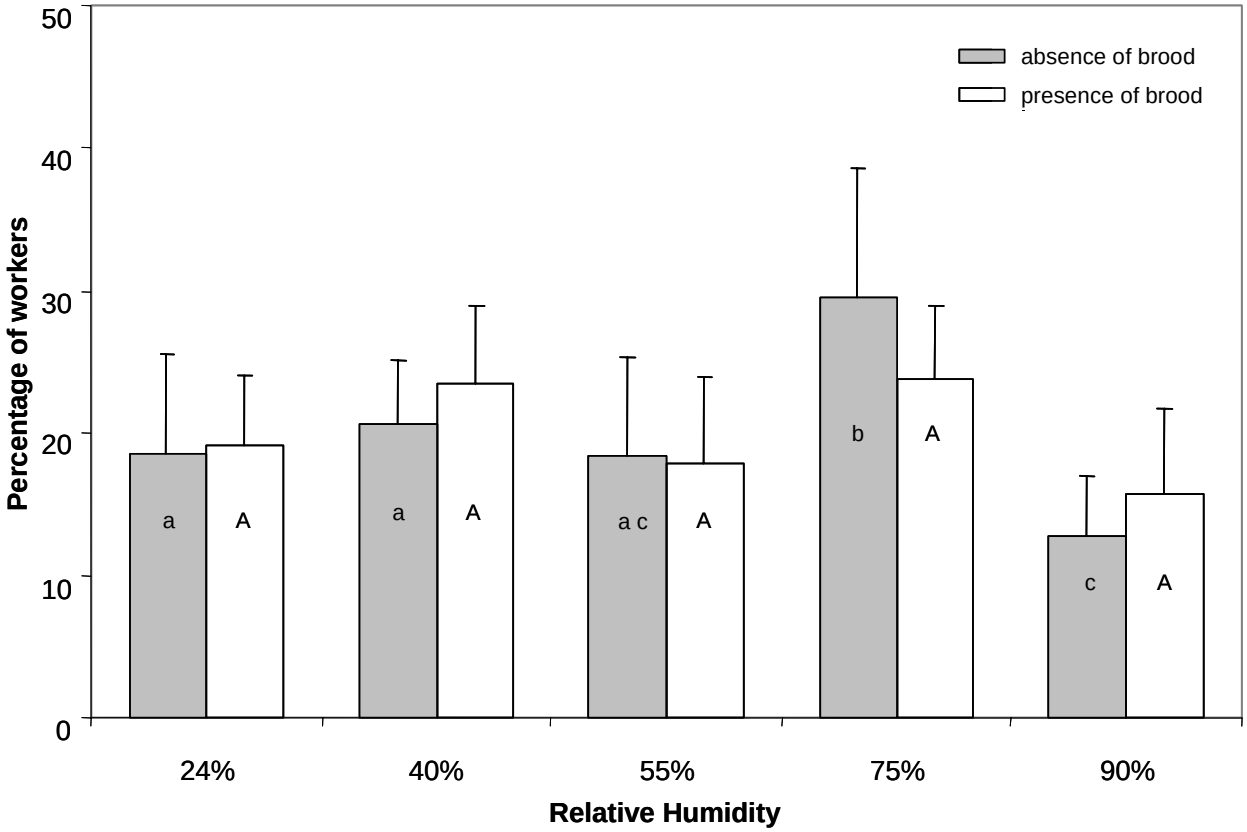


Fig. 3.2 The mean distribution of A. m. *scutellata* workers during the 3.5 h of exposure to a humidity gradient of 24-90 % RH in the absence (3 & 6 day old workers) of brood and the presence (6 day old workers) of brood. Means (±SD) for each humidity are presented (N=16) and letters indicate significant differences at $p<0.05$ (Wilcoxon Matched Pairs test). No significant differences were found between the number of workers in the different humidities in the presence of brood.

3.2 Hygropreference of workers in the presence of brood

The distribution of workers in the presence of eggs did not differ significantly from that of workers in the presence of larvae (Mann-Whitney test: U = 11.00, N = 5, N.S.). The data for eggs and larvae were therefore pooled and showed that workers were unevenly distributed among the chambers (Friedman ANOVA x^2 = 20.6, d.f. = 4, $p<0.05$). However a pairwise comparison of the chambers yielded no significant differences (Wilcoxon matched pair test: Z>3, N = 8, N.S., Fig. 3.2).

3.3 The effect of humidity on fanning behaviour and worker mobility

The distribution of fanning workers was not dependent on the position of the chamber in the linear setup since their number was not significantly different between the five 55% RH chambers (Friedman ANOVA x^2 = 5.17, d.f. = 4, N.S). This number was consistently low in

all chambers with a mean (±SD) of 0.3±0.1 workers fanning per chamber during an observation time.

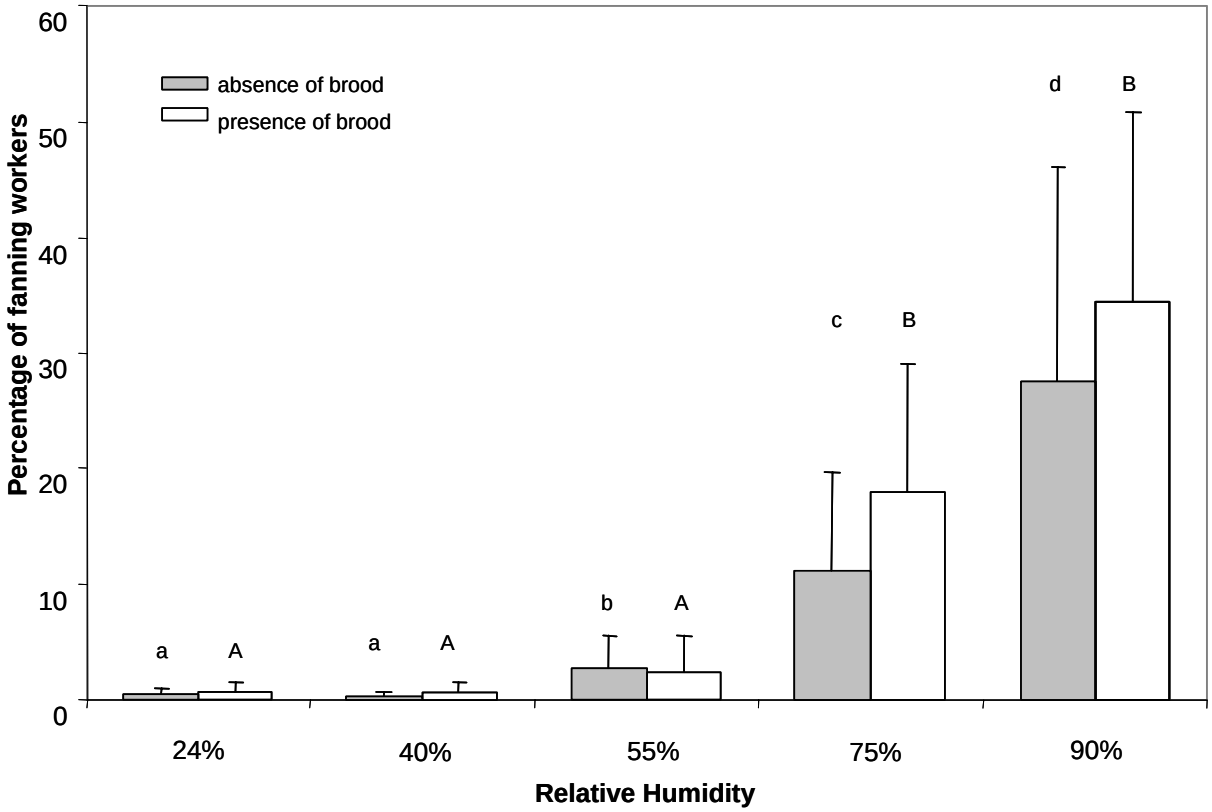


Fig. 3.3 Percentage of fanning workers given as mean (±SD) per chamber in a RH gradient (24 to 90% RH). Letters a - d indicate significant differences in the presence (6 day old workers) of brood and letters A - B indicate differences when in the absence (3 & 6 day old workers) of brood ($p<0.05$, Wilcoxon Matched Pairs test).

The number of fanning workers in the 24% to 90% RH gradient was strongly influenced by the humidity in the chambers (Friedman ANOVA $x^2 = 53.03$, d.f. = 4, $p<0.01$, Fig. 3.3) and showed a steady increase with increasing humidity. The mean (±SD) number of fanning workers in each chamber during an observation was 0.1±0.04, 0.1±0.03, 0.4±0.24, 2.9±0.77 & 3.1±0.91, from low to high humidity chambers respectively. When the chambers were tested pairwise, all chambers differed significantly in the number of fanning workers (Wilcoxon matched pair test: $Z>2.07$, N = 16, $p<0.05$) except for the 24% RH versus 40% RH chambers (Wilcoxon matched pair test: Z = 1.34, N = 16, N.S., Fig. 3.3). In the presence of brood, a Friedman ANOVA showed an uneven distribution of fanners amongst the chambers (x^2= 26.29, d.f. = 4, $p<0.01$, Fig. 3.3). However, a combination of pairwise tests showed that the 24, 40 and 55% RH chambers contained a significantly lower number of fanners than the 75 and 90% RH chambers (Wilcoxon matched pair test: $Z>2.52$, N = 8, $p<0.05$). The 24, 40 and

55% RH chambers and the 75 and 90% RH chambers did not contain significantly different numbers of fanners (Wilcoxon matched pair test: Z<2.24, N = 8, N.S.). The number of fanning workers in the presence of brood was higher in the 75 and 90% RH chambers compared to the number of fanners in the absence of brood, but this difference was not significant (Mann-Whitney test: U = 756.5, N = 40, N.S.).

The number of mobile workers differed significantly between humidities in both the absence (Friedman ANOVA x^2= 26.8, d.f. = 4, p<0.01, Fig. 3.4) and presence of brood (Friedman ANOVA x^2= 25 . 7 , d.f. = 4, p<0.01) and showed a consistent decrease with increasing humidity. A significantly smaller number of workers was observed to be mobile when in the presence of brood (mean ±SD, 3.9±0.5) compared to the absence of brood (5.1±0.9, Mann-Whitney test: U = 508.0, N = 40, p<0.01, Fig. 3.4). The number of mobile workers was not dependent on the position of the chamber in the linear setup since the distribution of workers between the five 55% RH chambers was not significantly different from random (Friedman ANOVA x^2 = 3.68, d.f. = 4, N.S.).

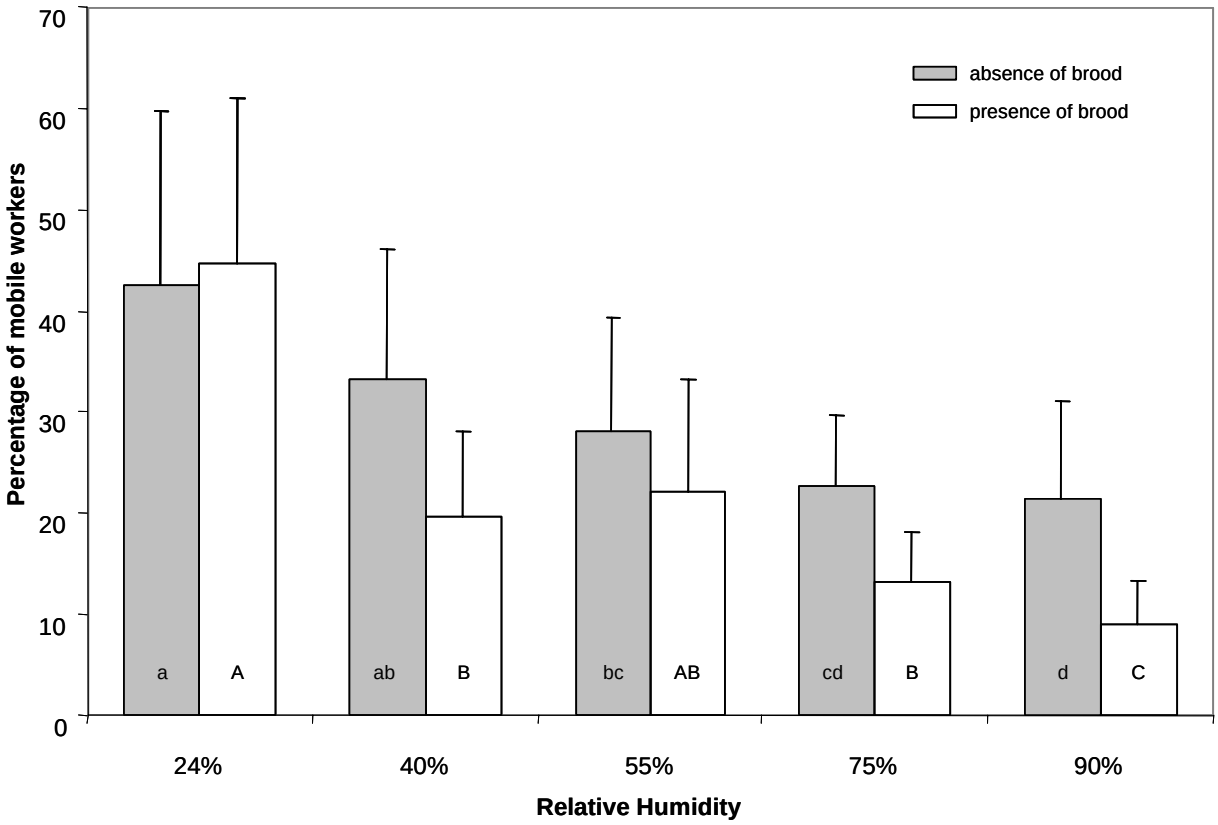


Fig. 3.4 Mean percentage (±SD) of mobile workers in a humidity gradient of 24 to 90% RH when in the absence (3 & 6 day old workers) of brood and in the presence (6 day old workers) of brood. Letters a - d indicate significance at p < 0.05 in absence of brood and letters A – C in the presence of brood (Wilcoxon Matched Pairs test).

