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Sleeping in a society:

Social aspects of sleep within colonies of honey bees

(*Apis mellifera*)

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Sleeping in a society:

Social aspects of sleep within colonies of honey bees

(*Apis mellifera*)

by

Barrett Anthony Klein, B.S., M.S.

Dissertation

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Dedication

Without compromising the love and support that comes with being phenomenal parents,
Arnold Klein and Karen Anne Klein have become my closest friends and consistent
collaborators, cultivating and sharing my connection with art and nature.
I dedicate this work to them and to future scientists
curious enough to unveil sleep's secrets.

Acknowledgements

It is only appropriate that while investigating social aspects of sleep I depend on a large social community to help guide and support me, even if only to relieve me of my own sleep deprivation. I am grateful to so many who have inspired and aided my studies, including my collaborators, assistants, family and friends, committee members, and mentors.

My identical twin brother, Arno Klein, has worked closely with me on three of the four studies reported in this dissertation. Arno applied his programming prowess and keen sense of graphics to elucidate some of the data for transcription, analysis, and visual display. Arno has infused our work with a level of technical sophistication that would otherwise be lacking, and takes time away from his brain studies at Columbia University (http://www.binarybottle.com) to contribute to bee sleep studies. Everyone should have a twin.

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While at UT, I have had the opportunity to forge brief and more extended collaborations. I have had the excellent fortune of teaching alongside John Abbott for field biology, aquatic entomology, and many semesters of general entomology, and eagerly await the publication of our damselfly field guide. Ryan Taylor and Mike Ryan introduced me to anuran studies, paving the path for our research with faux frogs. I have enjoyed having John and Mike as committee members and eagerly anticipate future collaborations with both of them.

Ulrich Mueller and Larry Gilbert have served as my co-advisors. Spending time with Larry in the wilderness is an unparalleled experience and I am grateful to him for showing me Sirena and welcoming me to Austin. Someday I will follow his persistent piece of advice to study sleep in *Heliconius* butterflies. Joining Ulrich's lab was not exactly an act of spontaneity. Nearly 20 years ago I filmed Ulrich's ants at Cornell, and I almost accepted his invitation to begin graduate studies in Panama back in 1996. I have enjoyed Ulrich's friendship, myrmecological outings, and our sporadic two-member sleep discussion dinners. Ulrich has supported my independence and varied interests without reservation.

Many undergraduates have assisted me in my research at UT, in upstate New York, and in Germany. Kathryn Busby, Clairissa Dewstow, Kristin Fabry, Laura Still, Katie Saunders, Uma Bhat, Asmi Trivedi, and Melody Latronico have each taken time from their studies at UT to work with me and with the bees. Katie Olzsowy assisted me in upstate New York and Martin Stiegler was of critical help in Germany. Study-specific collaborations and assistance are listed below. In addition to human assistance, I have benefited from financial assistance from several generous sources. I have particularly benefited from several UT sources of funding, including Dorothea Bennett Memorial Graduate Research fellowships, University Continuing fellowships, a Carl Gottfried

Hartman Fellowship, UTopia Grant, and Texas EcoLab grants. My work is possible thanks also to a Deutscher Akademischer Austausch Dienst (DAAD) Fellowship, funding from Jürgen Tautz at the University of Würzburg, and a Doctoral Dissertation Improvement Grant (NSF).

Research is motivated and inspired in large part by the accomplishments and guidance of others. Walter Kaiser and Tom Seeley have each inspired this dissertation. Walter Kaiser established a foundation for honey bee sleep studies by conducting meticulous behavior and physiology research that has allowed me to investigate sleep in bees within the bees' social setting. Tom Seeley first learned of my interest in honey bee sleep in 1992, and has facilitated and guided my work throughout my Ph.D. I could not have asked for a kinder, more supportive, and more accomplished mentor to inform my research.

Chapter 1

This work was previously published in the Journal of Experimental Biology (Klein et al. 2008) with Kathryn Olzsowy, Arno Klein, Katharine Saunders, and Thomas Seeley. K. Olzsowy helped me to collect behavioral data during round-the-clock observations, Arno employed his programming skills to facilitate analyses, K. Saunders edited video footage and assisted with data transcription, and Tom hosted my stay at Cornell, assisted with beekeeping, and guided my experimental design.

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Chapter 2

I am presently writing this manuscript in collaboration with Martin Stiegler, Arno Klein, and Jürgen Tautz. Martin enthusiastically joined me to collect all behavior data during marathon recording sessions and continued work in the USA to transcribe thermographic data we had collected. Arno computed all spatial mapping and assisted with graphics, and Jürgen Tautz generously hosted and facilitated research operations for each of my three summer stays.

I thank the University of Würzburg and all members of the BEEgroup for facilitating our research. Dirk Ahrens-Lagast was instrumental as our skilled and creative beekeeper. Hartmut Vierle, Sven Mayer, Rebecca Basile and Marco Kleinhenz offered assistance and guidance, and Walter Kaiser paid a special visit to discuss our research. Uma Bhat, Kathryn Busby, Clairissa Dewstow and Laura Still helped transcribe data and Samuel Scarpino and Sabrina Amador-Vargas offered statistical advice. I am also grateful to Jon und Hanna Ahrens-Lagast for teaching me some German, Anja Weidenmüller and Christoph Kleineidem for their friendship, and the honey bees for tolerating my meddling. Financial support came from the Deutscher Akademischer Austausch Dienst (DAAD), National Science Foundation's Doctoral Dissertation Improvement Grant, The University of Texas at Austin's Program in Ecology, Evolution and Behavior, and the Environmental Sciences Institute.

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I am presently writing this manuscript in collaboration with Thomas Seeley, who introduced me to Cranberry Lake, to honey bee research, and helped me with the experimental design of this study.

Margaret Kirkland Wray was instrumental in helping record visits to the feeder and with other aspects of the field work. Alex Weir and Lawrence Rathman graciously hosted and facilitated our research at CLBS. Samuel Scarpino contributed statistics guidance. Texas EcoLab, and the University of Texas at Austin's Program of Ecology, Evolution & Behavior provided funding.

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Kathryn Busby, Clairissa Dewstow, Uma Bhat, Kristin Fabry, Laura Still, Asmi Trivedi and Melody Latronico transcribed dance information, and Martin Stiegler, Greg Cocquyt, Heather Doviak, Ekaterina Kravchenko, Aleksandra Savchenko, and Anastasiya Zykova assisted with field work. Joey Stein and Terry Watts helped to design the insominator and Norman Gary provided experimental design advice for tagging bees. Preliminary magnetic tests were conducted at Liddell Field Station in Ithaca, NY and Texas A&M University, thanks to Tanya Pankiw, Ramesh Sagili and G. W. Burlin. Alex Weir and Lawrence Rathman facilitated our research at CLBS. Samuel Scarpino, Sasha Mikheyev, Nathan Marti, and Claus Wilke each offered statistics advice. Thanks to Andrew Schneider and Dosha Miranda for additional contributions. NSF DDIG, Texas EcoLab, and the University of Texas at Austin's Program of Ecology, Evolution & Behavior provided funding.

Sleeping in a society:

Social aspects of sleep within colonies of honey bees

(*Apis mellifera*)

Publication No._____

Barrett Anthony Klein, Ph.D.

The University of Texas at Austin, 2010

Supervisors: Ulrich G. Mueller and Lawrence E. Gilbert

Sleep is a behavioral condition fraught with mystery. Its definition—either a suite of diagnostic behavioral characters, electrophysiological signatures, or a combination of the two—varies in the literature and lacks an over-arching purpose. In spite of these vagaries, sleep supports a large and dynamic research community studying the mechanisms, ontogeny, possible functions and, to a lesser degree, its evolution across vertebrates and in a small number of invertebrates. Sleep has been described and examined in many social organisms, including eusocial honey bees (*Apis mellifera*), but the role of sleep within societies has rarely been addressed in non-human animals. I investigated uniquely social aspects of sleep within honey bees by asking basic questions relating to who sleeps, when and where individuals sleep, the flexibility of sleep, and why sleep is important within colonies of insects. First, I investigated caste-dependent sleep patterns in honey bees and report that younger workers (cell cleaners and nurse

bees) exhibit arrhythmic and brief sleep bouts primarily while inside comb cells, while older workers (food storers and foragers) display periodic, longer sleep bouts primarily outside of cells. Next, I mapped sleep using remote thermal sensing across colonies of honey bees after introducing newly eclosed workers to experimental colonies and following them through periods of their adult lives. Bees tended to sleep outside of cells closer to the edge of the hive than when asleep inside cells or awake, and exhibited caste-dependent thermal patterns, both temporally and spatially. Wishing to test the flexibility of sleep, I trained foragers to a feeder and made a food resource available early in the morning or late in the afternoon. The bees were forced to shift their foraging schedule, which consequently also shifted their sleep schedule. Finally, I sleep-deprived a subset of foragers within a colony by employing a magnetic "insominator" to test for changes in their signaling precision. Sleep-deprived foragers exhibited reduced precision when encoding direction information to food sources in their waggle dances. These studies reveal patterns and one possible purpose of sleep in the context of a society.

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INTRODUCTION

"Insects do breath and sleepe."
-Pliny the Elder (77-79 C.E., translated by Philemon Holland, 1601)

"Since less highly evolved animals never stop reacting to the outside world, these animals rest but do not truly sleep."
-J. Allan Hobson (1995)

The question of whether or not insects "sleep," engage in a "sleep-state," "profound rest," are "quiescent" or simply "rest" is a long-standing problem. The concept is steeped in semantics and has been tested with varying rigor. For some, the sleep phenomenon is limited to very human-like, electrophysiologically-defined sleep stages. For others, the term sleep is loosely applied to organisms exhibiting what superficially resembles some aspect of a presumed sleep-state. Loose applications are not limited to the lay literature, but can be found in peer-reviewed scientific journals. The term sleep has been applied without definition, when impossible to verify, or when only mildly suggestive in form, as in the discovery of a dinosaur with an avian-like "sleeping posture" (Xu and Norell 2004). This unfounded and ungrounded case of fossilized slumber is one impetus for me to begin this introduction and each chapter that follows with a definition of sleep–so as to distinguish between vague versus more scrupulous applications of the term.

Sleep behavior is broadly defined by a suite of characters that include a species-specific posture, relative immobility, reversibility, and, most importantly, an increased response threshold to external stimuli (Piéron 1913, Flanigan 1972, Flanigan et al. 1973). Tobler (1985) included the criterion of compensation following sleep loss, lending a functional implication to the behavioral definition of sleep. The earliest work in insect sleep was observational and descriptive (e.g., Fiebrig 1912, Rau and Rau 1916, Schulze 1924, Rau 1938) (Fig. 1) and more recent research has examined insect sleep experimentally, with species-specific distinctions for cockroaches (Tobler 1983, Tobler

and Neuner-Jehle 1992), the fruit fly *Drosophila melanogaster* (Hendricks et al. 2000, Shaw et al. 2000), a species of paper wasp (Klein 2003), and the European honey bee, *Apis mellifera* (Kaiser and Steiner-Kaiser 1983, Kaiser 1988, Sauer et al. 2004). In Chapter 1 I discuss the behavioral definition of sleep and extend it to my study subject.

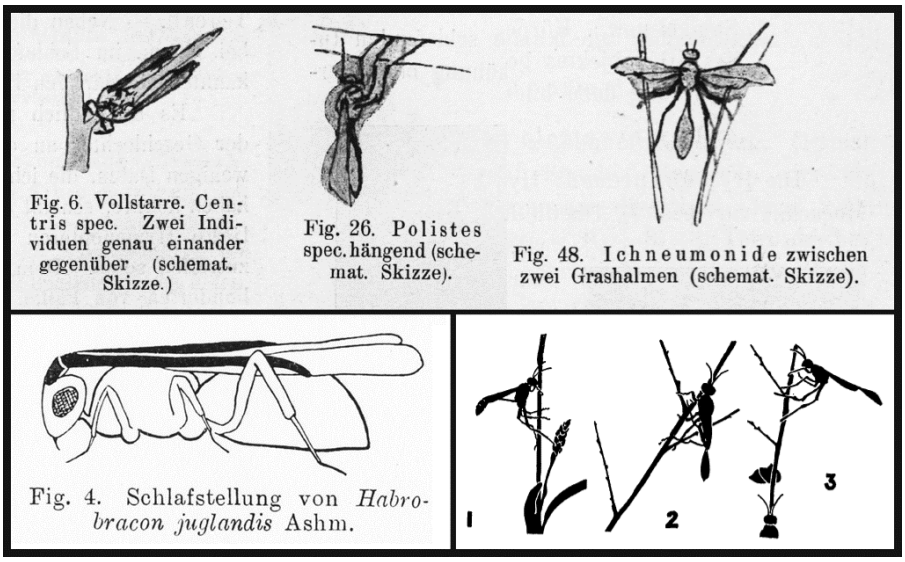

Fig. 1. Illustrations of sleeping insects, observed and described by Fiebrig (1912), Schulze (1924), and Rau (1938) (Figs. 6, 26, 48; 4; and 1-3, respectively).