4. Discussion

The results of this study show that young honeybee workers in the absence of brood exhibit a hygropreference for a humidity of approximately 75% RH at 34.5°C. When brood was present, this preference was expressed to a lesser degree and worker fanning and mobility levels were altered.

Control experiments with uniform RH showed that neither the distribution, fanning behaviour nor mobility of honeybee workers was dependent on the position of the chamber in the linear setup. All the differences in behaviour we observed were therefore due to differences in humidity. In the absence of brood, i.e. without the availability of tasks related to brood care that could lead to a differentiation of behaviour based on age polyethism, workers of 3 and 6 days of age showed the same humidity preference. This is in spite of the fact that these groups might have performed different tasks prior to their placement in the experimental setup. It is possible that, based on differences in humidity of the different nest regions (Human et al., 2006) where workers are active, individuals would show different hygropreference. Workers caring for brood (6 day old) and brood cell cleaners (3 day old) could have a preference for higher humidity compared to workers active in the drier region of nectar stores (Human et al., 2006). Due to workers being raised in cages their chronological age may have differed (3 vs 6 days) but their biological age, which is based on the physiological and behavioural interactions with other nestmates, may have been similar. In addition, we detected no difference in the behaviour of workers that were exposed to eggs or larvae, suggesting a similar sensitivity of these brood types to desiccation and no preference of 6-day-old workers for tending either of them.

Our results diverge from similar studies conducted on some ground dwelling ant species in that honeybee workers show a preference for a humidity of approximately 75% RH and not for an extremely high humidity (90% RH, Walters and Mackay, 2003; Roces and Kleineidam, 2000; North, 1991; Potts et al., 1984). The amount of water required to saturate the brood nest at 35°C is approximately 1.1 ml and this volume could evaporate from the abundant sources of moisture (e.g royal jelly, respiration of nest inhabitants, nectar) and saturate the nest's atmosphere with water vapour. The fact that such high humidity is not measured in hives (Human et al. 2006) and that a preference for a lower humidity level was detected in our study

suggests that it could be adaptive for honeybees to actively decrease humidity in the nest to a preferred level. Avoidance of high humidities could contribute to an increase in adult longevity and decreased microbial development. The results of this study suggest that a humidity of approximately 75% RH (higher than 55% and lower than 90% RH) is an optimal value in the brood nest. This value is higher than that measured by Human et al. (2006) in hives in the field during the dry South African winter, but comparable to other measurements in field colonies conducted simultaneously with the present study, in spring and summer. This discrepancy could thus be due to different measuring conditions (laboratory vs. field) or to seasonal or intercolonial variation in humidity regulation or preference.

These observations also differ from other similar studies in that the response of honeybee workers to a humidity gradient was weak, with a mean of 30% of the honeybee workers selecting the 75% RH chamber. By dispersing in the humidity gradient the individual workers curtail the benefit of clustering which could reduce water loss and ultimately metabolic work. It is likely that some characteristics of the experimental design (e.g. discontinuous gradients, absence of brood in the connecting tubes) limits the movement of bees between chambers and the expression of a strong preference. However, using similar designs, strong hygropreference was shown for many ant species with most of the workers gathering in the chamber with the highest humidity (North, 1991; Walters and Mackay, 2003). Rather than an experimental artefact, the weak preference observed could therefore correspond to a real biological phenomenon if honeybees can actively regulate humidity in their nest. Indeed, such regulation mechanisms would necessitate the dispersal of workers throughout a humidity gradient enabling them to actively counteract sub-optimal conditions where they occur. This idea is supported by the fact that fanning was consistently low below 55% RH and increased with relative humidity, indicating that this behaviour is aimed at removing humid air from the hive in a natural situation. Mobility increased with increasing vapour pressure deficit, which could also result from the workers trying to regulate humidity by other means than fanning, such as water collection and spreading.

In some ant species hygropreference is dependent on the presence of brood or fungal gardens (Potts et al., 1984; Roces and Kleineidam, 2000). In contrast, in the honeybee, the expression of hygropreference was further weakened when workers were exposed to brood. Although a Friedman ANOVA detected a non-random distribution of workers in our chambers with different RH, a pairwise test showed that these variations were not sufficient to result in

significant differences between the numbers of workers in each chamber. We hypothesise that workers dispersed throughout the experimental setup in an attempt to regulate humidity for the desiccation-sensitive brood that cannot be moved between chambers and that this resulted in an almost even distribution. This idea is supported by our observations that fanning activity by workers was higher in the presence of brood, albeit not significantly so. This suggests that the presence of brood further stimulates the workers to counteract adverse conditions by adjusting the humidity to optimal levels. In addition, significantly fewer workers were mobile in the presence of brood. This is likely to reflect the fact that the addition of brood resulted in some workers settling on the comb to care for the larvae or eggs. It is worth noting that the occurrence of fanning and mobile workers in the absence of brood shows that the presence of brood is not essential for workers to attempt to adjust adverse humidity conditions: the presence of other workers might be enough to trigger these behaviours.

In order to maintain stable nest homeostasis, honeybee workers are able to regulate various microclimatic parameters within the hive. For instance, when the brood nest temperature increases by 3 °C from 34 to 37 °C, the number of fanning workers increases ten fold (Lensky, 1964) and an increase in the CO_2 level in hives can cause a thirty fold increase in fanning workers (Seeley, 1974). Under our experimental conditions when all other factors are held constant, the number of fanning workers increases 10 times when humidity increases from 55 to 90% RH. This shows that a single behaviour, i.e. fanning, can affect several microclimatic parameters. In the same way, there are various behaviours involved in thermoregulation within the hive, such as water spreading and tongue lashing, which can also affect humidity. This can lead to the occurrence of tradeoffs in the adjustment of each optimum, as is evident in termite and ant nests (Korb and Linsenmair, 1999; Kleineidam and Roces, 2000) and can prevent honeybees from regulating some of these factors optimally (Human et al., 2006).

Several other factors are likely to influence humidity within the hive. Relative humidity is dependent on temperature and the thermoregulation of the colony is therefore directly linked to the relative humidity within the nest. Larval cocoons are hygroscopic and can provide a buffering effect on humidity fluctuations (Chauvin et al., 1979). Since larval cocoons accumulate in the cells in which the brood develops (Hepburn and Kurstjens, 1988), the physical properties of the comb can buffer humidity fluctuations (see chapter four). Water also evaporates from the nectar stores; however this source is seasonal and dependent on

floral availability and quality. Evaporative water losses from the bodies of the nest inhabitants can also increase nest humidity and the phenomenon of brood rearing in the winter cluster has thus been described as a strategy to reduce the water content of overwintering colonies (Omholt, 1987). Since so many factors influence humidity, detailed studies of their role and interactions will be necessary to understand the water economy of a honeybee hive. Our results provide the first demonstration that fanning can be triggered by an increase in humidity, suggesting that humidity is yet another microclimatic variable that is actively controlled by honeybee colonies. We suggest that the ability of honeybee workers to regulate this parameter weakens the expression of their preference for a particular RH.

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CHAPTER FOUR

Brood comb as a humidity buffer in honeybee nests

Abstract

Adverse environmental conditions can be evaded, tolerated or modified in order for an organism to survive. During their development, some insect larvae spin cocoons which, in addition to their protective function, modify microclimatic conditions, thus facilitating thermoregulation or reducing evaporative water loss. Silk cocoons are spun by honeybee *(Apis mellifera)* larvae and subsequently incorporated into the cell walls of the wax combs in which they develop. The accumulation of this hygroscopic silk in the thousands of cells used for brood rearing may significantly affect nest homeostasis by buffering humidity fluctuations. This study investigates the extent to which the comb may influence homeostasis by quantifying the hygroscopic capacity of the cocoons spun by honeybee larvae. When comb containing cocoons was placed at high humidity, it absorbed 11% of its own mass in water within four days. Newly drawn comb composed of hydrophobic wax and devoid of cocoons absorbed only 3% of its own mass. Therefore, the composition of the cocoons within comb may increase brood survivorship by maintaining a high and stable humidity in the cells, despite a lower humidity occurring in the nest atmosphere.

Keywords: honeybee, cocoon, larvae, water balance, nest homeostasis

1. Introduction

Many insects modify adverse microclimatic conditions rather than attempting to evade or tolerate them (Danks, 2002). For example, the larvae of numerous insect species spin cocoons which, in addition to their protective function (Otto, 1983), modify the local environment, thus facilitating thermoregulation or influencing water economy. The translucent cocoon of an arctic moth species acts as a micro-greenhouse by allowing sunlight to penetrate (Lyon & Cartar 1996). The larval cocoon of the moth *Tinea pellionella* reduces evaporative water loss (Chauvin et al. 1979). A similar effect was described by Nowbahari & Thibout (1990), who showed that the hygroscopic cocoon of the leek moth *Acrolepiopsis assectella* can absorb two thirds of its own mass in water. The presence of cocoons in this species was shown to have fitness implications in that it indirectly affects vitellogenesis and fertility in the adult. Cocoons also decrease water loss and increase survival in other invertebrates such as the spider *Mecynogea lemniscata* (Hieber, 1992), caddis larvae (Zamora-Münoz and Svensson, 1996) and the parasitoid wasp *Cotesia glomerata* (Tagawa, 1996). Epithelial cocoons of vertebrates are similarly known to prevent desiccation. Cocooned individuals of various Australian frogs exhibit 50 to 200 fold lower rates of evaporative water loss than non cocooned frogs (Withers, 1998).

Although it is not obvious, since they develop within the cells of their wax combs, honeybee *(Apis mellifera)* larvae spin a silk cocoon. The silk is formed in the labial glands of larvae as an a-helical fibroin of four strands which are coiled to form a silk thread (Rudall, 1962). This structure enables hydrophobic residues to be shielded in core positions and polar, charged residues to fill non-core positions, thus making the silk hygroscopic. The silk is ultimately incorporated into the cell walls (Jay, 1964). As an adult emerges from its cell, the cell is cleaned of debris before the queen lays another egg in it, but the silken cocoon remains. With successive generations, the cocoons therefore accumulate in a cell, eventually replacing the hydrophobic wax and causing the darkening of the comb (Hepburn & Kurstjens, 1988). The accumulation of cocoons in the thousands of cells used for brood rearing may have significant implications at the colony level. The cocoons could affect nest homeostasis by buffering humidity fluctuations and thus passively influencing the regulation of this parameter in the nest (Human et al, 2006). This study investigated the hygroscopic capacity of the cocoons spun by honeybee worker larvae. We hypothesised that an increase in silk content will cause an increase in the absorption capacity of the comb and a concomitant influence on nest homeostasis.

2. Methods

Nine pieces of light comb (i.e. containing no cocoons; Fig. 4.1a) and twenty pieces of dark comb (i.e containing silken cocoons; Fig. 4.1b) were selected from different honeybee colonies *(Apis mellifera scutellata).* The light comb had been used for nectar storage before being cleaned by the workers and dark comb had been used for brood rearing. These were cut into blocks of 8 by 8 cells and then placed for two months into a desiccation chamber containing silica gel. The chamber was placed in an incubator set at 35°C. The dry mass of desiccated combs was then determined with an analytical balance to 0.1g (Mettler Toledo, AG64, Switzerland).

After the desiccation period four light and five dark combs remained in the chamber containing silica gel. Humidity within the brood nest is often found to be around 60% RH (refer to chapter two and Human et al., 2006). For this reason, we placed five dark combs in a chamber containing a saturated salt solution of $NaNO_3$, generating 60% RH. With the presence of royal jelly or water in the cells, the cell's atmosphere is likely to be saturated with water vapour. To recreate these conditions, five light and ten dark combs were placed in a chamber containing a saturated salt solution of $K_2Cr_2O_7$, generating 90% RH. Relative humidity in the three chambers was recorded with two Hygrochron iButton data loggers (DS 1923, Dallas Semiconductor, USA) and a HOBO H8 data logger (Onset Computer Corporation, Pocasset, MA, USA). We thus confirmed that humidity was maintained at the required levels with little variation (silica gel: 1 ± 1.6% RH, $NaNO_3$: 60 ± 1.5 % and $K_2Cr_2O_7$: 90 ± 2.2% RH). The mass of each piece of comb was recorded every 12 h; care being taken that they were not exposed to ambient humidity for longer than 20 s. Microbial growth on the comb was observed after 120 h in the chamber with 90% RH and therefore the measurements of mass after 96 h were not used.

Wohlgemuth (1957) and Büdel (1948) mentioned that high humidity favours the growth of microorganisms on the combs. In order to monitor the development of such growth, combs were photographed with a digital camera from a height of 35 cm under controlled lighting conditions before being placed in the humidity chambers. Combs were left in these chambers for 9 days after the last weighing (on day 4) and were then photographed for a second time (on day 13). Since microbial growth modifies the appearance of the comb, the amount of

growth was assessed by measuring the mean brightness of each piece of comb, before and after exposure to the various humidities, using custom designed image analysis software. The mean brightness was calculated over an area covering seven entire cells (Fig. 4.1).

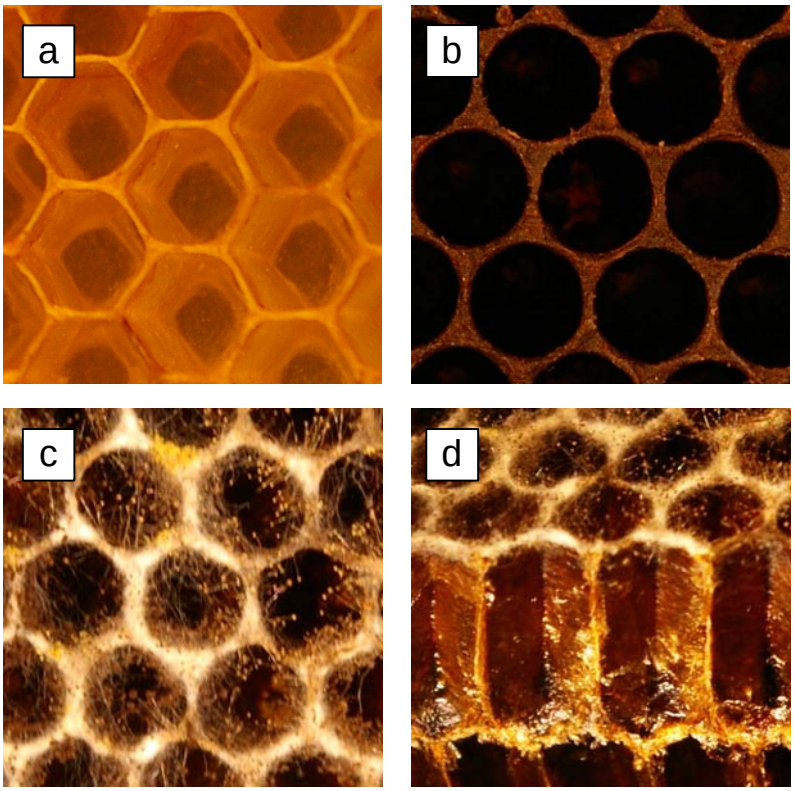

Fig. 4.1 a.) Light comb used for nectar storage and containing no larval cocoons **b.)** Dark comb used for brood rearing and containing larval cocoons **c.)** The dark comb shown in b. after being left in 90% RH for 13 days showing microbial growth **d.)** A cross-section through a dark comb after being left in 90% RH for 13 days showing microbial growth on the coping but not on the cell walls.

Mann-Whitney U tests were used to determine if differences existed between the water absorption capacity of light and dark comb and a sequential Bonferroni adjustment was applied due to multiple comparisons. Sign tests were used to determine if differences existed between the brightness of combs photographed before and after exposure and a Mann-Whitney U test was used to detect differences in brightness between light and dark comb. Data is presented as means ± standard deviation.

3. Results

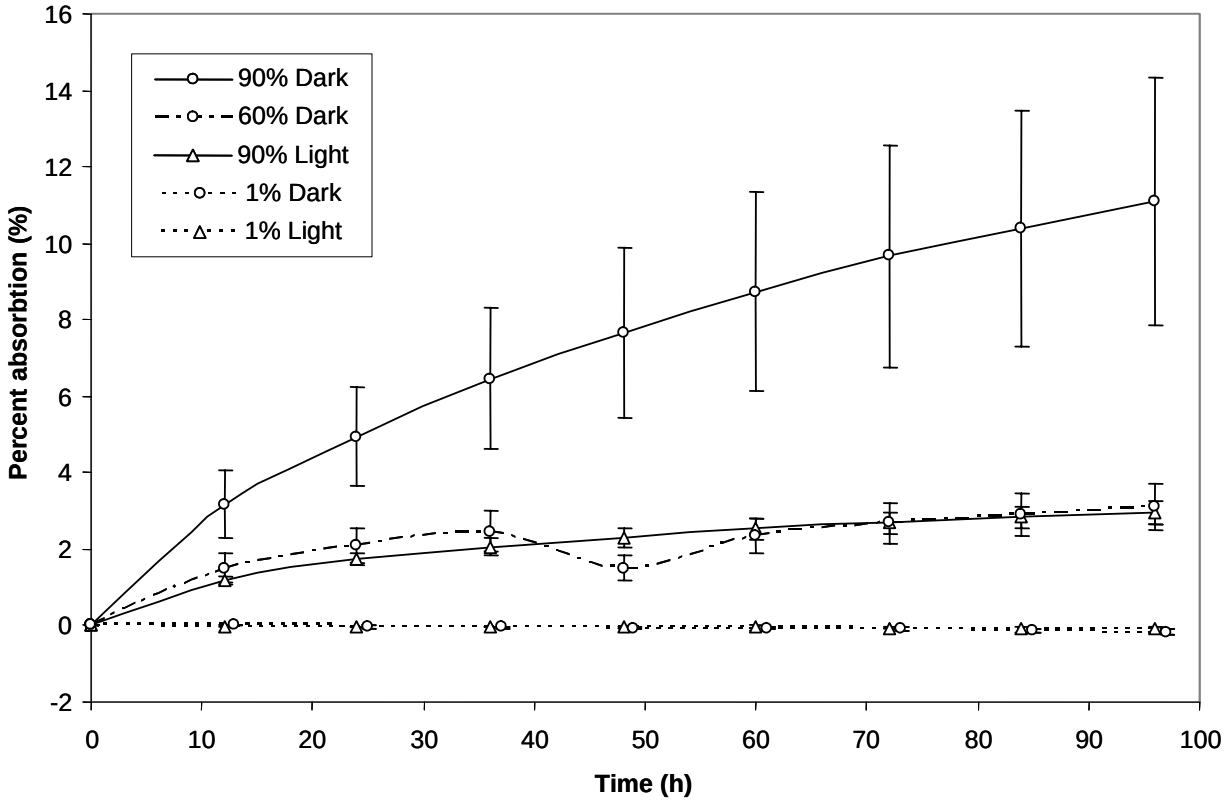

Fig. 4.2 Mean (±SD) water sorption by light (no cocoons) and dark (with cocoons) comb when placed in 1 %, 60% and 90% RH for 96 hrs, given as a percentage of the comb mass. Data points of the l% Dark comb are offset by 1 h for visual clarity.

When dark comb containing cocoons was placed in a 90% RH chamber for 96 h, it absorbed 11.1 ± 3.2% (0.4 ± 0.05 g) of its own mass in water (Fig. 4.2). The dynamics of absorption fits an asymptotic curve and half of the water was absorbed within the first 30 h. The decrease in absorption of the dark comb in 60% RH between 36 and 48 h (Fig. 4.2) was due to the lid of the chamber not sealing properly, causing the humidity to drop from 60 ± 1.5 % to 24.0 ± 0.51 % RH. At 48 h the lid was once again properly sealed and the comb resumed its increase in mass. After 96 h the dark comb had absorbed significantly less water at 1 % RH than at 60 % or 90 % RH (Mann-Whitney U: $z<-2.6$, $p<0.017$, $n = 4$, Fig. 4.2). However the amount of water absorbed by the dark comb at 1% RH is not significantly different from that absorbed by light comb at 1 % RH (Mann Whitney U: $z=-1.47$, $p>0.05$, $n_{dark}=5$ $n_{light}=4$). When light comb was placed at 90 % RH for 96 hrs, it absorbed 2.95 ± 0.3 % (0.06 ± 0.01g) of its own mass in water which is significantly more than when at 1% RH (Mann Whitney U: $z= -2.45$, $p=0.025$, $n_{90\%}=5$, $n_{1\%}=4$) but significantly less than absorbed by the dark comb in 90% RH (Mann Whitney U: $z=3.06$, $p=0.01$, $n_{light}=5$, $n_{dark}=10$).

Photographs taken before treatment show that light comb was approximately five times brighter than dark comb (Mann-Whitney U Test: $p<0.01$, $z=4.24$, $u=0.00$ $u_{light}=9$ $n_{dark}=20$). There was no significant difference between the brightness of comb before and after 13 days of exposure to various humidities (Sign test: $z<1,78$, $p>0.07$, $n_{light1\%}=4$, $n_{dark60\%},=5$, $n_{dark1\%}=5$, $n_{light90\%}=5$) except for the dark comb placed in the 90 % RH chamber (Sign test: $z=2.85$, $p=0.00$, $n=10$). These combs became brighter as a result of microbial growth on their coping (Fig. 4.1c).

4. Discussion

When placed in high humidity, dark brood comb absorbed a mean of 11 % of the pre-desiccated comb mass of water. Microbial growth could be observed on these combs after 120 h exposure to 90 % RH. In contrast, under the same condition, freshly produced wax only absorbed a mean of 3 % of its mass in water.

The higher amount of water absorbed by dark combs suggests that the hygroscopic cocoons act as water reservoirs. The large standard deviations in the mean mass observed for dark comb in 90 % RH (Fig. 4.1) were not due to variation in absorption by individual combs over time but to consistent differences between individual combs. The disparity between these absorption curves is likely to be due to differences in age of the combs (i.e. number of cocoons) which was not controlled in our experiment. Our results also indicate that brood comb responds rapidly to changes in RH. When the 60 % RH chamber did not seal properly, causing the RH to decrease for 12 h, the comb lost 30 % of its total absorbed water. These results suggest that the hygroscopic cocoons spun by honeybee larvae can rapidly release the absorbed water and can therefore have a substantial buffering effect on humidity within the hive. This may be the reason why Berry and Delaplane (2001) found better survivorship of brood reared in older, darker comb. Light comb should have a low buffering capacity since it was shown to absorb 3 % only of its mass in water. Although this is a relatively small amount of water, it is nonetheless unexpected that a comb composed of hydrophobic wax would absorb any water at all. It is possible that hygroscopic sugar or pollen residues which remained on the comb subsequent to cleaning by the workers could be responsible for the slight absorption.