Although sleep has been defined and measured in hundreds of species (Campbell and Tobler 1984, Rattenborg and Amlaner 2002), few studies have explored concepts relevant to sociality, despite the obvious and testable functions of sleep relating to division of labor, communication, or group predation avoidance. Sociality, or the combined properties and processes of social existence (Wilson 1975), plays an important role in nature–as is demonstrated by the number of phylogenetically diverse social species, and the sheer number of social organisms that have evolved ecologically influential predatory, agricultural, and architectural feats. By simultaneously examining aspects of sleep and sociality, new insights could emerge into organismal behavior, ecology, and evolution.

I chose to investigate social aspects of sleep within honey bees (*Apis mellifera* Linnaeus, 1758) by examining patterns of sleep within their colonies and addressing potential functions of sleep unique to their societies. As adult honey bees age, they change tasks, and my studies report changes in sleep duration, constitution and periodicity across these castes (Chapter 1). Older workers, for example, slept for longer uninterrupted bouts, slept with day-night periodicity, and slept more often outside cells. Younger workers slept arrhythmically and most often inside cells. Honey bees slept most during the first few days of adulthood, with no difference in total sleep duration among nurse bees, food storers and foragers. These results confirmed and extended our knowledge of ontogenetic sleep patterns in worker bees.

After establishing temporal differences in sleep among the worker castes, I applied remote thermal sensing to aid in distinguishing spatial differences in sleep among the castes (Chapter 2). I introduced individually marked, newly eclosed adult workers into colonies and recorded their sleep sites and temperatures as they matured and changed castes. The goal was to introduce "sleep maps," or scientific visualizations of sleep locations with respect to bee caste and physical features of the hive. Behavior correlated with temperature (of bees and of their surroundings), age of bees, presence of brood, and distance from the edge of the hive. These correlations suggest that thermography may offer one means of helping to characterize colony-wide sleep patterns across castes.

The first two chapters demonstrate that patterns of sleep, both temporal and spatial, differ among age-based castes of worker honey bees. My next objective was to test the flexibility of honey bee sleep (Chapter 3). I trained foragers to a food resource either early in the morning or late in the afternoon. Observing bees' behavior for 24-hour periods following each training, I discovered that bees slept, groomed, and engaged in every behavior the same total amount of time, but differed in *when* they slept. Individual bees exhibited a shift in their sleep schedule in response to shifting resource availability, which demonstrates temporal plasticity of sleep under ecologically relevant conditions.

Sleep is flexible, but is it necessary? In the spirit of sleep deprivation studies aiming to address functional aspects of sleep, I tested whether or not sleep-deprived

foragers encode signals less precisely (Chapter 4). Foragers can impart direction and distance information to a food source by performing a waggle dance. I sleep-deprived a subset of foragers within their hive and analyzed subsequent signaling by sleep-deprived and non-sleep-deprived foragers. Sleep-deprived foragers exhibited reduced precision when encoding the direction information in their waggle dances. As with all previous studies, I staged experiments inside hives and without cages so as to maximize normalcy and avoid confounds associated with testing social animals in isolation. In each chapter I discuss the implications of sleep specifically for honey bees and in the context of societies. Here, I posit the testable hypothesis that improving communication could be one of sleep's key benefits shared by social organisms.

Understanding sleep in honey bees may seem like a remote exercise, or a new enterprise. For the sake of understanding nature, applied apiculture, and ourselves, the concept of sleep in bees dates back at least to Pliny the Elder (1601, Fig. 2), who in 77-79 C.E. asserted:

> From the mids of winter unto the rising of Arcturus, for 60 daies Bees are nourished only with sleepe, without any other food. But from that time unto the Spring equinoctiall, and namely, where the weather is more warme, they are awake. Howbeit, they lie stil in their hive, & then fall to their victuals which they laid up in store against that time. But in Italie they do the like indeed after the rising of the star Vergiliæ: howbeit, untill then they do nothing but sleep.

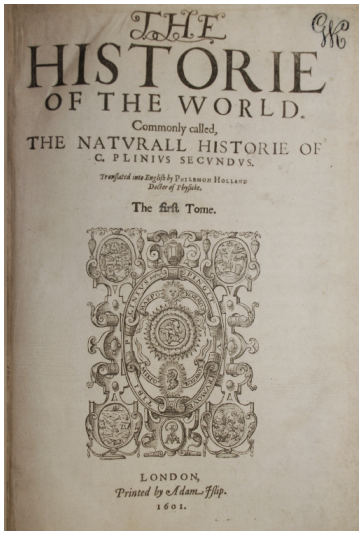
THE
HISTORIE
OF THE WORLD.
Commonly called,
THE NATVRALL HISTORIE OF
C. PLINIVS SECVNDVS.
Translated into English by PHILEMON HOLLAND
Doctor of Physicke.
The first Tome.
LONDON,
Printed by Adam Islip.
1601.

Fig. 2. Pliny the Elder's Naturis Historia, in which insects are purported to sleep, as well as to lack bones, teeth and tails. An observation of honey bees dubiously reports: "In the night they rest untill the morning: by which time, one of them awaketh and raiseth all the rest with two or three bigge hums or buzzes that it giveth, to warne them as it were with sound of trumpet… Toward evening their noise beginneth to slacke and grow lesse and lesse: untill such time as one of them flieth about with the same lowd humming wherewith shee waked them in the morning, and thereby giveth a signall (as it were) and commaundement to goe to rest: much after the order in a campe. And then of a suddaine they are all husht and silent."
From the library of William Close.

Thanks to the careful work of Walter Kaiser and others (e.g., Kaiser & Steiner-Kaiser 1983, Kaiser 1988, Sauer et al. 2004), we have a more informed foundation on which to study sleep in honey bees. We need not follow the star Vergiliæ as it rises in spring to know that honey bees are sleeping, but questions remain as to *why* bees sleep and whether universal aspects of sleep, homologous or analogous, exist across organisms. The future of elucidating sleep's mechanisms, ontogeny, evolutionary basis and functional relevance offers promise in honey bees, with their genome sequenced and a rekindled interest in seeking model organisms to further the field of sleep medicine and sleep biology.

> Thus the study of sleep in all its phases is a difficult subject; the path of approach is uncertain and the technique is one of pioneering. Thus far, no one with a comprehensive view of this field has been lured to attempt to correlate and integrate the materials with a view of bringing the problem to a fruitful conclusion. Such a subject requires the talents of a scholar versed in the branches of physiology, ecology, and animal behaviour; and especially one who appreciates the significance of the border-line sciences where these fields overlap one another. The materials thus far accumulated are merely fragments of information which may perhaps one day attract a scientist with an ability for digesting and correlating facts; one who will resurrect them from the dust-bins of time and from them bring the various aspect of sleep into clear focus. Then only will we know the meaning of the phenomenon of sleep in the world of plants and animals.
>
> -Phil Rau (1938)

Although sleep is still a wondrous mystery at every level of analysis, we now know that sleep can play a vital role with respect to various forms of learning, memory consolidation, vigilance against threats, and maintaining health and brain plasticity. Examining sleep behavior has contributed to our understanding of different facets of organisms' biology, including their social biology. Rau (1938) was correct that sleep presents a multi-disciplinary challenge and to understand its patterns and implications may require novel techniques. Although there can be no "conclusion" to the problem of understanding sleep, I present the following as an additional attempt at disentangling the complexities of sleep in one remarkable and social species of insect.

CHAPTER 1

Caste-dependent sleep of worker honey bees

ABSTRACT. Sleep is a dynamic phenomenon that changes throughout an organism's lifetime, relating to possible age- or task-associated changes in health, learning ability, vigilance and fitness. Sleep has been identified experimentally in many animals, including honey bees (*Apis mellifera*). As worker bees age they change castes, typically performing a sequence of different task sets (as 'cell cleaners', 'nurse bees', 'food storers' and 'foragers'). Belonging to a caste could differentially impact the duration, constitution and periodicity of a bee's sleep. I observed individually marked bees within observation hives to determine caste-dependent patterns of sleep behavior. I conducted three studies to investigate the duration and periodicity of sleep when bees were outside comb cells, as well as duration of *potential* sleep when bees were immobile inside cells. All four worker castes I examined exhibited a sleep state. As bees aged and changed tasks, however, they spent more time and longer uninterrupted periods in a sleep state outside cells, but spent less time and shorter uninterrupted periods immobile inside cells. Although cell cleaners and nurse bees exhibited no sleep:wake rhythmicity, food storers and foragers experienced a 24 h sleep:wake cycle, with more sleep and longer unbroken bouts of sleep during the night than during the day. If immobility within cells is an indicator of sleep, this study reveals that the youngest adult bees sleep the most, with all older castes sleeping the same amount. This in-cell potential sleep may compensate for what would otherwise indicate an exceptional increase of sleep in an aging animal.

INTRODUCTION

Sleep is a flexible behavior that can change in duration, constitution, and periodicity throughout an organism's lifetime. Duration, constitution, and periodicity of sleep depend on many factors, including age (Roffwarg et al. 1966, Jenni et al. 2005). Humans, for example, exhibit far more rapid eye movement (REM) sleep and sleep in

general, but less circadian organization to the timing of sleep during infancy than during adolescence or maturity. The same pattern of reduced sleep with increasing age exists in rats, cats, and guinea pigs (Jouvet-Mounier et al. 1970) and is generally thought to exist in all mammals (Frank and Heller 2003).

Sleep ontogeny has also been investigated in two invertebrate species—the fruit fly *Drosophila melanogaster* Meigen, 1830, and the honey bee *Apis mellifera* Linnaeus, 1758. Fruit flies, like mammals, sleep less as they age (Shaw et al. 2000). Periods of sleep and wake become less consolidated in aging fruit flies, but not in aging humans (except for the elderly) (Koh et al. 2006). As for honey bees, data on the ontogeny of sleep duration and periodicity are limited to work conducted on rest:activity rhythms measured in isolated laboratory individuals (Spangler 1972, Sauer et al. 1998, Sauer et al. 1999, Toma et al. 2000) and ambiguous measures of sleep of workers within the hive (Lindauer 1952, Moore et al. 1998).

Definition of sleep in honey bees

Although the set of characters considered diagnostic of sleep ranges widely and no set of characters has been universally adopted in the literature, several "sleep signs" are deemed critical by most researchers when defining sleep behaviorally. A sleeping organism exhibits a specific *posture* during *easily reversible* bouts of *relative immobility*, during which its *arousal threshold is increased* (Flanigan 1972). According to Tobler (1985), such a state should be *internally controlled*. The definition of sleep, initially behavioral, expanded to include correlative electrophysiological measures and the combination of behavior and electrophysiological recordings has often been used to identify sleep in vertebrates (Flanigan et al. 1973). Individually, behavior and electrophysiology present limitations when used to define sleep. Brain states often differ between sleeping and wakeful organisms, but relying on electrophysiology alone can result in misidentification of sleep in mammals and birds, and is less informative for other animals (Campbell and Tobler 1984). Some attempts to electrophysiologically distinguish between wakeful and

quiescent states in invertebrates have been performed (Kaiser and Steiner-Kaiser 1983, Schuppe 1995, Nitz et al. 2002, Ramón et al. 2004), but these gross measures require coincident behavioral characters to reliably establish sleep. Alternatively, relying exclusively on a subset of behavioral characters can also be misleading. Immobile animals can have low arousal thresholds, and animals with high arousal thresholds can be awake but reluctant to move (e.g., habituated to disturbance). A cautious application of operational definitions relying on correlations of sleep signs is often a necessity when identifying a sleeping animal.