The magnitude of the hygroscopic effect of brood comb in the context of a colony can be calculated from our results: the pieces of dark comb used contained 64 cells (8x8) and absorbed 0.4 g of water when placed at 90 % RH. This implies that an established colony containing 14 140 to 23 000 brood cells (Otis & Wearing-Wilde, 1992; McNally & Schneider, 1992; Winston et al, 1981) lined with cocoons can store between 87.4 and 142.1 g of water. This amount is sufficient to saturate 2215 – 3603 litres of air at 35°C. Given that the mean volume of a colony of *A. m. scutellata* is 44 ± 14 litres (Schneider and Blyther, 1988), the quantity of water stored in the cocoons lining the cell walls is easily sufficient to maintain the hive atmosphere at high humidity.

High humidity of the nest atmosphere is, however, not beneficial to honeybees since it would prevent nectar evaporation during the honey preparation process (Reinhardt, 1939) and favours the growth of microorganisms on the comb (Wohlgemuth, 1957; Büdel, 1948). This is consistent with our observation of microbial growth on the brood comb when it is exposed to high humidity for more than 4 days. Microbial growth took place extensively on the copings of the cells, whereas little growth was observed within the cells (Fig. 4.1d). Absence of growth within the cell is unexplained, but is likely to be linked to the composition of the cell wall. Since the coping appears to be a good substrate for microbial growth, high humidity in the hive atmosphere is detrimental to colony hygiene. Fanning by honeybee workers, which expels damp air and replaces it with drier ambient air, is seemingly important in preventing this microbial growth and facilitating the evaporation of nectar (Ellis et al, 2008). This is consistent with humidity measured by Human et al. (2006) between brood combs which varied from approximately 40% to 60% RH; no growth was observed in our experiment at such low humidity levels.

Growth does not take place on the cell walls, even at 90% RH (Fig. 4.1d) and high humidity could therefore be maintained within the cell for optimal brood development. Indeed, Doull (1976) showed that brood requires a relative humidity of between 90 and 95% in order to hatch successfully. Such a microclimate within the cells would have no adverse effects on colony hygiene or nectar processing.

Exchange of air between the cell and hive atmosphere would decrease the cell's humidity due to the hive atmosphere being drier than the cell. This could occur passively through the cell opening or actively as a worker enters the cells to care for the brood. A worker's body is

approximately the same volume as a cell and would thus expel most of the air from the cell as it enters; this air would subsequently be replaced from the hive atmosphere as the worker exits the cell. Several factors could, however, ensure the persistence of high humidity within the cells. The jelly provided to the larvae as food has high water content (Dietz and Haydak, 1971) and may generate high RH in the cell. Workers are also known to spread water (when the temperature is above 32°C) onto the interior surfaces of brood cells, thereby increasing the water content within the cell (Lindauer, 1954). In addition, larvae and developing pupae release water through cuticular and respiratory evaporation. Furthermore, the internal surface area of a cell is approximately 235 mm^2, in comparison to the outer coping of the cell with a surface area of 2.1 mm^2 (calculated from Hepburn, 1986). This means that there is a substantially larger surface area from which water can directly evaporate into the cell atmosphere compared to the relatively small surface of the coping that is directly exposed to the nest atmosphere. The coping is constructed from wax and propolis (Ribbands, 1953) which further reduces evaporation directly from this surface.

We have shown that the honeybee brood comb absorbs a large amount of water and can release it when humidity decreases. Fanning by workers could ensure the hive atmosphere remains within a humidity range that is favourable for evaporation of nectar and prevents microbial growth, whereas high humidity necessary for brood development is maintained only where it is important, i.e. within the cell. The hygroscopic cocoons that are incorporated into the cell walls could play an important role in buffering humidity fluctuations. However, the direct relationship between brood survivorship and a comb's ability to absorb and release water remains to be confirmed. Humidity should be measured within the cells to confirm whether it is indeed elevated above hive atmosphere, thus favouring brood development. Elucidating the water economy of the colony will allow further understanding of the complexity of nest homeostasis regulation in honeybees.

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CHAPTER FIVE

General Conclusion

This dissertation describes some of the complexities of humidity and water relations in the homeostatic nest environment of *Apis mellifera* colonies. It provides evidence of both active and passive regulatory mechanisms responsible for the observed humidity patterns in honeybee nests. Earlier studies of nest humidity were challenging due to the low accuracy and large size of recording devices; however, these challenges are being overcome with the smaller modern conductance type humidity sensors (Human et al., 2006). Chapter two of this dissertation (after the introductory chapter) provided a detailed description of nest humidity on a daily, seasonal and two dimensional scale, with over 1.8 million humidity recordings. Chapter three then explored the possibility of active regulation of humidity by groups of workers and determines whether they exhibit a hygropreference. Chapter four examined a passive regulatory mechanism in honeybee nests by determining the hygroscopic capacity of the cocoons spun by the larvae. It then explained the apparent contradiction between the high humidity requirements of eggs and larvae and the observed low humidity in the brood nest of honeybee colonies. Here I elaborate on some key findings of this study and outline possible future research on colonial water balance.

1. Honeybee nest humidity in context

Honeybee colonies are self-organised and do not rely on a centralised control system (Seeley, 1995). In this self organised system the individual worker responds to the immediate conditions defined by her local environment without having a global understanding of the condition of the colony (Moritz and Fuchs, 1998). Therefore, on the basis of very simple rules, a complex homeostatic balance emerges as the result of seemingly "intelligent" cooperation. Regulation of humidity in the nest takes place by such a mechanism. Chapter two shows that the level of humidity in the brood nest is highly correlated with the amount of uncapped brood. This is not merely a consequence of evaporation from the desiccation sensitive brood but in chapter three, this study shows that the behavioural response of workers in a humidity gradient is dependent on the presence of brood. The hygropreference of workers in the presence of brood is weakened, suggesting that workers tend to the brood by distributing evenly in the humidity chambers. This response contrasts with some ant species

that relocate their brood to the chamber with the highest humidity (North, 1991; Walters and Mackay, 2003).

Although one might expect that humidity is maintained near saturation in the honeybee brood nest where the desiccation sensitive larvae and eggs are located, it is not. Vapour pressure deficit in the brood nest is high, resulting in a large evaporative capacity of the air. The high VPD will prevent the moulding of the comb (Wohlgemuth, 1957; Büdel, 1948), but could be detrimental to the brood. This apparent contradiction can be explained if a high RH is maintained in each brood cell. Honeybee larvae spin silk cocoons, which are subsequently incorporated into the cell walls of the wax combs in which they develop. Chapter four shows that the accumulation of this hygroscopic silk in the thousands of cells used for brood rearing may significantly affect nest homeostasis by buffering humidity fluctuations. This study shows in chapter four that comb that contains cocoons can absorb 11 % of its own mass in water, whereas newly drawn comb does not. Therefore, the composition of the cocoons within comb may increase brood survivorship by maintaining a high and stable humidity in the cells, despite a lower humidity occurring in the nest atmosphere. In a recent theoretical study, Humphrey and Dykes (2008) calculate the thermal conductance of honeybee comb and chapter four proposes a similar passive mechanism for the transport of water between comb cells. The comb in newly established colonies does not contain cocoons and a question, which arises from chapter four is whether such a colony will need to work harder in order to prevent brood desiccation.

2. Trade-offs in social insect nests

Microclimatic parameters in a social insect nest cannot be viewed independently and regulation of one parameter will affect another. Although temperature in the honeybee brood nest remains constant, the level of carbon dioxide varies in a cyclic fashion (Hess, 1926; Lindauer, 1954; Southwick and Moritz, 1987). Chapter two of this study, in support of Human et al. (2006), shows that nest humidity is not constant and in fact a cyclic pattern is evident even in the nest entrance. Although the brood nest could easily be saturated from the numerous sources of water in the nest, humidity fluctuates within certain limits and is seldom found above 80 % or below 40 % RH. Brood nest humidity does not show a daily pattern even though ambient temperature and humidity fluctuate substantially throughout the day. Surprisingly all colonies measured during this study, whether in Africa or Europe, have a

median RH in the brood nest of between 50 and 60 % and a VPD of between 20 and 30 mb. These findings indicate that although humidity is not regulated at a constant value, it occurs within limits which maximise a colony's energy expenditure. This expenditure is based on trade-offs between temperature, carbon dioxide and humidity regulation. If humidity is too low it may cause an increase in adult and brood mortality, if too high microbial growth may ensue and maintenance at a constant theoretical optimum would impede the regulation of temperature and carbon dioxide.

Another example of a trade-off that maximises the energy expenditure of a social insect colony is found in the harvester ant, *Pogonomyrmex occidentalis.* These ants clear all the vegetation in the immediate vicinity of their nests and this influences the time window for activity by increasing soil temperature: midday activity time is lost during summer when the temperature is above the ants thermal tolerance, but a substantial gain is evident during the cooler months (Bucy and Breed, 2006). The thermal trade-off due to the removal of shade leads to a net energy gain when calculated over all the seasons.

The regulation of respiratory gases in honeybee colonies is unlike that of many other social insects, in that it is internally and not externally driven. In *Macrotermes michaelseni* colonies, the regulation of respiratory gases is driven by a complex interaction between nest architecture and kinetic energy of the wind (Turner, 2001). To a lesser degree the natural convection induced by metabolism within the nest influences ventilation but not to the extent described by Lüsher's (1961) thermosiphon model. In essence, the termites expend energy to build the nest and then rely on external factors to drive ventilation. In the nests of the leaf cutting ant, *Atta vollenweideri*, ventilation is also driven primarily by an external factor (i.e. wind). These ants construct turrets on the central mound channels which probably enhance nest ventilation by viscous entrainment and by Bernoulli's effect (Kleineidam et al., 2001). The ventilation of honeybee nests is primarily due to active fanning by workers (Hazelhoff, 1954). Numerous authors have shown that fanning is used to regulate carbon dioxide and temperature in the nest (Hazelhoff, 1954; Seeley, 1974; Southwick and Moritz, 1987). Chapter three shows that fanning is also used to regulate humidity, which supports an internally and not externally driven ventilation system. This idea is further strengthened by finding that nest humidity is not strongly correlated with any ambient parameters, including wind direction and speed.

3. Future research

I began this thesis with a quote from Jean Giraudoux and now I can agree that water does certainly reveal much about the earth or at least one organism that inhabits it. However, in the case of the honeybee there is still much that needs to be revealed and brought to our very lips. I end this thesis with ideas for research that can fill some more gaps in our knowledge of homeostasis and water balance in honeybee colonies.

Little is known of the interaction between the moisture content and the thermodynamics of a nest. Thermal loss is influenced by moisture in the nests of *Formica polyctena* wood ants (Frouz, 2000) and vapour pressure gradients induce circadian adsorption and desorption cycles which stabilise temperature in the paper nests of *Vespa crabro* (Klinger et al., 2005). It would therefore be very interesting to understand the effect that moisture has on thermoregulation in honeybees, especially in the context of an actively ventilating and metabolising colony.

This study has shown that humidity can be actively decreased in the nest, specifically by fanning, but it is not known whether workers attempt to actively raise the level of humidity in the nest. This could be achieved by behaviours such as droplet extrusion and water spreading, although low humidity may not be a problem in the nest due to the numerous sources of water. For instance, when concentrating nectar from 20 % to 82 % workers must evaporate 0.75 g of water for every 1 g of nectar collected (Nicolson, 2009). Although some preliminary data was collected during this study, there is no conclusive evidence that honeybees attempt to actively raise the level of humidity in the nest (Appendix E).

An interesting behaviour that influences the water balance of the colony is the uptake of condensed water at the nest entrance (personal observation). Workers were observed to drink large quantities of condensed water in the nest entrance of both a field colony and an observation hive in a climate room (with an artificially cooled entrance). These workers were then observed offloading their water load to workers within the nest. This mode of water gain could be the superorganisms' equivalent of the water recycling due to counter current heat exchange in the nasal passages of some mammals and birds (Huntley et al, 1984; Geist, 2000). It would be interesting to know under which circumstances and how often water condenses in the nest entrance and what quantity of water is returned to the colony by such activities.