Versions of the behavioral definition of sleep have been measured and reported in many vertebrate species and in a handful of invertebrate species (Rattenborg and Amlaner 2002). Behavioral sleep has been identified in a cuttlefish (Duntley and Morrissey 2004), an octopus (Brown et al. 2006), a crayfish (Ramón et al. 2004), species of scorpions (Tobler and Stalder 1988), cockroaches (Tobler 1983, Tobler and Neuner-Jehle 1992), and a paper wasp (Klein 2003). The most extensive invertebrate sleep research has been conducted with the fruit fly *D. melanogaster* (Hendricks et al. 2000, Shaw et al. 2000) and the honey bee, *A. mellifera* (Kaiser 1988, Sauer et al. 2004).

Apis mellifera workers exhibit age polyethism, or the changing of task sets with age. A worker honey bee begins life as an egg laid within a beeswax cell. After passing through the developmental stages of larva and pupa, the freshly eclosed worker (called a callow) spends the first days of her adult life as a member of the "cell cleaner" caste, spending much of her time oriented headfirst in cells—occasionally cleaning these cells (Seeley 1982, Seeley and Kolmes 1991, Moore 2001). After three days as a cell cleaner, the typical worker spends days 4-12 of adulthood as a "nurse bee," feeding and tending brood and the queen, followed by days 13-20 as a "food storer" (or "middle-aged bee"), receiving and storing fresh nectar (Seeley 1982, Johnson 2008). A worker bee spends her remaining days in the "forager" caste, exiting the hive in the search for and acquisition of nectar and pollen to feed her colony. The age polyethism schedule of worker honey bees is flexible, and depends on variables ranging from genetic predisposition (Calderone and Page 1989) to colony needs (Seeley 1995) and the caste demographics within a colony

(Huang and Robinson 1996). Task sets performed by bees may profoundly influence bee sleep, as demonstrated when Bloch and Robinson (2001) induced foragers to perform the tasks of nurse bees, resulting in a reversion from rhythmic back to arrhythmic behavior.

Kaiser and Steiner-Kaiser (1983) first discovered the potential for sleep in *A. mellifera* by tethering isolated foragers under constant light conditions and recording circadian sensitivity of optomotor interneurones to moving visual stimuli. Kaiser (1988) and Sauer et al. (2004) followed this electrophysiological work by performing a series of meticulous studies on isolated foragers that examined behavioral and physiological characters associated with sleep. Having satisfied the criteria defined above as diagnostic of sleep, worker honey bees appear to be sleeping when relatively immobile, with body and appendages slumping in the direction of gravity (Fig. 3; for more descriptions see Kaiser 1988). "Relative immobility" refers here to bees that are immobile except for exhibiting occasional, apparently spontaneous antennal, tarsal, or leg twitches or proboscis extensions, or discontinuous respiratory pumping motions of the gaster (posterior body tagma of hymenopterans). This postural state can be sustained for extended periods, but is easily reversed, often by the physical contact of a neighboring bee (see Arousal Video in supplementary material, http://jeb.biologists.org/cgi/content/full/211/18/3028/DC1).

With a definition of sleep and an understanding of the typical sequence of age-correlated behaviors in worker honey bees, we can pose questions about how sleep duration, constitution, and periodicity change with respect to caste and age. How long do members of each caste sleep, when do they sleep, and at what point in an adult bee's lifetime do sleep:wake rhythms take shape? Worker bees are the subject of numerous studies regarding circadian rhythmicity, some that explicitly relate to sleep (Kaiser 1988, Sauer and Kaiser 1995, Sauer et al. 1998, Sauer et al. 1999, Bloch and Robinson 2001), but many more that ambiguously address sleep biology, either by examining "presumptive inactive behaviors" (Moore et al. 1998), or by examining circadian *activity* rhythms (Spangler 1972, Southwick and Moritz 1987, Toma et al. 2000, Meshi and Bloch 2007).

Honey bee foragers follow very strong diurnal rhythms of activity, including visiting flowers and dance communication (von Frisch 1967). Lindauer (1952) observed one pollen forager continuously for two days and, although he did not distinguish sleep from a state of being "Müβig" (idle), he found that the forager rested more at night than during the day (although grooming was included in this measure). Kaiser (1988) followed up on this observation with extensive studies that consistently supported a circadian sleep:wake cycle of experimentally isolated foragers. Sauer and Kaiser (1995) confirmed that pollen foragers also exhibit circadian rest within an observation hive. The younger nurse bees, in contrast, do not show diel rest-activity rhythms (Lindauer 1952, Crailsheim et al. 1996, Moore et al. 1998), although they do show non-circadian sleep signs (Sauer et al. 1999). Spangler (1972) reported no circadian activity in a freshly eclosed and isolated worker and contrasted this with an older worker, which exhibited circadian activity cycles. Moore et al. (1998) recorded various in-hive tasks, all of which were performed arrhythmically except "resting," which increased at night relative to the day as workers aged. Sauer et al. (1998) and Sauer et al. (1999) specifically examined sleep signs within developing adults living in isolation and found an increasing circadian organization to the timing of sleep as the bees aged.

Our aim[1] is to determine whether or not worker bees living in a natural colony setting engage in more rhythmic and shorter sleep as they grow older. To do so, we observed worker honey bees for caste-dependent sleep behavior, recording duration and periodicity of sleep when bees were outside of comb cells, and immobility when bees were inside cells. Some preliminary results have been previously presented as an abstract (Klein 2006).

[1] This research was a collaborative effort and my co-authors are listed in the Acknowledgements.

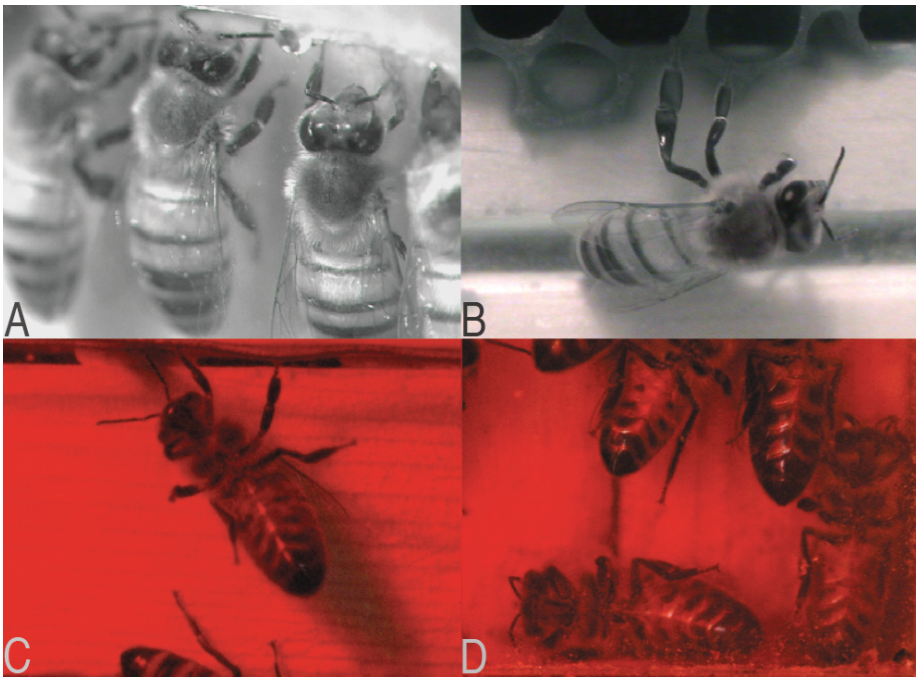

Fig. 3. Worker honey bees displaying typical sleep postures while relatively immobile, with limbs and body drooping in the direction of gravity. Bees exhibit a sleep state while **(A)** in groups, **(B)** isolated, **(C)** dangling motionless from tarsal claws, or **(D)** leaning against the observation hive wall or floor. Photographs taken with a Panasonic AG-DVC30 video camera in infrared-sensitive mode (A, B) and Nikon D70 under red lamplight (C, D).

METHODS

Our data were obtained from three studies on two colonies, featuring scan sampling and focal sampling of subjects categorized by behavior or known age. I chose different sampling methods in order to balance the tradeoff between obtaining a relatively complete picture of each bee and a representative picture of each caste. We scan-sampled bees in one hive, recording behavior for three days (Scan:72h), we focal-sampled bees selected pseudo-randomly within a known age group in a second colony for periods throughout their adult lives (Focal:life), and we selected one representative of each age caste from this second colony and recorded her behavior for one day (Focal:24h); see details, below (Fig. 4).

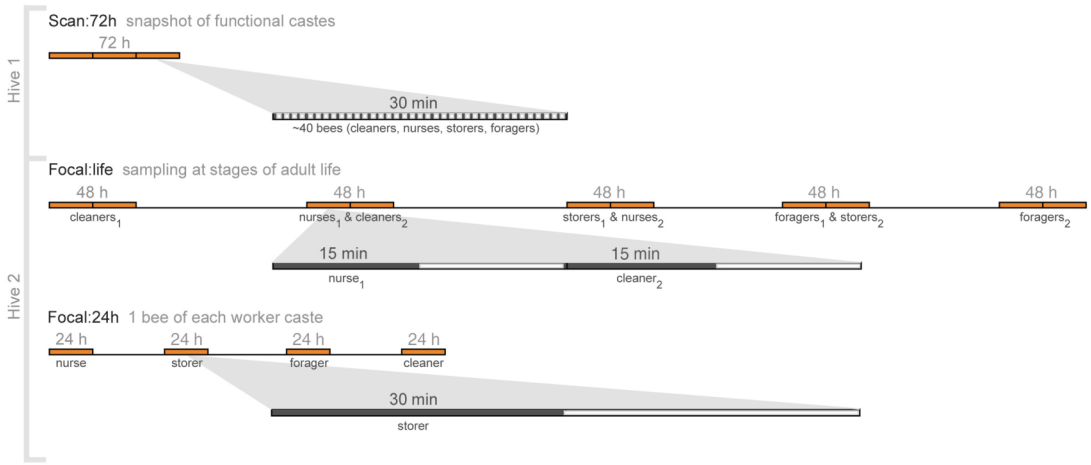

Fig. 4. Timeline of each sampling method. (Scan:72h) Scan sampling of behavior of ~40 worker bees every 30 min for 72 h. Enlarged view represents one of 144 consecutive 30-min periods, and each dark bar signifies a single observation of one of the ~40 bees. Bees were selected by the function they performed (or by evidence of their recent eclosion, in the case of cell cleaners) prior to the 72-h study. (Focal:life) Focal sampling of worker bees at important stages of their adult lives. Two groups of cell cleaners were introduced (represented by the subscripts 1 and 2) and two randomly selected bees were each observed for 15 min each hour (enlarged view) for 48-h periods. (Focal:24h) Focal sampling of a representative of each worker caste. Each bee was observed for 30 min every hour for 24 h. Enlarged view represents one hour of 24 consecutive hours during which a single food storer was observed for 30 min.

We conducted separate studies to control for colony differences and environmental fluctuations while obtaining information that was representative of worker bee sleep behavior. Scan sampling and focal sampling honey bee behavior have been shown to produce "indistinguishable" results (Kolmes 1984). Using both methods enabled us to control for environmental fluctuations by condensing our observations into a three-day snapshot or extending our observations across adult lifespans. Furthermore, I introduced cell cleaners twice during the Focal:life study, allowing us to examine each age caste's behavior within the same colony on different dates. This, along with conducting the Scan:72h study at a different time and with a different colony than the Focal:life study, decreased the possibility that the behavior patterns we found were

idiosyncratic, a function of the particular colonies, or a result of weather conditions during our study.

Experimental design

We set up two 2-frame observation hives (Seeley 1995), each hive containing approximately 2000 New World Carniolan honey bees (*Apis mellifera carnica*; queen breeder: C. F. Koehnen and Sons, Inc., Glenn, CA) on 23 and 25 June 2005 at Cornell University's Liddell Field Station (Ithaca, New York, USA). We placed the two observation hives in separate rooms of the field station, gave the bees free access to the outdoors, and restricted each colony's queen to the bottom frame by inserting a queen-excluder between the two frames. Bees collected food primarily from wildflowers surrounding the field station.