The role of excretion in the water balance of honeybee colonies is not known. Workers of the giant honeybee, *Apis dorsata*, engage in mass defaecation flights which have been misinterpreted as "yellow rain" (Marshall, 1986). These flights are timed so as to minimise the negative effects of disturbing the protective curtain of bees which surround the exposed comb of these bees (Woyke et al., 2004). In cool conditions, flights occur around midday, when the ambient temperature is highest, to prevent cooling the nest. In contrast, at high ambient temperatures the flights occur in the cooler morning and afternoon hours to prevent overheating of the nest. During these flights the workers excrete 20% of their body mass (Mardan and Kevan, 1989). Although these mass flights probably serve numerous purposes, quantification of the amount of water excreted is important in furthering our understanding of colonial water balance.

Indeed there are still questions to be answered on colonial water balance and homeostasis of honeybee colonies. However, when discussing the honeybee dance language the great ethologist and Nobel Prize winner Karl von Frisch said, "A question answered usually raises new problems, and it would be presumptuous to assume that an end is ever achieved" (von Frisch, 1974). To me, this statement epitomises the joy that is to be found in science.

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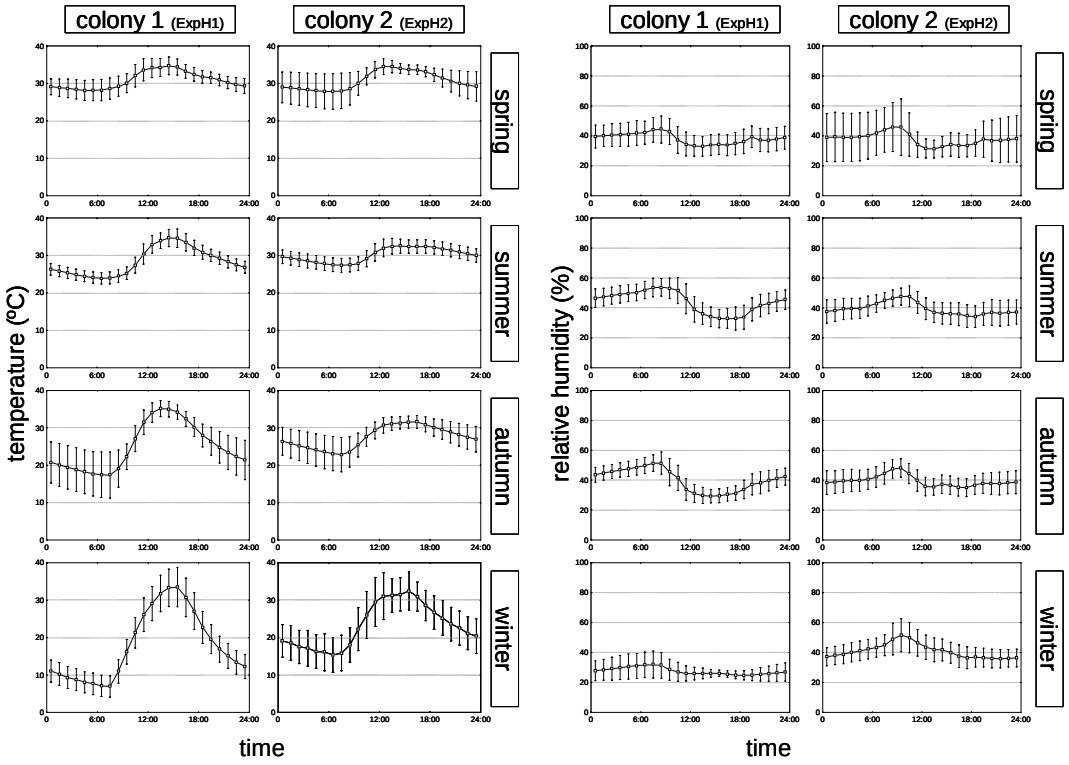
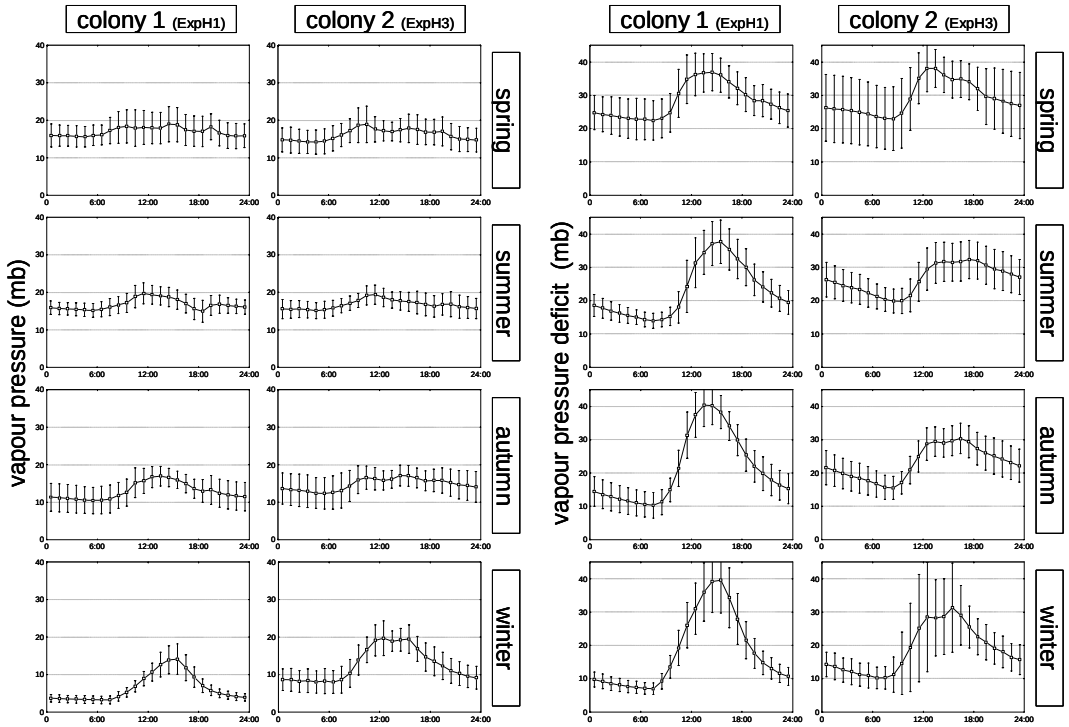
APPENEDIX A

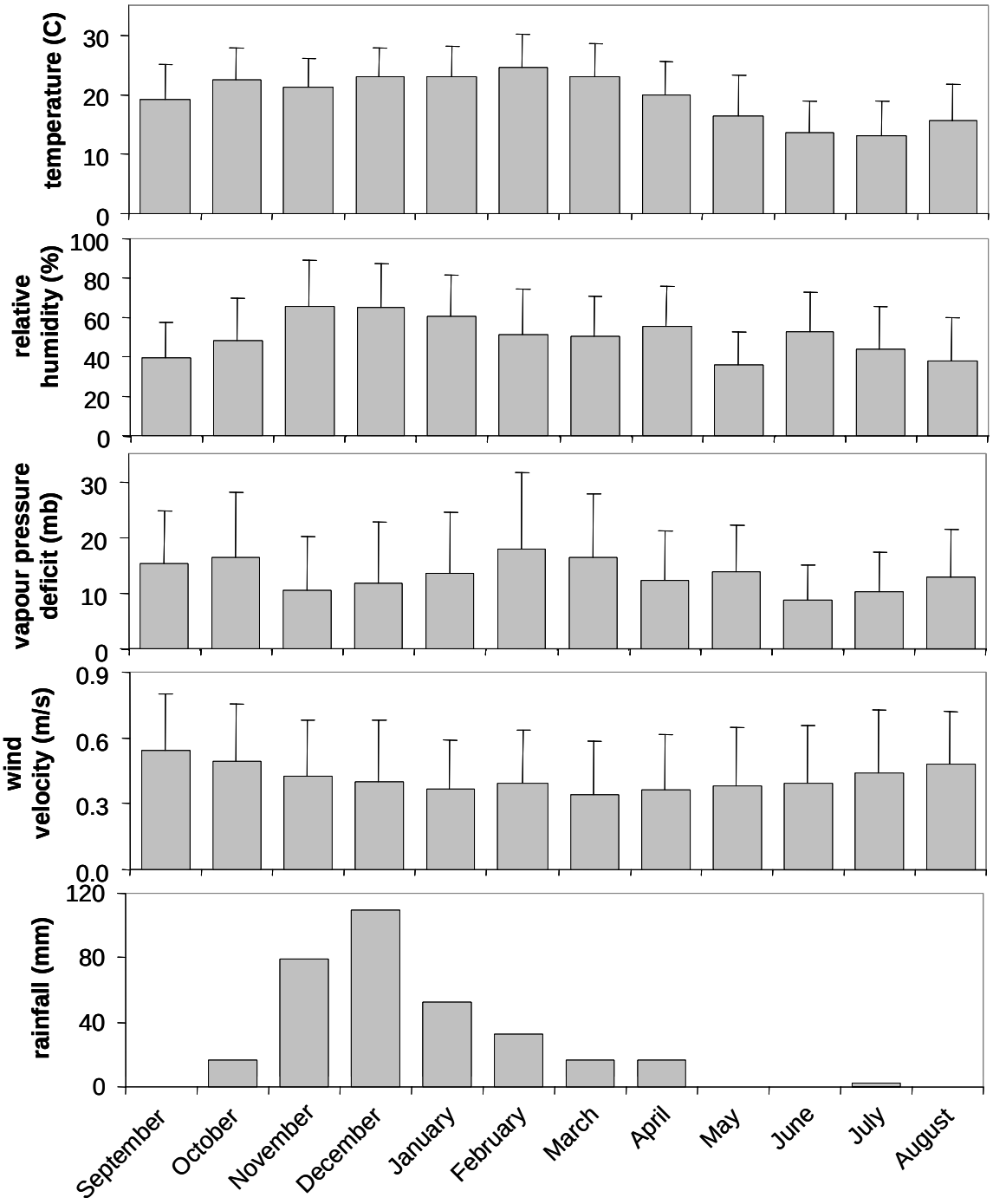
Daily Temperature and humidity patterns per season in the nectar store of colony 1 (ExpHl) and 2 (ExpH2). Each point represents an hourly mean ± SD of measurements taken at 12 min intervals over a three month seasonal period. Grey bars indicate night time.





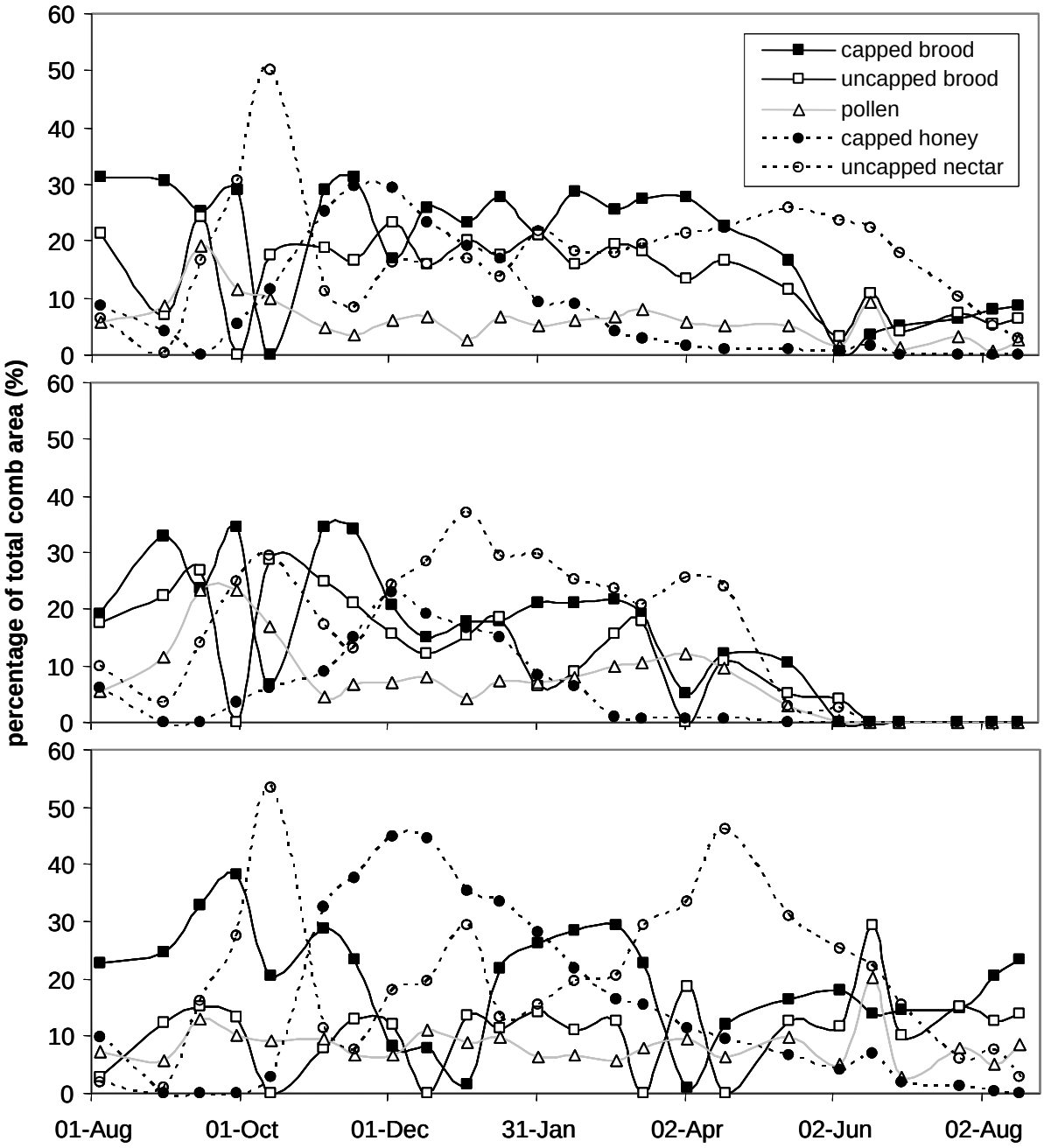
APPENEDIX B

Monthly weather patterns (mean ± SD) and total monthly rainfall at the University of Pretoria apiary from September 2006 to August 2007. This time period coincides with the recording of temperature and humidity in the nests of three honeybee colonies (ExpH1,2&3) in the apiary.