K. Olzsowy and I observed adult worker honey bees labeled for individual identification and belonging to different age or functional castes for 72-h (Hive 1), and 48-, and 24-h durations (Hive 2) between 17 July and 15 August 2005 under ambient light (daytime) or red light (nighttime), to which honey bees are less visually sensitive (von Frisch 1967). Red light shone from a lamp mounted to each side of the observation hives and these lamps remained on for the duration of each study. After we recorded a bee's behavior, we shone a handheld LED light when necessary to clarify a marked bee's identity. The sun was visible ca. 0545h and the sun set ca. 2015 – 2030h, so for the purposes of this study daytime is defined as 0600–2000h and nighttime as 2000–0600h and "periodicity" refers to the day versus night presence-absence of sleep behaviors. Indoor temperature remained between 22-25.5ºC. Ambient sunlight probably did not strike the inside of our observation hives via the hive entrance, but indirectly shone through a window that was perpendicular to the plane of each observation hive. Levels of indirect light changed during the course of each day, but curtains prevented any direct sunlight from striking either hive. *Apis mellifera* have been known to colonize sites

exposed to ambient light, although this behavior is not common and effects of ambient light on diurnal sleep patterns are unknown.

Marking

I marked bees with unique combinations of color, repeated on the dorsal and ventral sides of the gaster to facilitate identification of a bee when she was in a cell or dorsally obscured (e.g., while clinging to the glass pane of hive). I repeated the color combinations on the dorsal alitrunk (midsection of hymenopteran body; Scan:72h study), or attached numbered plastic labels with Canada balsam (Focal:life and Focal:24h studies). I chilled bees prior to marking, except in the case of callows, which are more sensitive to such treatment. The marked bees acclimated for 2.5-3 h (1 h for foragers) within holding cages adjacent to the observation hives, then spent 2-7 h (3 h for Scan:72h study, 7 h and 2.5 h for Focal:life study, and 2 h for callow in Focal:24h study) within the hives prior to behavioral recordings.
Colors: shellac+dry artists' pigments for Scan:72h study, or shellac+dry artists' pigments combined with Sharpie oil-based marker, Prismacolor marker or Pentel correction Presto! for Focal:life and Focal:24h studies.

Behaviors recorded

For bees in a relaxed state, we recorded three forms of relative immobility (bee without motion except for spontaneous leg or tarsal twitching, proboscis extension, or respiratory pumping of gaster): (1) with antennae immobile; (2) with minute twitching of antennae; or (3) with larger, usually swaying motions of antennae (see Videos 1-3, respectively, in supplementary material, http://jeb.biologists.org/cgi/content/full/211/18/3028/DC1). We also recorded whether each relaxed, relatively immobile bee was inside or outside a cell. When inside a cell, a bee's antennae were not visible, rendering the three forms of relative immobility indistinguishable.

We distinguished the relative immobility of relaxed bees, just described, from the active state (bee locomotes, grooms, lifts or turns body, or fans wings), and from the relative immobility of non-relaxed bees (bee exhibits an alert stance, is groomed by another bee, antennates rapidly between bouts of locomotion, lifts a leg, turns her head, engages in trophallaxis, or processes wax with her mandibles). Movements associated with wakefulness while in cells include turning or rhythmic motions of the body (Sakagami 1953), or continuous pumping of the gaster, including when bees are heating adjacent brood cells (Kleinhenz et al. 2003).

Scan:72h

We collected and marked four sets of 13 adult workers, presumed to belong to the four worker castes: cell cleaners from the original source colony, nurse bees within the observation hive's brood comb cells, food storers from within the upper frame's (brood-free) cells, and foragers netted prior to reentry into the observation hive (Hive 1). Following a predetermined path, we visually scanned the hive's frames for marked bees and observed each marked bee 3-5 sec to determine its behavioral state. If the bee was immobile but jostled by another bee during this time, we waited 5 sec and observed the bee again. We recorded the behavior of each visible, marked bee every 30 min for 72 h (3-5 sec per bee x ~40 bees/30 min x 72 h; Fig. 4).

Focal:life

We introduced 30 and 40 recently eclosed adult bees (callows) to Hive 2 on 17 and 23 July, respectively, and began recording the callows' behavior 14 h and 9 h after they had been collected (7 h and 2.5 h after introduction into hive, respectively). We selected two of these bees per hour (from a non-repeating pseudo-randomly generated list of numbers) and recorded the behavior of each bee continuously for 15 min. We made continuous, real time recordings by typing keys on a laptop as we observed behaviors, each key representing a different behavior, using JWatcher 0.9, a freeware behavior-recording and

analysis program (Blumstein et al. 2000). We repeated this recording of bees' behavior for 15 minute periods every hour, two bees per hour, for 48 h. Each week, as the bees aged and changed tasks, we repeated this method (15 min per bee x 2 bees/h x 48 h/week x 4 weeks; i.e., 48 h on, 4-8 days off, 48 h on, etc.; Fig. 4). We began our 48-h study periods on July 17, 23, 29 and August 8, 13.

Focal:24h

We selected four bees from Hive 2, one bee representing each worker caste. We recorded the behavior of one bee continuously for 30 min each hour for 24 h (30 min/h x 1 bee of each worker caste x 24 h) and repeated this recording regimen for each of the other three bees on separate days (Fig. 4). Continuous recordings were, again, made using JWatcher 0.9. Of the four bees examined, the nurse bee, food storer and forager were selected from among the subjects used in the Focal:life study and the cell cleaner was newly marked and reintroduced for this study, conducted on August 3, 7, 10 and 13, respectively. We drew maps indicating cell visits that lasted longer than several seconds by the cell cleaner and by the food storer, as well as sleep sites of the forager, recorded within the hourly 30-min observation periods.

Analysis

I conducted one-way *F*-tests for every analysis distinguishing day vs. night behavior and two-way *F*-tests for every analysis with the additional factor of worker caste. I followed up statistically significant results by making pairwise comparisons using the Tukey-Kramer HSD test, or, when interactions were analyzed, I decomposed significant interactions using a simple main effects test ("Test Slices"). I treated multiple observations on the same individual as independent observations. I conducted an additional analysis of results from the Focal:life study eliminating all data except for one observation per bee per caste to address the assumption of independence of data. I report summary statistics of continuous variables as means $\pm$ standard error (s.e.m.). I set alpha

at 0.05 for all tests and all tests are two-tailed. Due to the non-normal distribution of our data, I also analyzed behavioral data (without interactions) using nonparametric tests. I analyzed data with two levels (day vs. night) using the Wilcoxon Test and four levels (worker castes) using the Kruskal-Wallis Test. I analyzed all data with the JMP IN (version 5.1.2; SAS Institute Inc. 2004) computer package and I conducted all analyses on a Windows XP machine with a Pentium-IV processor.

A. Klein tested for rhythmicity of behaviors in the Scan:72h data by using integrated analytical tools (flytoolbox) developed by Levine et al. (2002) using MATLAB (version 7.4; The MathWorks 2007). A. Klein plotted correlograms using the simple signal processing functions of flytoolbox to nonlinearly detrend the data with a 72-hour high-pass Butterworth filter while applying the autocorrelation function. Levine et al. (2002) discuss these methods and the precedence and value of using the autocorrelation function to assess rhythmicity.

RESULTS

Worker bees from each caste exhibited sleep signs. Results, below, include data for bees that were relatively immobile (defined above and referred to hereafter as "immobile") and were observed either outside or inside cells. We report antennal states associated with sleep in bees as either "antennae immobile" (i.e., antennae motionless) or "antennae variable" (i.e., antennae motionless, slightly twitching, or exhibiting larger, usually swaying motions). Data representing these two categories allow for additional analyses of 'deep sleep' [as Kaiser (Kaiser 1988) was tempted to call the sleep state during which antennae are immobile] *versus* total sleep exhibited outside cells, respectively. Results from nonparametric tests are consistent with parametric test results, with one possible exception, noted below.

Scan:72h

Every 30 min we recorded the behavior of each of the 13 marked bees per worker caste that we could find, resulting in observations for approximately 6 foragers and 11 of each of the other three castes (average = 11.1 ± 0.1 cell cleaners, 11.3 ± 0.1 nurse bees, 11.4 ± 0.1 food storers, 5.8 ± 0.2 foragers; n = 144 observations per caste, including 28 observations per day and 20 per night during each 24-h period). In all four castes, some workers exhibited a sleep state outside of cells, or were immobile inside cells (see average percentages over the entire study or with respect to day or night, Table 1).

Table 1. Immobility of bees determined during the course of two studies.

	Scan:72h study (% observations)				Focal:life study (% time)			
	Outside cell, antennae immobile	Outside cell, antennae variable	Inside cell	Out + in cell	Outside cell, antennae immobile	Outside cell, antennae variable	Inside cell	Out + in cell
Cell cleaners								
Total	3.9±0.6	6.5±0.7	31.8±1.4	38.4±1.5	1.0±0.4	2.3±0.6	39.4±3.7	41.7±3.6
Day	3.1±0.6	5.4±0.9	34.2±1.9	39.7±2.0	1.1±0.5	1.9±0.6	36.5±4.9	38.5±4.7
Night	5.1±1.0	8.1±1.3	28.4±1.8	36.6±2.3	0.9±0.6	2.9±1.1	43.7±5.8	46.5±5.4
Nurse bees								
Total	4.8±0.6	9.1±0.8	19.8±1.0	28.9±1.2	2.8±0.9	4.9±1.3	17.5±2.7	22.3±2.7
Day	4.7±0.8	8.5±1.1	21.1±1.4	29.6±1.6	2.7±1.0	4.8±1.6	18.5±3.7	23.3±3.7
Night	5.0±0.8	10.0±1.2	17.9±1.6	28.0±1.9	2.9±1.6	4.9±2.0	16.0±3.7	20.9±3.8
Food storers								
Total	8.3±0.7	12.5±0.8	15.8±0.9	28.4±1.3	11.2±2.0	16.5±2.5	4.3±1.3	20.8±2.5
Day	6.8±0.9	9.8±1.0	16.0±1.2	25.8±1.6	6.6±2.1	10.6±2.7	4.1±1.6	14.6±2.9
Night	10.3±1.3	16.4±1.3	15.6±1.5	32.1±1.9	18.0±3.6	25.0±4.4	4.6±2.2	29.6±4.3
Foragers								
Total	19.2±2.0	28.5±2.4	2.5±0.6	31.0±2.5	15.1±2.0	23.6±2.7	1.6±1.1	25.2±2.8
Day	5.3±1.5	10.9±2.4	2.0±0.8	12.9±2.3	9.9±2.1	14.6±2.9	2.7±1.8	17.3±3.2
Night	38.8±2.6	53.3±2.4	3.1±0.9	56.4±2.7	22.4±3.4	36.3±4.5	0.0±0.0	36.3±4.5

Values are means ± s.e.m.
Immobile was regarded as relaxed posture with no movement except for respiratory gaster pumping or twitch of leg or tarsus, or proboscis extension.
Antennae variable = antennae immobile, slightly twitching or exhibiting larger, usually swaying motions; Scan:72h = antennae immobile, scan sampling bees' behavior across 72h; Focal:life = antennae immobile, focal sampling of bees' behavior at important stages of their lives – across the entire study period (Total), throughout the day (Day), or throughout the night (Night).

Sleep outside of cells:

Older bees slept longer and with greater 24-h periodicity outside cells than did younger bees. The percentage of observations in which relaxed, immobile bees exhibited antennal immobility did not differ between cell cleaners and nurse bees, but was greater in food storers and greatest in foragers. The same relationships held when antennal states were

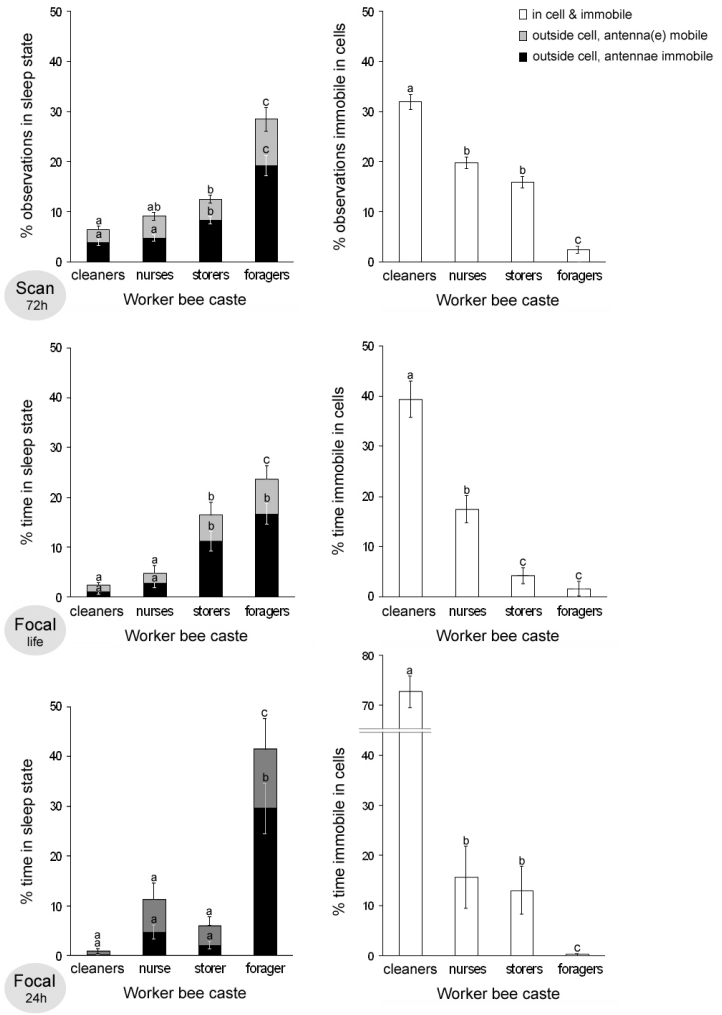

Fig. 5. Relative immobility in relaxed state with respect to worker caste. Immobile bees were observed outside of comb cells with motionless antennae (black bars), with antennae twitching or exhibiting larger motions (gray bars), or bees were observed inside cells and immobile (white bars). Patterns of sleep and immobility within cells remained fairly consistent across the studies, with older bees sleeping more outside cells and younger bees spending more time immobile inside cells. Error bars reflect s.e.m. margins associated with black bars, black + gray bars, or white bars. Different letters indicate statistically significant differences among the castes with respect to these measures. Castes are ordered by presumed (nurse bees, food storers and foragers in Scan:72h study) or actual (cell cleaners in Scan:72h study, and all bees in Focal:life and Focal:24h study) age,, from left to right. (Scan:72h) % observations of ~40 randomly selected marked bees; scan sampling: 3-5 sec per bee every 30 min for 72 h. (Focal:life) % time, 4 bees randomly selected from same age group; focal sampling: 15 min per bee per h for 48 h every week for four weeks of their lives. (Focal:24h) % time, 1 bee of each worker caste; focal sampling: 30 min per hour x 24 h.

variable, except that nurse bees did not significantly differ from cell cleaners or from food storers (Fig. 5).

Sleep outside of cells did not significantly differ between night and day for cell cleaners or for nurse bees (antennae immobile: $F_{1,568} = 1.15, 0.03$, $P = 0.28, 0.87$, with respect to worker caste; antennae variable: $F_{1,568} = 1.38, 0.50$, $P = 0.24, 0.48$, respectively), although nonparametric results may show a day-night difference with nurse bees' "antennae variable" measure ($c^2_1 = 3.86$, $P = 0.05$). Food storers and foragers, however, both slept outside of cells more during the night than during the day (antennae immobile: $F_{1,568} = 3.79, 351.66$, $P = 0.05, < 0.0001$, respectively; antennae variable: $F_{1,568} = 8.87, 365.52$, $P = 0.003, < 0.0001$, respectively; Figs 6, 7). Examining sleep rhythmicity using autocorrelations, we found no evidence for diurnal or ultradian rhythms in cell cleaners or nurse bees. Food storers exhibited a perceptible 24-h periodicity when antennae were variable, and foragers displayed a strong and clear 24-h periodicity when antennae were immobile or variable (Fig. 8A,B). Sleep outside of cells was impacted by worker caste, day vs. night and the interaction of worker caste by day vs. night (antennae immobile: $F_{3,568} = 86.91$, $F_{1,568} = 120.33$, $F_{3,568} = 78.76$, respectively, $P < 0.0001$ for each; antennae variable: $F_{3,568} = 107.26$, $F_{1,568} = 143.74$, $F_{3,568} = 77.51$, respectively, $P < 0.0001$ for each).

Immobile inside cells:

The percentage of observations of bees immobile *inside* cells decreased across castes, with cell cleaners found most often inside cells and immobile, nurse bees and food storers found less often, and foragers found least inside cells and immobile, in contrast to the pattern of increasing worker sleep outside of cells (Fig. 5). Although we observed some bees immobile inside cells more frequently than other bees, this difference existed only with respect to caste ($F_{3,568} = 133.66$, $P < 0.0001$) (Fig. 5) and day vs. night ($F_{1,568} = 4.10$, $P = 0.04$), and not with respect to interaction of caste by day vs. night ($F_{3,568} = 2.21$, $P =$

0.09). Autocorrelations revealed no diurnal or ultradian rhythmicity of in-cell immobility for any worker caste (Fig. 8C).

When we summed the recordings of sleep outside cells with those of immobility inside cells, we found that cell cleaners exhibited more sleep than their older siblings. Nurse bees and food storers slept slightly less than foragers and foragers slept less, although not statistically so, than cell cleaners. Cell cleaners and nurse bees were as immobile during the day as they were during the night ($F_{1,568} = 1.13, 0.31, P = 0.29, 0.58$, respectively), but food storers and foragers were immobile more often at night than during the day ($F_{1,568} = 4.48, 218.89, P = 0.03, < 0.0001$, respectively; Figs 6, 7). Autocorrelations revealed no rhythmicity in cell cleaners or nurse bees, the food storers' 24-h periodicity was dampened by lack of rhythmicity while immobile in a cell, and the foragers' 24-h rhythm remained significant (Fig. 8D). Sleep outside cells + in-cell immobility were impacted by worker caste, day vs. night, and interaction of caste by day vs. night ($F_{3,568} = 9.70$, $F_{1,568} = 58.48$, $F_{3,568} = 55.44$, respectively; $P < 0.0001$ for each).

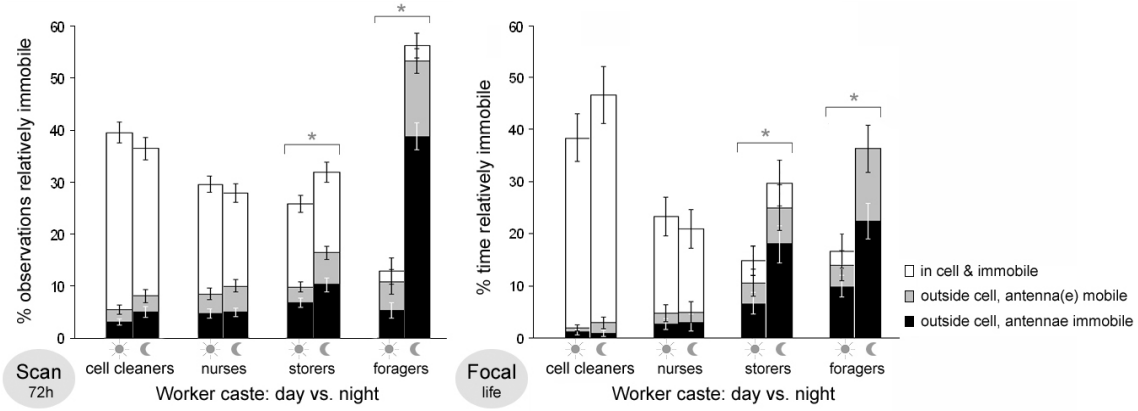

Fig. 6. Relative immobility in relaxed state during the day ☀ versus during the night ☾ with respect to worker caste. Food storers and foragers exhibited more sleep, by any cumulative measure, during the night than during the day. Error bars reflect s.e.m. margins associated with black bars, black + gray bars, or black + gray + white bars. The asterisk signifies a statistically significant difference between daytime and nighttime measurements with respect to these measures. All worker castes spent the same duration immobile inside cells during the night as during the day, so measure of "in cell & immobile" did not alter night-day differences for any worker caste. (Scan:72h) % observations. (Focal:life) % time.

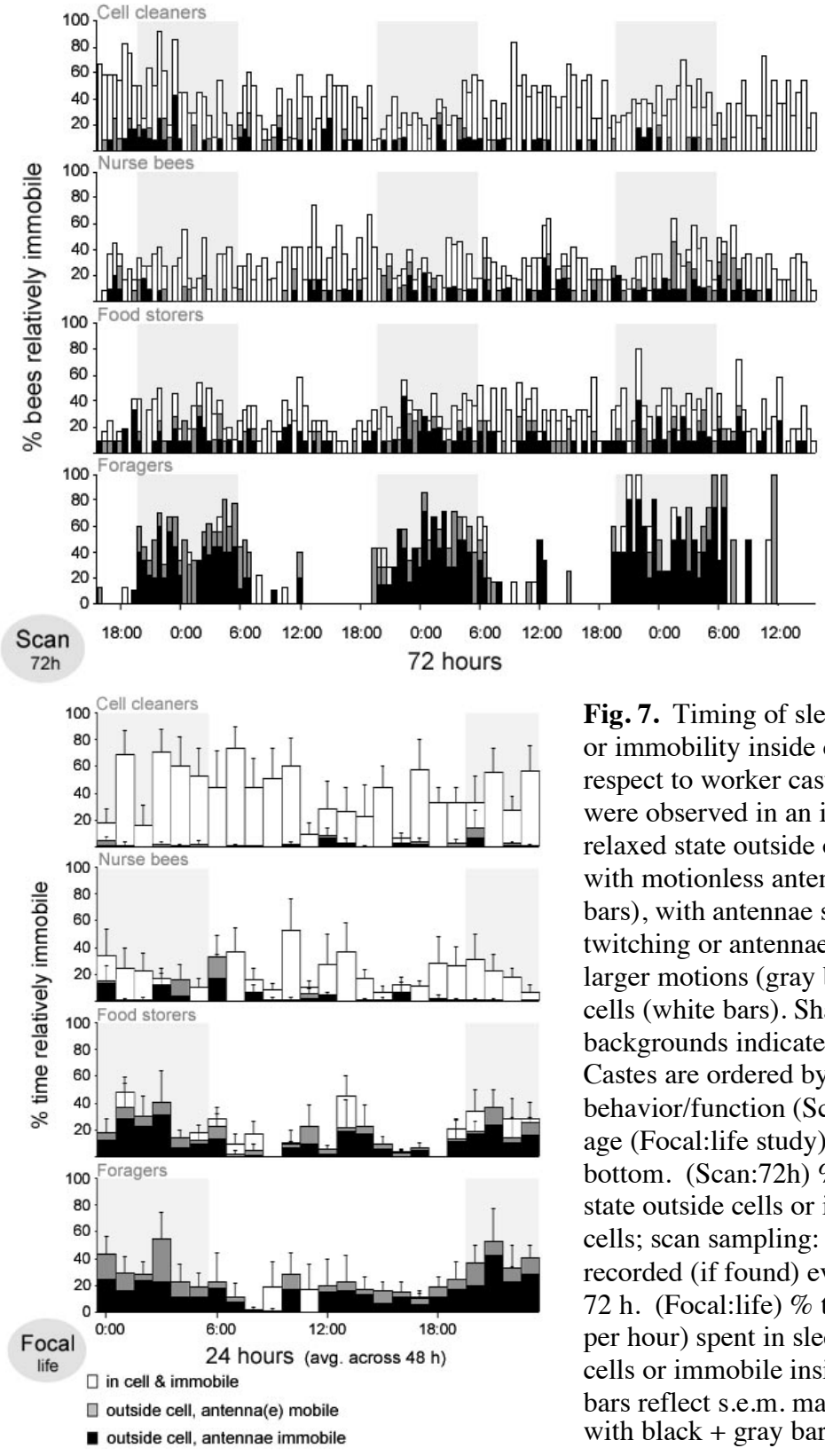

Fig. 7. Timing of sleep outside cells or immobility inside cells with respect to worker caste. Worker bees were observed in an immobile, relaxed state outside of comb cells with motionless antennae (black bars), with antennae slightly twitching or antennae exhibiting larger motions (gray bars), or inside cells (white bars). Shaded backgrounds indicate nighttime. Castes are ordered by behavior/function (Scan:72h study) or age (Focal:life study), from top to bottom. (Scan:72h) % bees in sleep state outside cells or immobile inside cells; scan sampling: each bee recorded (if found) every 30 min for 72 h. (Focal:life) % time bees (n = 4 per hour) spent in sleep state outside cells or immobile inside cells. Error bars reflect s.e.m. margins associated with black + gray bars, or white bars.