APPENEDIX C

The colony condition of three *A. m. scutellata* colonies in the University of Pretoria apiary from August 2006 to September 2007: ExpHl (top), ExpH2 (middle) and ExpH3 (bottom) Colony condition is given as a percentage of total comb area and divided into utilisation categories (Capped brood, uncapped brood, pollen, capped honey and uncapped nectar).



APPENEDIX D

Recording dates, devices, placement, sampling interval of all colonies used to investigate humidity and temperature patterns in honeybee colonies during this study.

colony	start date	end date	device	placement	sampling interval (s)
ExpH1	2006/09/01	2007/04/01	iButton & Hobo	brood & nectar	720
ExpH2	2006/09/01	2007/04/01	iButton & Hobo	brood & nectar	720
ExpH3	2006/09/01	2007/04/01	iButton & Hobo	brood & nectar	720
BomaH4	2007/06/19	2007/06/22	Senserion	brood & nectar	15
FlaH1	2007/07/12	2007/07/17	Sensirion	brood & nectar	15
FlaH2	2007/07/12	2007/07/17	Sensirion	brood & nectar	15
FlaH3	2007/07/12	2007/07/17	Sensirion	brood & nectar	15
LaesoH7	2007/07/07	2007/07/14	iButton & Hobo	brood & nectar	180
LaesoH12	2007/07/07	2007/07/14	iButton & Hobo	brood & nectar	180
CYB34H	2006/07/15	2006/07/19	iButton	natural nest	90
CYB50H	2006/07/16	2006/07/20	iButton	natural nest	90
DEEPC2	2006/07/17	2006/07/21	iButton	natural nest	90
OLIRAN	2006/07/15	2006/07/19	iButton	natural nest	90
OLIWB	2006/07/15	2006/07/19	iButton	natural nest	90

Ambient climatic parameters (Mean ± SD) during recording of nest microclimate of the honeybee colonies used during this study.

		AMBIENT		
colony	subspecies	temperature (ºC)	relative humidity (%)	vapour pressure deficit (mb)
ExpH1	*A. m. scutellata*	19.7 ± 6.8	50.6 ± 23.2	13.4 ± 10.4
ExpH2		19.7 ± 6.8	50.6 ± 23.2	13.4 ± 10.4
ExpH3		19.7 ± 6.8	50.6 ± 23.2	13.4 ± 10.4
BomaH4		12.1 ± 12.9	36.8 ± 4.9	12.5 ± 11.5
FlaH1	*A. m.* (buckfast)	16.3 ± 3.2	81.9 ± 12.5	3.4 ± 3
FlaH2		16.3 ± 3.2	81.9 ± 12.5	3.4 ± 3
FlaH3		16.3 ± 3.2	81.9 ± 12.5	3.4 ± 3
LaesoH7	*A. m. mellifera*	no data	no data	no data
LaesoH12		no data	no data	no data
CYB34H	*A. m. scutellata* (natural nests)	20.8 ± 4.6	49 ± 15.5	13.8 ± 7.7
CYB50H		21 ± 8.1	51.1 ± 20.6	15.9 ± 13.8
DEEPC2		17.9 ± 5.4	55.9 ± 15	10.4 ± 6.7
OLIRAN		19.8 ± 4.8	47.7 ± 15.1	13.3 ± 7.1
OLIWB		19.8 ± 6.5	46.2 ± 19.4	14.9 ± 10.4

Details of the natural honeybee nests studied in the Kruger National Park, South Africa, including specifications of nest entrance size, dimensions, volume, coordinates and site. Colonies used for temperature and humidity measurement are marked with an asterisk (*).

Colony name	latitude	longitude	nest site	no. of entrances	entrance size (cm^2)	propolis (cm^2)	entrance orientation (º)	breadth (cm)	length (cm)	volume (l)
BAOBE3	S22 33' 49.5	E31 11' 37.8	*Adansonia digitata*	1	11.3	518.8	120	-	-	-
BAOBE5	S22 25' 10.7	E31 16' 56.4	*Adansonia digitata*	1	6.2	27.1	-	-	-	-
BAOBEE	S22 24' 57.1	E31 16' 41.4	*Adansonia digitata*	1	-	131.9	40	-	-	-
OLIRAN*	S24 00' 12.7	E31 44' 30.9	*Acacia nigrescens*	1	1.5	37.6	250	24	215	97
OLIWB*	S23 59' 54.6	E31 43' 56.5	*Sclerocarya caffra*	2	8.8	46.7	90 & 350	30	62	44
SATABI	S24 23' 57.5	E31 47' 03.7	-	1	3.6	28.2	90	21	88	30
CYB50H*	S24 24' 28.1	E31 46' 29.1	*Sclerocarya caffra*	2	4.6	16.5	40	28	100	62
CYB38H	S23 56' 00.6	E31 38' 23.0	*Colophospermum mopane*	1	1.8	25.0	120	-	-	-
CYB24H	S23 18' 20.6	E31 30' 06.5	*Combretum imberbe*	2	2.4	9.1	10	24	80	36
CYB34H*	S23 59' 49.9	E31 45' 07.3	*Sclerocarya caffra*	3	6.9	48.6	190	30	122	86
CYB62H	S23 53' 16.1	E31 32' 30.5	Rock crevis	-	-	-	-	-	-	-
CY122H	S24 00' 47.9	E31 36' 21.1	*Colophospermum mopane*	3	-	10.6	50 & 220	23.5	193	84
CY100H	S23 56' 08.5	E31 42' 36.9	*Diospyros mespiliformis*	1	3.4	17.2	90	22.5	90	36
CY112H	S23 19' 17.8	E31 30' 05.6	*Combretum imberbe*	2	-	14.5	40	15	67	12
DEEPC2*	S24 59' 33.1	E31 35' 22.3	Dead tree-trunk	2	-	-	100 & 200	15	66	12
Mean (±SD)					**5.0 ± 3.2**	**71.7 ± 138.1**		**23.3 ± 5.3**	**108.3 ± 53.8**	**49.9 ± 30.8**

APPENEDIX E

When is wet too wet and dry too dry: a honeybee colony's response to unfavourable nest humidity

Most organisms can sense whether the atmosphere surrounding them is wet or dry. Many of these organisms will relocate if conditions are unfavourable and will negatively affect their survival or that of their young. Some organisms are able to alter the humidity in their immediate surroundings: humans can switch on a humidifier but is it possible for a honeybee colony to do something similar? This study aims at determining the response of a colony to being subjected to unfavourable nest humidity. This appendix presents preliminary results of two colonies at different environmental temperatures.

Methods and Materials

Experimental colonies

Two *Apis mellifera scutellata* colonies, each consisting of approximately 2500 workers and a viable queen, were obtained from the University of Pretoria apiary. The queens were marked and her wings were clipped to hinder absconding. Two brood frames and one honey frame were selected from each colony and placed with the workers and queen into an observation hive. Experimental trials were conducted in a dark climate-controlled room which was heated by two heater fans (Tempadait, Fan Heater, Johannesburg, SA) regulated by a thermistor (A419, Johnson Controls, Milwaukee, USA, ±1°C). One experimental colony was maintained at 35 °C and the other was maintained at 32 °C.

Observation hive

Experimental colonies were housed in a perspex observation hive with the internal dimensions of 45 x 480 x 720 mm, designed to hold three full size frames. The hive contained 11 holes (0: 10 mm) for placement of humidity probes (SHT75, Sensirion, Zürich, Switzerland, ±1.8% RH, interval: 30 s); three were placed in the centre of each frame and one between the frames. The top plate of the hive had 42 aeration holes (0: 3 mm) for inlet of gases from a buffering chamber directly above the hive (dimensions: 45 x 480 x 55 mm). The entrance of the observation hive was 35 x 120 x 15 mm and contained three holes for insertion of a Sensirion humidity probe, carbon dioxide sub-sampler (V1.0 sub-sampler, Sable Systems, Henderson,

USA) and a hot wire anemometer (HD2103.2 with AP471S2 probe, Delta Ohm, Padova, Italy, interval: 15 s). The sub-sampler was adjusted to sample air from the entrance tube at a flow rate of 200 ml/min and the carbon dioxide concentration was analysed with an infrared gas analyzer (Li6262, Li-Cor, Nebraska, USA, interval: 5 s).

Humidity adjustment

Hive humidity was adjusted with a flow through system (fig. 1), which was set to produce three different relative humidities (20 %, 50 % and 90 %). Using a pump (DDA-P 136, Gast, Benton Harbour, USA), air was drawn from outside the climate room, through a fibreglass wool filter. A gas flowrator (1003237N, Fisher and Porter, Workington, England) was used to ensure that a flow rate of 15 litres/min was maintained and this was calibrated using a bubble flow meter. Air was then passed into a coiled copper tube in a water bath with the temperature maintained at 37 °C with a heater element (R14013, Lauda, Germany). The air then passed through a silica gel scrub and/or a bubble humidifier and the mixing ratio between the two was controlled to enable the desired RH to be maintained. Prior to entering the hive, the air flow passted through an in-line humidity and temperature sensor (HMP41 with HMP42 probe, Viasala, Helsinki, Finland) to ensure that the correct input humidity was maintained. Colonies were exposed to the various humidities (20 %, 50 % or 90 %) at 15 litres/min for 3 h from 11:00 am to 14:00 pm.

Behavioural observations

The number of fanning and droplet extruding workers were counted and the amount of water spreading was observed. Workers were classified as fanning if they moved their wings rapidly while themselves being stationary. This behaviour was not confused with scenting (i.e. fanning with abdomen in the air). Droplet extruding workers were defined as those bees which rapidly extend and retract their proboscis to form a visible droplet of fluid between their mouthparts. The amount of water spreading was determined by counting the number of droplets in the hexagonal depression between capped brood cells.

The initial observations took place half an hour before (i.e. 10:30 am) the treatment (air flow at various humidities) was connected to the hive. This allowed determining the behaviour of workers in non manipulated situation. Observations stopped one hour after (i.e. 15:00 pm) the end of the treatment in order to follow the return to normal conditions. Observations took place every 10 minutes from 10:30 am to 12:00 pm and from 14:00 pm to 15:00 pm. From

12:00 pm to 14:00 pm observation were made every 20 minutes. Behavioural observations were recorded for a single side of the hive which was divided into 6 sections (120 by 450 mm), except for water spreading with was determined both sides. The behaviour of fanning workers in the entrance tube was recorded separately.

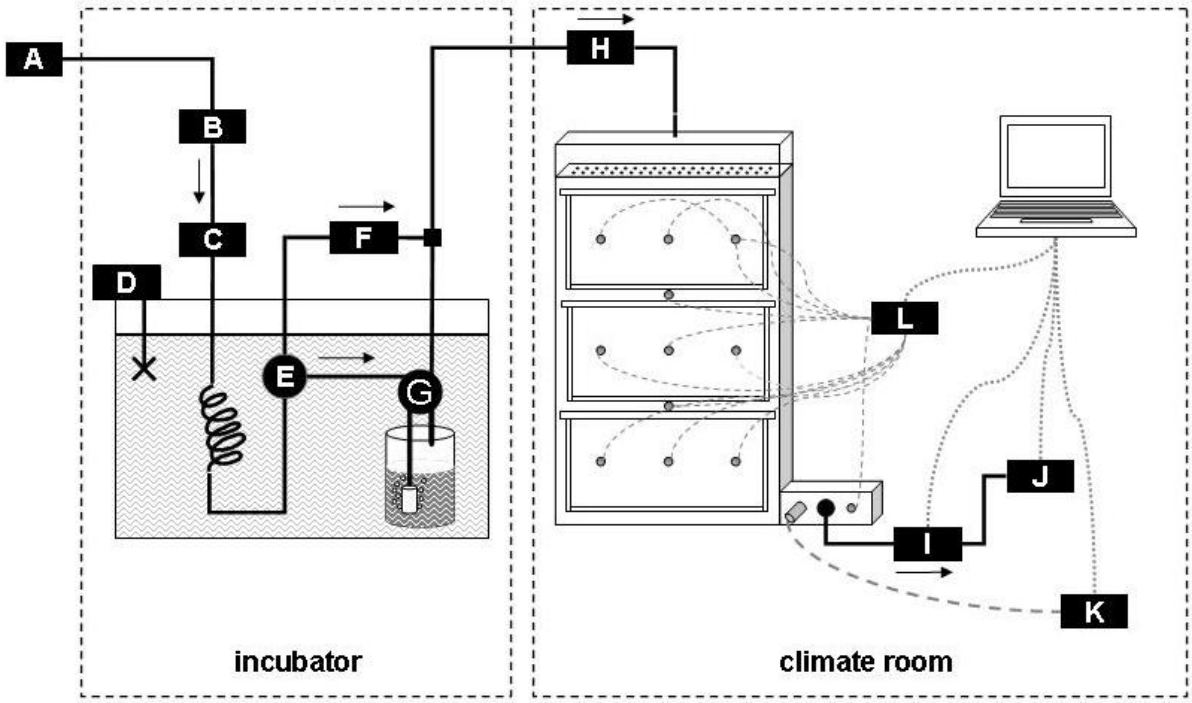


Fig. 1 Humidity in the observation hive was altered using a gas flow through system **A)** Fibreglass wool filter **B)** Air pump **C)** Flowrator **D)** Heater and stirrer **E)** Tap to control mixing ratio **F)** Silica scrub **G)** Bubble humidifier **H)** Inline humidity meter **I)** Sub-sampler **J)** CO_2 analyser **K)** Hot-wire anemometer **L)** Sensirion humidity sensor

A water collection point was placed 5 m from the hive entrance. Water collectors were labelled individually with small numbered and coloured tags (Opalithplättchen). When the colony was initially placed in the observation hive, some workers were trained to the water point to ensure that it was used exclusively for water foraging. A video camera (WV-CP242E, Panasonic System Solutions, Suzhou, China) was set to record at the water point. The number of water collectors was later counted by viewing the 3 min video clips: one of these clips every 15 min was observed. On 22 August 2007 and 28 August 2007 the entire days' video footage was observed and the number of collection visits recorded for each individually marked worker.

Results

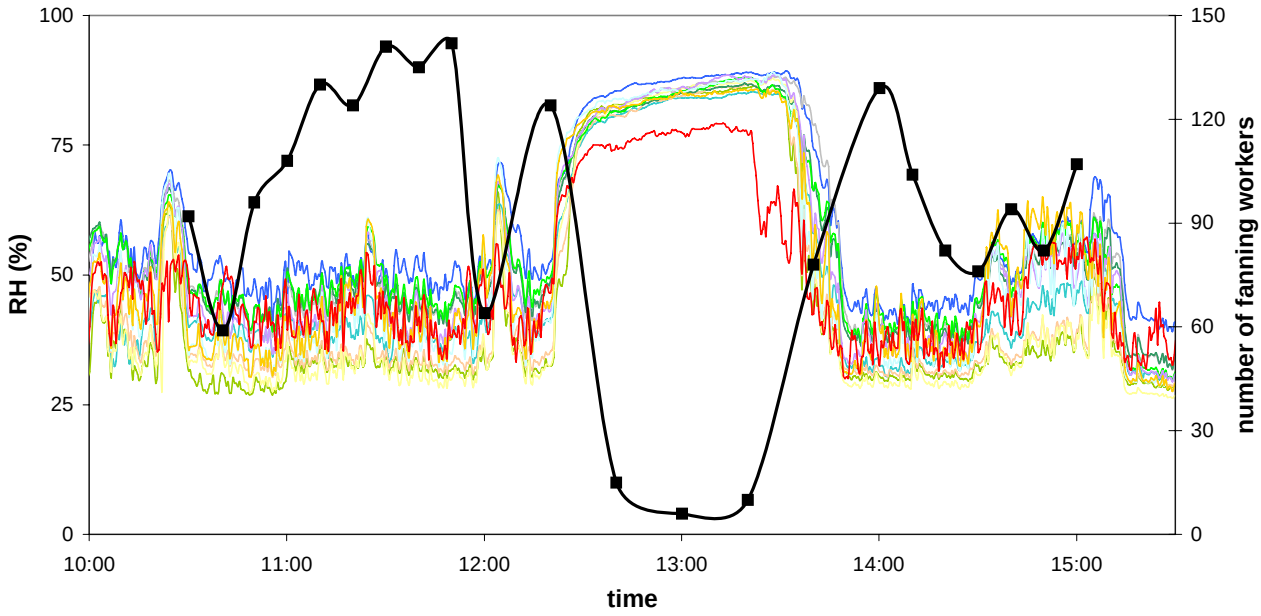


Fig. 2 An example of a 3 h experimental run at a room temperature of 35 °C and with a flow of 90 % RH air into the hive. This particular run shows an abnormal response during which the workers stopped fanning for over an hour (12:30 pm to 13:30 pm). The reason for the cessation of fanning is unclear but it caused hive RH to immediately increase to 80% RH. Humidity recordings were obtained by eleven humidity probes (grey lines) placed in the observation hive and the number of fanning workers (black line) was determined by observation. Arrows indicate start and end of flow through.

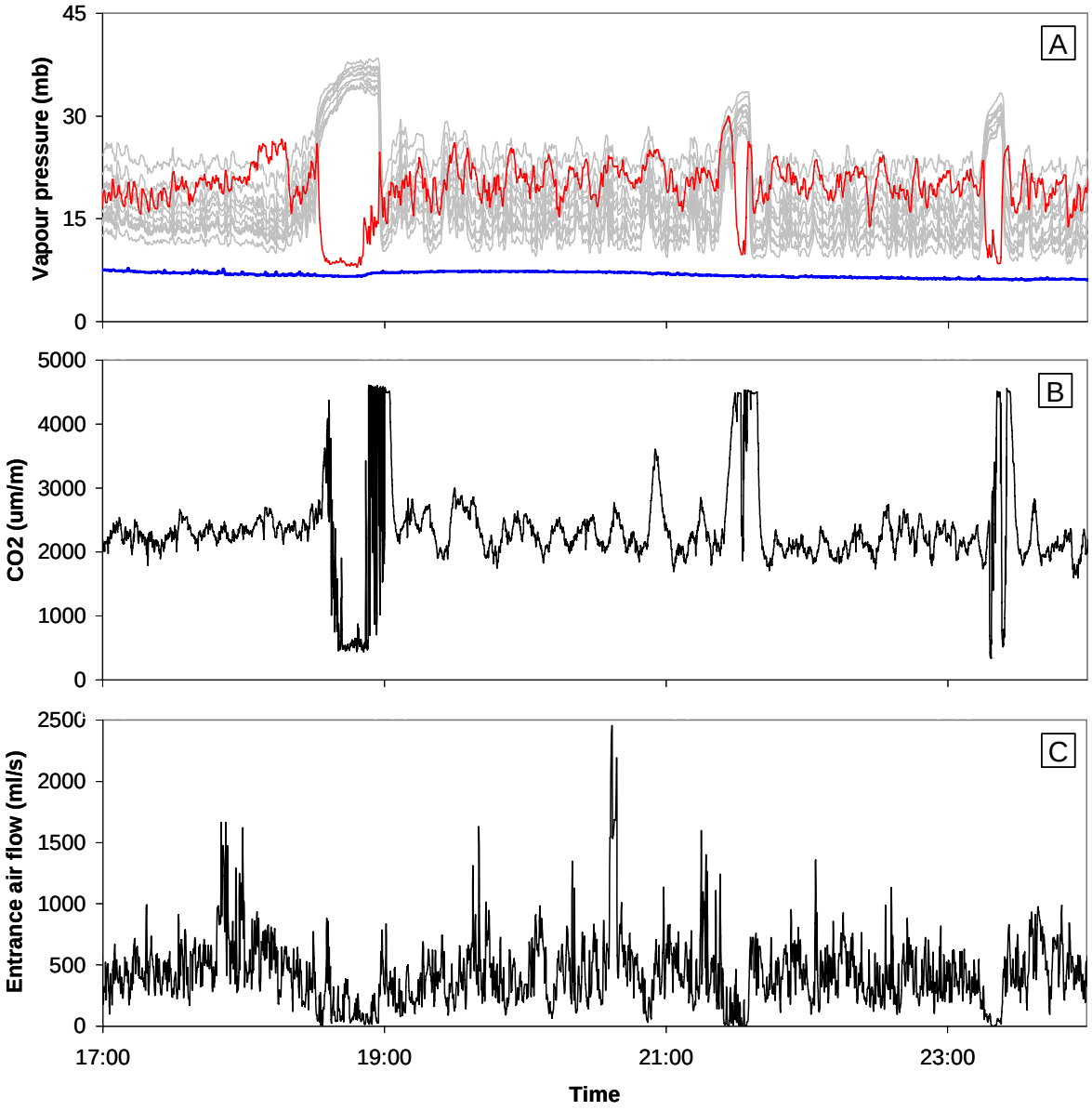


Fig. 3 A seven hour period of normal regulation, without the flow through system connected at a room temperature of 35 °C. A) Hive vapour pressure (mb) recorded from eleven sensors in the hive (grey lines), one in the entrance (red line) and one in the ambient air (blue line). Apnea phases are seen when entrance P_w approaches ambient and hive P_w increases. B) The CO_2 trace for the same period shows a Ca) peak before and after the apnea phase indicating increased ventilatory effort before and after apnea. C) Anemometer readings (ml/s) for the same period showing low flow rate during apnea.