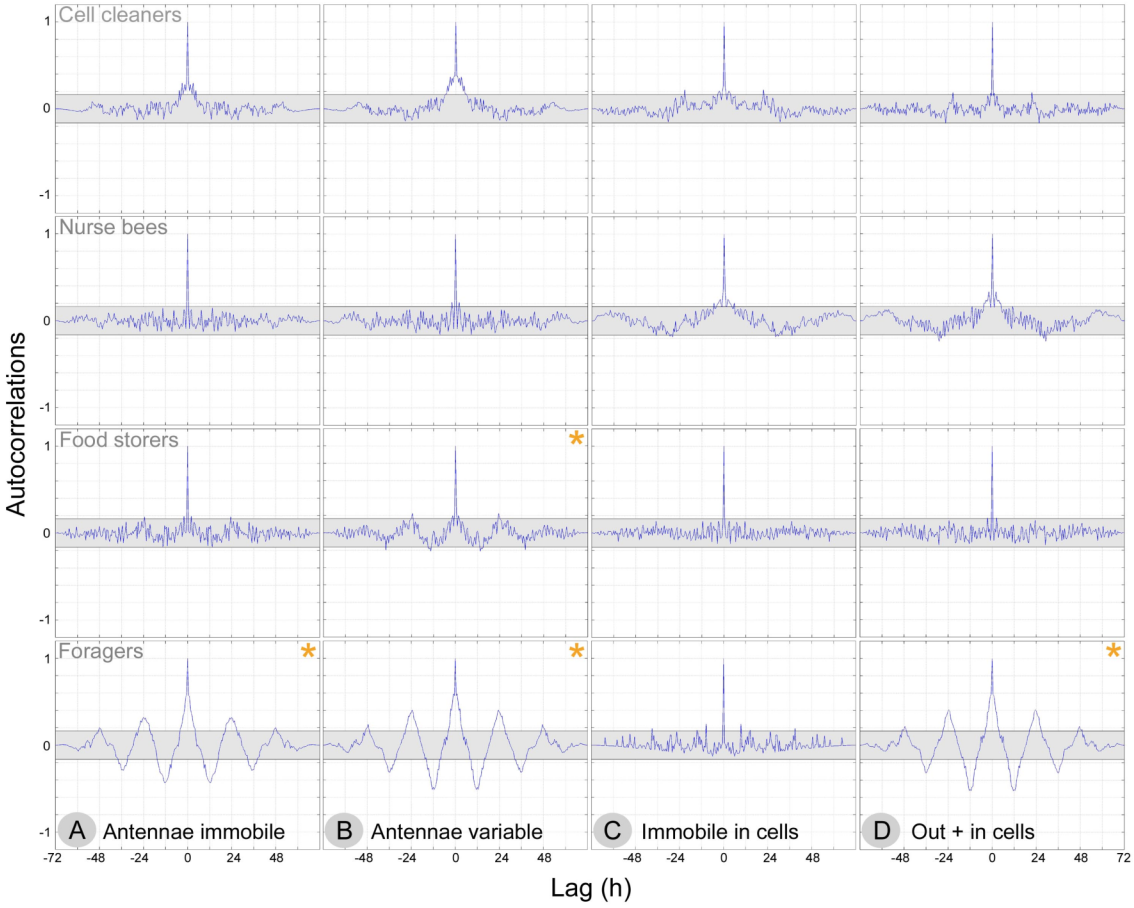

Fig. 8. Autocorrelations conducted on Scan:72h data, assessing rhythmicity of worker castes in relaxed posture exhibiting relative immobility when **(A)** antennae were immobile, **(B)** antennae were variable, **(C)** immobile inside cells, and (**D**) the sum of A, B, and C. Food storers exhibited a 24-h rhythm when antennae were variable, and foragers exhibited strongly significant 24-h rhythmicity when antennae were immobile, variable, and when all measures were summed. There was no evidence for ultradian or circadian rhythms (i.e., large oscillations were absent) for cell cleaners, nurse bees, or any in-cell immobility. An asterix signifies periodicity and gray bars represent 95% confidence intervals. A peak at the center of each graph indicates zero lag (amount of shift) and therefore perfect correlation.

Focal:life

We continuously recorded the activity of bees at different stages of their lives: 54 as cell cleaners, 33 as nurse bees, 30 as food storers and 21 remaining foragers—two bees per hour for 15 min each bee (x 2 trials), resulting in an average of 96 15-min observation sessions of each worker caste as the bees aged. When we eliminated all data except for one observation per bee per caste, statistical significance was retained in all analyses, unless noted below.

Cell cleaners exhibited a sleep state, as did each of the subsequent age castes (see average percentages over the entire study or with respect to day or night, Table 1). There was great variation in behaviors exhibited within each age caste. Some bees of typical nurse bee age exhibited behavior typical of cell cleaners (extended periods in empty or egg-containing cells without obvious body movement). Some bees of typical food storer and forager age did not neatly fall into their respective functional classifications. Callows introduced into Hive 1 were rejected by the hive, so all data from the Focal:life study refer to Hive 2.

Sleep outside of cells:

Consistent with the Scan:72h study, young cell cleaners and nurse bees spent less time in a sleep state with antennae immobile than after the transition from nurse bee to food storer, but time spent in this sleep state did not increase after food storers became foragers. When antennae were variable, the same pattern of age-dependent sleep increase occurred, except that foragers spent more time in a sleep state than any younger age caste ($F_{3,379} = 36.91$, $P = < 0.0001$; Fig. 5). This statistical increase from food storer to forager was not retained after we eliminated all data except for one observation per bee per caste.

Sleep outside of cells did not significantly differ between night and day for cell cleaners or for nurse bees (antennae immobile: $F_{1,379} = 0.00, 0.01$, $P = 0.95, 0.94$, respectively; antennae variable: $F_{1,379} = 0.06, 0.00$, $P = 0.80, 0.98$, respectively). As food storers and foragers, however, bees slept outside of cells more during the night than during day (antennae immobile: $F_{1,379} = 15.18, 18.56$, $P = 0.0001, = 0.00002$, respectively; antennae variable: $F_{1,379} = 14.37, 32.81$, $P < 0.0001, = 0.0002$, respectively;

Figs 6, 7). Sleep outside of cells was impacted by worker caste, day vs. night and the interaction of worker caste by day vs. night (antennae immobile: $F_{3,379} = 25.62$, $F_{1,379} = 17.03$, $F_{3,379} = 5.65$, $P < 0.0001$, < 0.0001, $= 0.0009$, respectively; antennae variable: $F_{3,379} = 33.14$, $F_{1,379} = 24.14$, $F_{3,379} = 7.80$, $P < 0.0001$ for each, respectively). When we eliminated all data except for one observation per bee per caste, one difference was lost: foragers did not show a night-day difference.

Immobile inside cells:

The percentage of time spent in cells (immobile or not) decreased when cell cleaners became nurse bees and decreased again when nurse bees became food storers (45.7 ± 3.9%, 29.5 ± 3.2%, 8.7 ± 1.7%, respectively). There was no significant change in time spent in cells when food storers became foragers (4.0 ± 1.7%). Time spent *immobile* inside cells followed the same caste-dependent pattern ($F_{3,379} = 49.40$, $P < 0.0001$; Fig. 5). Time immobile inside cells was not impacted by day vs. night, or by the interaction of caste by day vs. night ($F_{1,379} = 0.06$, $F_{3,379} = 0.84$, $P = 0.81$, 0.47, respectively). When we eliminated all data except for one observation per bee per caste, time spent immobile inside cells decreased with age, but the statistical difference between nurse bees and food storers, and food storers and foragers was not retained.

Nearly all results pertaining to the combined measure of sleep outside cells and immobility inside cells are consistent with the Scan:72h study. Bees spent more time immobile (outside + inside cells) as cell cleaners than during any subsequent stage of their adult lives (Figs 5, combining outside + inside cell percentages). Cell cleaners and nurse bees spent as much time immobile (outside + inside cells) during the day as they did during the night ($F_{1,379} = 1.92$, 0.17 and $P = 0.17$, 0.68, respectively). As food storers and foragers they spent more time immobile at night than during the day ($F_{1,379} = 6.50$, 10.56, $P = 0.01$, 0.001, respectively; Figs 6, 7). Worker sleep or in-cell immobility were impacted by caste, day vs. night, and interaction of caste by day vs. night ($F_{3,379} = 10.94$, $F_{1,379} = 11.51$, $F_{3,379} = 2.59$, $P < 0.0001$, $= 0.0008$, 0.05, respectively). Differences in day versus night were not retained after we eliminated all data except for one observation per bee per caste.

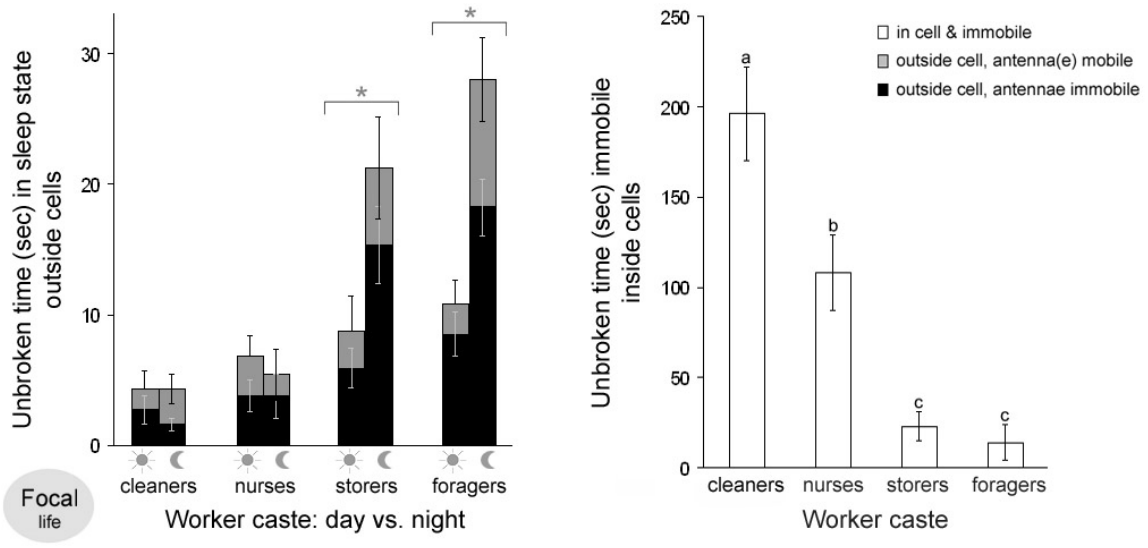

Fig. 9. Uninterrupted bouts of relative immobility with respect to worker caste. Outside cells: Food storers and foragers exhibited longer unbroken sleep bouts during the night than during the day , as measured by antennal immobility (black bars) or in combination with antennae slightly twitching or exhibiting larger motions (gray bars). Inside cells (white bars): Unbroken bouts of relative immobility decreased with age. No difference existed between night and day, so data were collapsed for each caste. Worker bees were observed for 48 h during each of four stages of their adult lives (cell cleaners to foragers, respectively). An asterisk indicates a significant difference between daytime and nighttime measurements of black and black + gray bars and different letters indicate statistically significant differences among white bars. Error bars reflect s.e.m. margins associated with black bars, black + gray bars, or white bars.

Sleep bouts:

Uninterrupted sleep bouts were not only longer in the older bees (food storers or foragers > cell cleaners or nurse bees), but lasted longer during the night than during the day (Fig. 9). Unbroken bouts of immobility inside cells decreased as bees aged, both when cell cleaners became nurse bees and again when nurse bees became food storers (Fig. 9), although when we eliminated all data except for one observation per bee per caste the only statistical difference retained was between cell cleaners and older castes. Maximum durations of unbroken sleep bouts while outside cells were 89, 180, 330, and 333 sec for cell cleaners, nurse bees, food storers, and foragers, respectively. Of these periods, 89, 162, 330, and 303 sec were maximum unbroken periods with antennae immobile. Immobility inside cells sometimes exceeded entire 900-sec census periods for cell cleaners and nurse bees and lasted at most 463 and 653 sec for food storers and foragers,

respectively. Unbroken bouts of sleep spent outside of cells were impacted by caste, day vs. night, and the interaction of caste by day vs. night (antennae immobile: $F_{3,379} = 20.54$, $F_{1,379} = 14.74$, $F_{3,379} = 6.05$, $P < 0.0001$, $= 0.0001, 0.0005$, respectively; antennae variable: $F_{3,379} = 19.66$, $F_{1,379} = 19.24$, $F_{3,379} = 8.11$, $P < 0.0001$ for each).

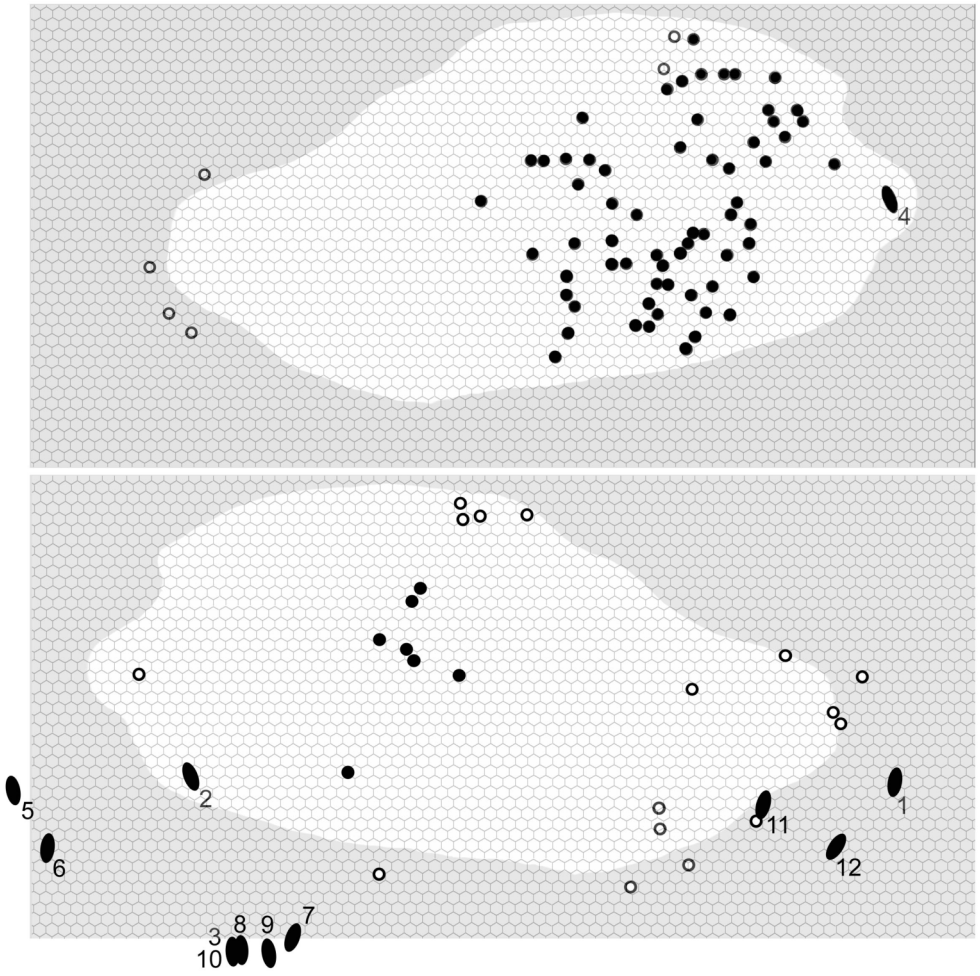

Fig. 10. Activity of three bees, mapped on opposite sides of the observation hive's bottom frame, indicating that the forager slept on the periphery of the hive, the cell cleaner visited cells within the brood comb area, and the food storer visited cells on the edge of the brood comb. White space roughly outlines brood comb on 13 August; ● = cell cleaner's cell visits, ○ = food storer's cell visits, ◥ = 12 nighttime sleep sites of the forager, numbered chronologically. The three bees were observed on separate days continuously for 30 min per hour for 24 h.

Focal:24h

The cell cleaner spent 74% of her 24 h (30 min observation per hour) in at least 70 different cells, including 54 egg, 1 larval, 6 empty, and 9 unknown, all positioned within the centrally located brood comb (Fig. 10). The nurse bee spent almost all of her time in the brood area and tended the queen, but she also spent time in three egg cells and a pollen cell. The food storer spent time (range: 16 – 1520 sec) in at least 22 different cells (10 pollen, 3 larva, 7 egg, 2 empty; Fig. 10). The forager spent no time in cells and was either immobile, engaging in trophallaxis, locomoting, or foraging (every hour from 0700–1100h). The forager exhibited no sleep site fidelity, although she spent all of her sleep state periods close to the periphery of the brood comb (Fig. 10).

Sleep outside of cells:

The forager spent more time outside of cells in a sleep state than the cell cleaner, nurse bee, or food storer (antennae immobile: $F_{3,92} = 25.18$, $P < 0.0001$; antennae variable: $F_{3,92} = 26.61$, $P < 0.0001$; Fig. 5).

Immobile inside cells:

The cell cleaner spent more time immobile inside cells than her older sisters; the forager spent no time in cells. As with the Scan:72h and Focal:life studies, the cell cleaner spent more time immobile, in or out of cells, than older bees, due to her extended immobile periods spent inside cells ($F_{3,92} = 20.73$, $P < 0.0001$; Fig. 5).

Sleep bouts:

While the cell cleaner spent no time outside of cells in a sleep state, the nurse bee and food storer spent an average of 5 sec with antennae immobile and 9-13 sec with antennae variable. The forager spent the most time outside of cells in a sleep state: 40 ± 13 sec (antennae immobile) and 49 ± 12 sec (antennae variable). The reverse pattern occurred with respect to immobility inside cells: the cell cleaner exhibited more than the nurse bee and food storer (361 ± 56, 123 ± 65, 119 ± 43 sec, respectively) and the forager spent no time in cells.

DISCUSSION

Our investigation of sleep in honey bees revealed differences in sleep among the four worker castes, expanding on previous work distinguishing sleep duration, constitution, and periodicity in honey bees.

Duration and constitution: Patterns of sleep and immobility were consistent across the studies, with bees sleeping more outside cells when older, and spending more time immobile inside cells when younger. This increase in sleep outside cells with respect to age/caste held true for total antennal immobility, a state correlated with high arousal threshold and speculated to be the deepest state of sleep (Kaiser 1988). Cell cleaners and nurse bees exhibited more sleep and more deep sleep outside of cells when they became food storers and, in the case of variable antennae, again as food storers became foragers (Fig. 5). As younger bees aged and changed tasks, they also experienced longer unbroken bouts of sleep outside cells, increasing as nurse bees became food storers, and again as food storers became foragers, but experienced shorter bouts of immobility inside cells (Fig. 9). Our caste-dependent sleep and immobility data are consistent with some, but not all of Moore et al.'s (1998) findings.

Periodicity: We detected no diurnal or ultradian sleep cycles in cell cleaners or nurse bees, but did detect a 24-h sleep:wake periodicity in food storers and, as expected, a strong 24-h sleep:wake periodicity in foragers (Fig. 8). Food storers and especially foragers spent more time asleep, and had longer unbroken bouts of sleep, during the night than during the day (Figs 6, 9, respectively). We found no evidence of rhythmicity of in-cell immobility for any worker caste (Fig. 8). See below for comparisons with other studies.

For all of our work we used a proxy for the set of behavioral characters deemed diagnostic of sleep. Because it was impossible to examine all sleep signs simultaneously, we recorded relative immobility (defined above) when bees were inside cells or outside cells. Bees may perform wakeful actions that are undetectable under normal conditions, like voluntary head and mouthpart movements inside cells, or wing muscle contractions for heat production either inside or outside cells (Esch, 1960). Fortunately, bees performing certain wakeful actions can be distinguished from resting bees by the

temporal periodicity of their gaster pumping movements (discontinuous in resting bees; continuous in "heating" bees). Cell cleaners, for instance, have been shown to spend almost their entire time discontinuously ventilating (and staying relatively immobile) while inside cells (Sauer et al. 1998, B. A. Klein unpublished). Heating bees can also be distinguished from resting bees by the heat generated from their alitrunks (Kleinhenz et al. 2003) or by their posture (Bujok et al. 2002). Discontinuous ventilation covaries with antennal immobility (Sauer et al. 2003), so respiratory rate could potentially serve as a proxy, or even exclusive indicator of sleep in bees inside cells.

Our categories of sleep differ from Moore et al.'s (1998) categories of presumed inactivity and differ somewhat from Kaiser's (1988) categories of honey bee sleep. Moore et al. recorded observations of workers either "standing" (i.e., motionless, not in cell), or "motionless in cell" (i.e., bee remains in cell for longer than 3 min). These categories differ from our categories in that Moore et al. did not distinguish between non-rest immobile behavior and relaxed immobility and they did not distinguish between antennal immobility and mobile states of antennae. Also, Moore et al. measured in-cell immobility by duration (> 3 min) inside cell, not solely by lack of movement within cell. Kaiser's classification of "immobile" antennae included "sporadic, minute movements." We have divided this into "antennae immobile" and "antenna(e) twitching" (see Videos 1, 2 in supplementary material, http://jeb.biologists.org/cgi/content/full/211/18/3028/DC1), although our distinction was based on close observation and not via automated infrared detection in tethered subjects. Kaiser also noted separate categories of smaller and larger antennal movements, which we speculate falls under our designation of "antennae variable." Our conclusions likely are unaffected by this potential discrepancy, although future work should be careful to define precisely each behavioral category recorded. Clarity on this will facilitate replication of experiments and interpretation of results, which could be especially important should new insights reveal an important functional difference between the two antennal states.

Our research does not definitively address the importance of age versus task in determining sleep behavior. The most informative experiment to distinguish the effects of age and task occurred when Bloch and Robinson (2001) induced a reversion of "rest"

behavior in foragers. They found that by inducing foragers to re-engage in nurse bee behavior, they produced bees that no longer exhibited circadian "rest," although this work did not report on possible changes in duration of sleep or on behavior within cells. While we can be certain of the relatively young age of cell cleaners in all of our studies, and of the age of all bees in the Focal:life and Focal:24h studies, we cannot for the nurse bees, food storers and foragers in the Scan:72h study because they were chosen based on their behavior, not their age. Most of the nurse bee-, food storer- and forager-aged bees within the Focal:life study fell within the behavioral categories defined by Sakagami (1953) and Seeley (1982).

Until now, no study has explicitly examined the sleep:wake patterns of recently eclosed bees in the context of the colony. Lindauer's (1952) observations of cell cleaners confounded other behaviors with sleep, and other studies have measured *activity* rhythms of newly eclosed cell cleaners while maintained in isolation. Spangler (1972), Sauer et al. (1998), Sauer et al. (1999), and Moore et al. (unpublished) measured the locomotor activity of recently eclosed worker bees kept in constant darkness by using either vibration sensors (Spangler 1972) or infrared sensors. Sauer et al. (1998) and Sauer et al. (1999) placed each bee within a cuvette, provided her with a small piece of comb containing food, and reported that these bees, like those in our study, exhibited behavioral sleep signs and gradually gained a 24-h rest:activity rhythm as they grew older. Contrary to our autocorrelation results, however, Sauer et al.'s analyses identified and quantified an ultradian rhythm of rest:activity within the first 24 h, and subsequent days, following eclosion.