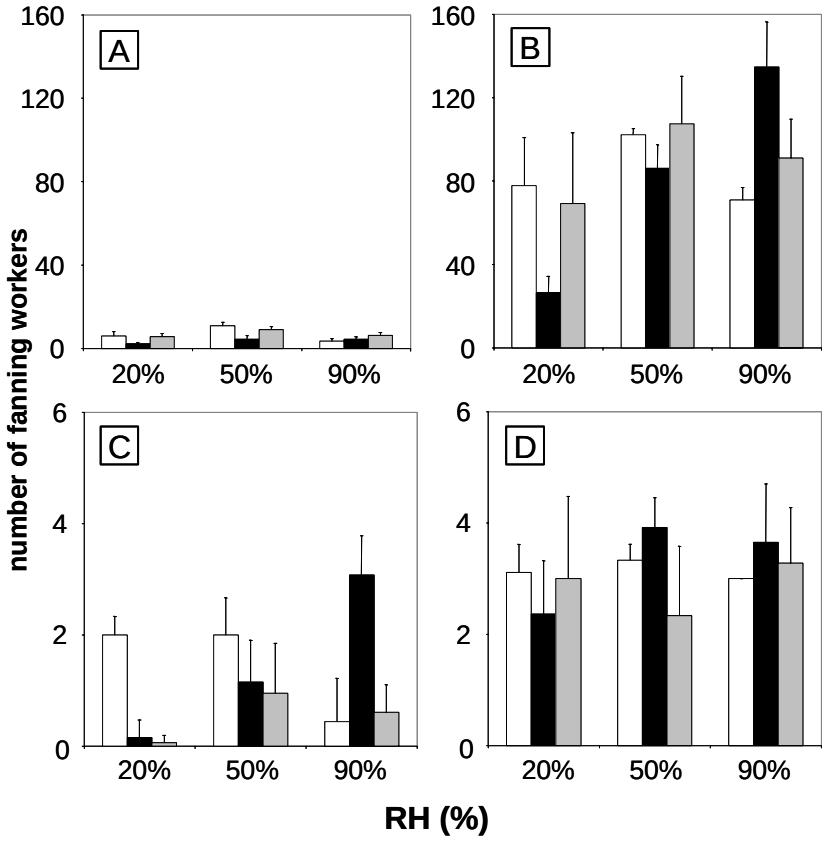


Fig. 4 The mean number of fanning workers (±SD) before (white bar), during (black bar) and after (grey bar) a 15 litres/min flow of 20%, 50% or 90% RH air was directed through the hive for 3 h. Each bar indicates the mean of three runs with the same colony. **A)** Hive fanners at a room temperature of 32 °C **B)** Hive fanners at a room temperature of 35 °C showing much higher numbers than at 32 °C and the black bars show an increasing number of fanners with increasing humidity **C)** Entrance fanners at a room temperature of 32 °C **D)** The number of entrance fanners at a room temperature of 35 °C reaches a peak that is indicated by the similar number of fanners during the 50 % and 90 % treatment. This could be due to the limited space available in the entrance tube.

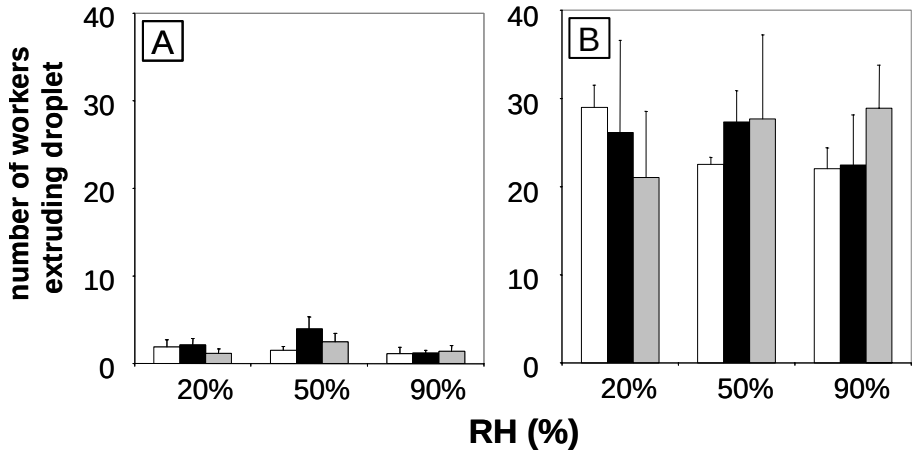


Fig. 5 The mean number of workers (±SD) extruding droplets before (white bar), during (black bar) and after (grey bar) a 15 litres/min flow of 20%, 50% or 90% RH air was directed through the hive for 3 h. Each bar indicates the mean of three runs with the same colony. **A)** At a room temperature of 32 °C **B)** At a room temperature of 35 °C. These preliminary data show that droplet extrusion is not depended on RH but, as supported by other studies, it is altered by changing temperature.

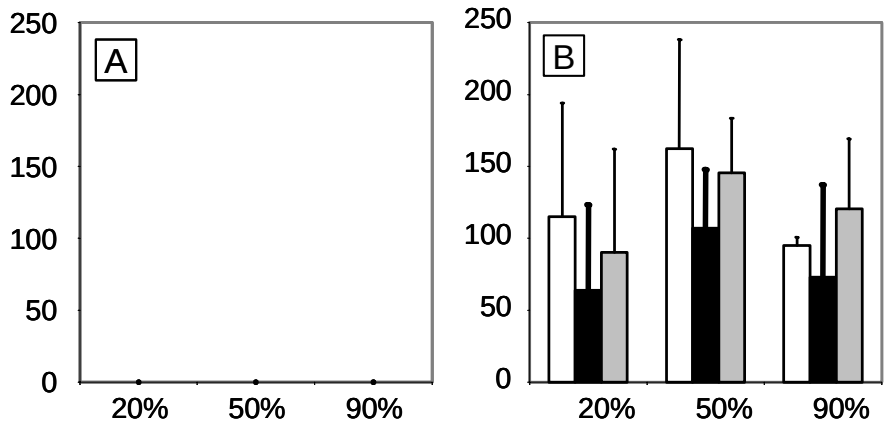


Fig. 6 The mean number of droplets of water spread into the hexagonal depressions of the brood comb, before (white bar), during (black bar) and after (grey bar) a 15 litres/min flow 01 20%, 50% or 90% RH air was directed through the hive for 3 h. Each bar indicates the mear of three runs with the same colony. **A)** At a room temperature of 32 °C **B)** At a room temperature of 35 °C. As with droplet extrusion, water spreading on the surface of the comb not dependent on RH but is depended on temperature.

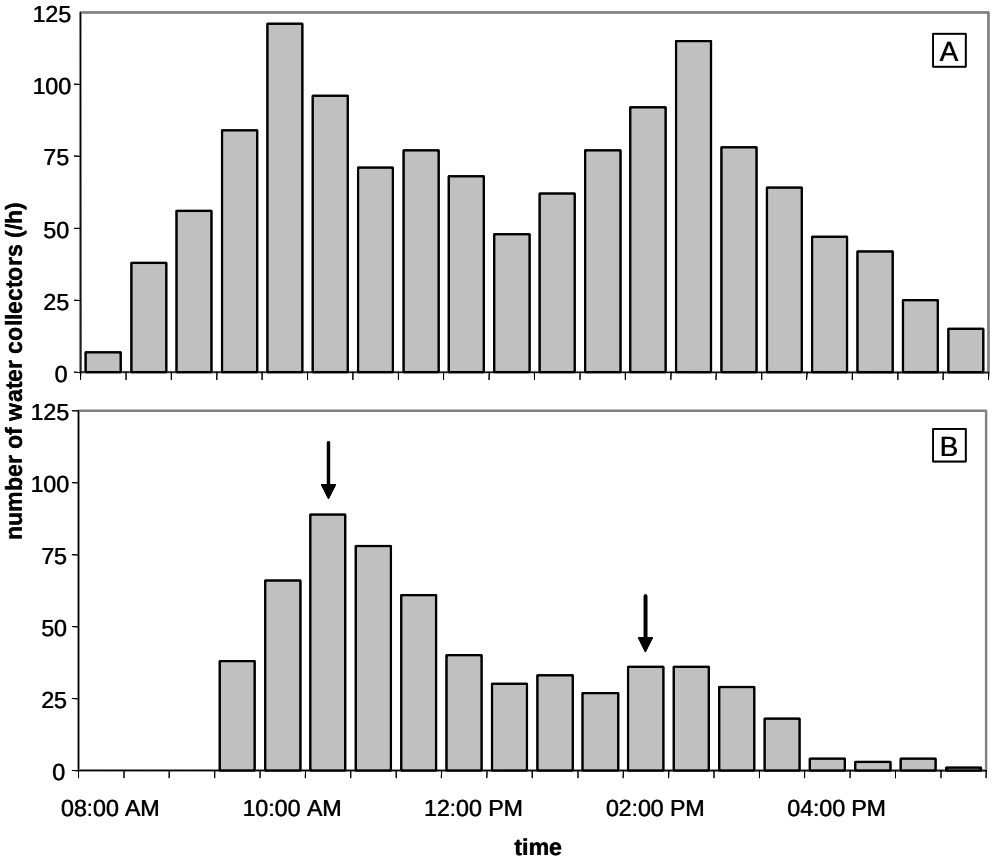


Fig. 7 The total number of water collectors that visited the water point given per hour on A) 22 August 2007 with no experimentally manipulated air flow into the hive, and B) 28 August with a 15 litres/min flow of 90% RH (room temperature: 35 °C) air into the hive (arrows indicate when the flow was turned on and off). The day with the flow through of 90 % RH air had a lower number of water collectors than the day without, however, this can not be taken as a conclusive difference.

Table. 1 The number of visits by individually marked water collectors to the water point on 22 and 28 August 2007. Only the six workers with the highest number of visits (lowest average cycle time) are presented for each day.

No flow (22 August 2007)				**80% RH flow** (28 August 2008)		
water collectors code	**no. visits per day**	**average cycle time (m)**		**water collectors code**	**no. visits per day**	**average cycle time (m)**
P-7	167	3.59		P-33	98	6.12
P-22	173	3.47		P-41	65	9.23
P-24	100	6.00		P-54	89	6.74
P-33	143	4.20		P-60	79	7.59
P-41	116	5.17		P-61	89	6.74
P-49	99	6.06		P-87	48	12.5

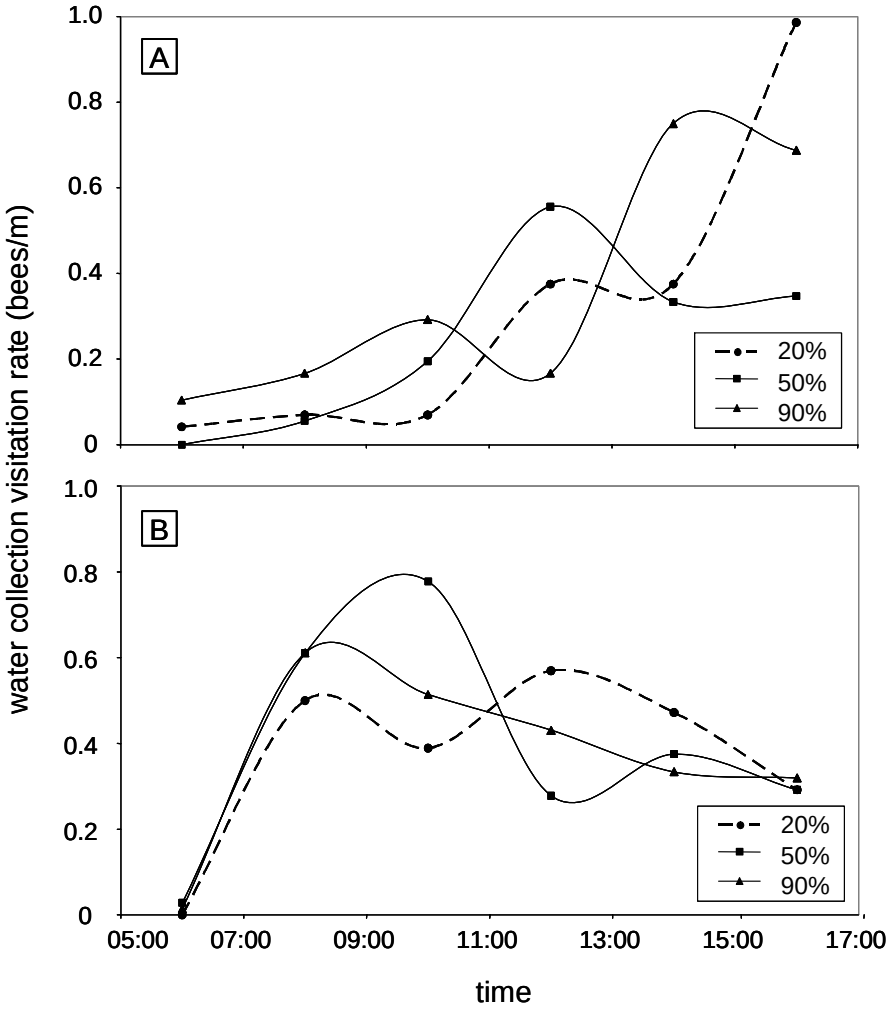


Fig. 8 The number of water collectors per minute observed at the water point. During these periods the colonies were maintained at a room temperature of A) 32 °C and B) 35 °C. Each point is an average over 2 h for three experimental runs at a certain input flow RH (20%, 50% or 90%). Water foragers peak in the morning at higher temperature (35 °C) due to the water stress incurred by a night in the hive but at low room temperature (32 °C) they only peak after the midday ambient high temperature. There does not appear to be an immediate or obvious water collection response to altering hive humidity with a gas flow though system.