Having examined bees older than cell cleaners ($\geq$ 4 days after eclosion), Moore et al. (1998) reported more in-cell immobility during the night than during the day—across age bins and in both genotypes of honey bee studied. They also reported more "standing" during the night than during the day, albeit only in one of the two genotypes. These data, suggesting greater rest during the night than during the day in worker bees, are consistent with Lindauer's (1952) observations and our autocorrelation data for older worker bees, but not consistent with our data for nurse bees.

One of Sauer et al.'s (1998) bees, observed for 21 days, developed a 12-h rest:activity cycle during food storer age and developed a circadian rhythm within 15-21

days after eclosion. If Sauer et al.'s measurements of rest:activity reflect sleep:wake cycles, our data do not support a 12-h sleep:wake cycle in food storer aged bees; instead our food storer data fall along a sinusoidal path that suggests 24-h periodicity of sleep (Fig. 8B). Although oscillations were not large in these food storer data, Levine et al. (2002) argue that autocorrelation data that follow a clear pattern but may not achieve statistical significance due to small sample size (e.g., < 180), should be interpreted as circadian/periodic.

A major component of our study focused on duration and (lack of) periodicity inside comb cells. Little is known about what honey bees do inside cells. Cell cleaners enter for periods that can last over an hour and a half, often engaging in no discernible activity (Sakagami 1953, Sauer et al. 1998). Kolmes (1984) referred to periods lasting more than three minutes inside a cell simply as "in cell." Lindauer (1952), by examining young bees entering cells abutted against glass in a specially designed observation hive, determined that callows occasionally clean cells. This behavior typically vanishes after the third day of adulthood, sometimes accounting for <1% of observations (Moore 1998). In spite of some ambiguity, callows are frequently referred to as "cell cleaners" throughout the literature ("Zellenputzen" by Lindauer 1952, Sakagami 1953, Seeley 1982, Seeley 1991).

While some cell cleaning appears to be performed by callows, few studies have attempted to distinguish or quantify what they may spend the majority of their time doing within cells: resting (or sleeping). Lindauer (1952) recorded "idlers" spending 30 min or longer inside an empty cell or egg-containing cell, but his extended observations of two bees did not distinguish rest from active grooming and did not quantify rest inside cells. Kleinhenz et al. (2003) distinguished resting bees from heating bees inside cells, but did not report durations or timing of in-cell rest, or which bees engaged in this behavior. Kaiser (1988) was well aware that bees appeared to rest while in cells, but found that isolated bees supplied with a small piece of comb did not always rest within the cells, so he chose to study only bees outside of cells to increase the visibility and continuity of measurements of his subjects. Sauer et al. (1998) reported that isolated bees were frequently observed in cells when young and that between brief alterations of body positions bees in cells were always observed discontinuously ventilating. Moore et al.

(1998) quantified "motionless in cell," beginning on the bees' fourth day of adulthood and discovered in-cell immobility to be consistently rhythmic, showing greater exhibition of this "presumed inactive" behavior during the night than during the day. Our data do not demonstrate this day:night difference with respect to in-cell immobility for any caste (Fig. 6, 8). Moore et al.'s data also show no consistent age-dependent differences with regard to duration spent motionless in cells, in contrast to our data, which show a decrease with respect to age (Fig. 5). As a result of this discrepancy, Moore et al.'s data show that forager-aged bees remained motionless in cells dramatically more than we found to be the case for our foragers. Moore et al. (1998), by recording rhythmicity and duration of immobility in cells, Sauer et al. (1998), by suggesting that all in-cell time was spent discontinuously ventilating, and Kleinhenz et al. (2003), by distinguishing measures of body temperature and respiratory movement between resting and heating bees, may have produced the only prior work, following Lindauer's (1952) observations, specifically addressing potential sleep occurring inside cells. If relative immobility within cells is an indicator of sleep, then our study demonstrates that the youngest adult bees (cell cleaners) exhibit the most sleep (Focal:life and Focal:24h studies), or more sleep than nurse bees and food storers and at least as much as foragers (Scan:72h study). This in-cell measure of potential sleep could compensate for what would otherwise indicate an exceptional increase of sleep in an aging animal (when sleep outside cells, and not immobility inside cells, is considered).

It is possible that "cell cleaners" are less a functional caste and more a transitional state between subimago and mature adulthood, during which bees primarily sleep. And why sleep within cells? Unlike foragers, which may sleep more frequently on the periphery of the comb (see Kaiser 1988 for his observations of unmarked bees presumed to be foragers, and Fig. 10 for our data on sleep sites in one forager), cell cleaners spend nearly all of their time within the bustling brood comb (Seeley 1985, p. 34; Fig. 10 for our 24-h examination of cell visits by a cell cleaner). Slipping into brood cells to sleep may help tired, young bees avoid disturbance by active colony mates and it may offer protection and warmth that could conceivably contribute to regenerative processes or cognitive development. Kaiser et al. (2002) and Schmolz et al. (2002) independently discovered that the voluntary sleep sites of foragers along an artificial thermal gradient

(and within an observation hive; Schmolz, 2002) exceeded ambient temperature and concluded that reasons other than energy conservation may be linked to this preference. Schmolz et al. speculate that the reduced thermoregulatory behavior of sleeping bees could serve as a compromise between energy conservation and regenerative processes.

Flexibility in sleep behavior may be one more hallmark of the adaptive flexibility exhibited by honey bees. Within-colony variation and individual plasticity with respect to sleep:wake schedules could enhance a colony's ability to effectively exploit changing resources (Klein and Seeley 2007) and respond to brood fluctuations, parasite invasions, or predator attacks. Nurse bees, for example, might respond to the colony's arrhythmic demand for hygiene and brood care by exhibiting a lack of rhythmic sleep, while food storers might sleep in response to a nocturnal lull in the foragers' food collecting, but then awaken when incoming food needs to be unloaded and processed.

Kaiser (1988) has compared sleep in bees to sleep in mammals, referring not only to the behavioral characters defining sleep, but also to differences with respect to timing of "deep" sleep, specifically in humans. Sauer et al. (1998) reported that honey bee workers exhibited more circadian organization to the timing of sleep as they aged—a phenomenon shared by humans, but not by *D. melanogaster* (Koh et al. 2006). Later, Sauer et al. (2003) reported another sleep measure shared with humans: foragers exhibited less sleep as they age. They partly attributed the reduction in sleep as foragers age to a possible influence of the stress of experimental isolation. In spite of the lack of control and precision, there is value in examining sleep behavior within the context of a society. Future investigations of sleep in its natural setting might further reveal the similarities and differences between honey bee sleep and human sleep. For instance, while little is known about prenatal human sleep, sleep in immature stages of insects remains a complete mystery. Holometabolous insect development features changes through immature stages (egg, larval stadia, pupa) that may serve as important periods during which a sleep-like state could play a critical role in proper development and function of the organism, as has been proposed for prenatal humans.

Understanding the frequency and degree to which individuals of different castes within an insect society sleep is the starting point from which one can test and understand

the nature of activity patterns, sleep, and sleep's impact on the behavior and ecology of societies, as well as societally-based purposes of sleep.

CHAPTER 2

Mapping sleep across a society:

Spatial and temporal analysis of worker honey bee sleep

ABSTRACT. Spatial patterns relevant to societies have long been visualized and interpreted by the use of maps, with examples ranging from the advance of invasive ant populations to the spread of disease within human demographics. I introduce the concept of mapping sleep across an insect society, and provide an empirical example by using thermography to aid in the mapping of sleep patterns within colonies of honey bees (*Apis mellifera*). Known to exhibit sleep that changes as they age and change caste, honey bees face variables such as surrounding temperature and position of resources within their hive that may impact their sleep, both spatially and temporally. I mapped sleep behavior and temperature of worker honey bees and produced maps of a hive's comb contents as the colony grew and the contents changed. Individual bees slept in many locations over the course of a day, but patterns of caste-dependent sleep emerged, with bees sleeping in different areas of the hive relative to position of brood comb and surrounding temperature. Worker bees generally slept outside comb cells when closer to the edge of the hive, but slept inside cells when farther from the edge of the hive. Older bees slept in colder regions of the hive, their temperatures decreased when asleep, and this lower sleep temperature did not differ between day and night. Surface temperature of sleeping foragers was lower than the surface temperature of their surroundings (averaged across comb and bees). Behavior depended in part on presence of brood; while the youngest worker bees spent more time asleep surrounded by uncapped brood, the eldest bees spent more time awake. I propose mechanisms that could generate caste-dependent sleep patterns and discuss implications for visualizing sleep within a society.

INTRODUCTION

Maps serve a vital purpose in biology by visualizing spatial or temporal information relevant to living organisms. Mapping social phenomena can reveal the spread of disease (Hay et al. 2009), routes of migration (Witteveen et al. 2009), foraging

paths (Noser and Byrne 2010), organization with respect to division of labor (Jandt and Dornhaus 2009), or spatial dynamics of competing colonies (Adams and Tschinkel 1995). Social insect colonies, and honey bee colonies in particular, lend themselves well to mapping of behavior. Honey bee activity has been visualized outside the hive with respect to flight paths (Menzel et al. 2005, Riley et al. 2005) and simulated landmark maps (Cartwright and Collett 1987), and inside the hive for spatial organization of waggle dance information (von Frisch 1967) and patterns generated by removal rates of comb contents (Camazine 1991). Seeley (1982) created maps depicting twelve of the most commonly performed tasks within a nest of honey bees. Conspicuously absent, however, are maps depicting where bees reside when *not* performing tasks. A behavior that has never been mapped across an insect society, in spite of its potential ecological and evolutionary significance, is sleep. Our aim was to visualize and quantify caste-dependent patterns of sleep behavior across colonies of honey bees.

Sleep behavior is broadly defined by a suite of characters that include specific posture, relative immobility, reversibility of the condition and, most importantly, an increased response threshold to external stimuli (Piéron 1913, Flanigan 1973). Tobler (1985) more recently included the criterion of compensation following sleep loss, and others have deemed decreased body temperature (Kaiser 1988) and sleep site fidelity (Clark and Gillingham 1990, Hendricks et al. 2000) important criteria that characterize sleep behavior. It is impossible to simultaneously confirm all features diagnostic of sleep in a subject, so identifying sleep behavior requires selecting a representative subset of sleep signatures. Honey bee workers exhibit all behavioral criteria defining sleep (Kaiser 1988, Sauer et al. 2004) and, fortunately, display features that robustly distinguish sleep from a wakeful state. Ventilation during sleep is always discontinous, co-occurring with increased antennal immobility and other measures indicative of sleep (Sauer et al. 2003), and is visible whether the bee is inside or outside of comb cells. Discontinuous ventilation consists of several pumping motions of the posterior body region (metasoma), followed by an extended ventilatory pause (Kaiser et al. 1996, Chapman 1998, Kleinhenz et al. 2003, Sauer et al. 2003). Klein et al. (2008) used relative immobility and discontinuous ventilation to serve as an operational definition for sleep in honey bees, with specific posture (Fig. 11) as a complementary indicator of sleep when exhibited

outside of comb cells. Reduced antennal mobility means higher response thresholds, and antennal immobility for extended periods may be suggestive of a deeper sleep state (Kaiser 1988).

Honey bees are eusocial animals, ultimately rendering the study of honey bee sleep as a study of sleep dynamics involving many interacting individuals engaged in a division of labor. Somewhat analogous to unihemispheric sleep in the brain of a bird or cetacean, which involves the simultaneous control of sleep and wakefulness by the organism (Rattenborg et al. 1999), differential sleep by castes within a colony of honey bees might be considered sleep by the superorganism. Honey bee workers typically progress through a chronological sequence of task-based castes, beginning adulthood as cell cleaners (Seeley and Kolmes 1991, Moore 2001), later tending brood and queen as nurse bees, then receiving and storing nectar as food storers (Johnson 2008) and ultimately serving as the colony's foragers (Seeley 1982). The sequence of age-based tasks is flexible, resulting in performance of several tasks at each age (Lindauer 1952, Sakagami 1953, Seeley 1982) and even accommodating a reversal of certain caste duties (Bloch and Robinson 2001). Typically, however, the patterns of caste-dependent sleep and wake are consistent across colonies: younger adult workers (cell cleaners and nurse bees) sleep primarily inside cells (Klein et al. 2008) and exhibit no sleep-wake periodicity (Lindauer 1952, Spangler 1972, Crailsheim et al. 1996, Moore et al. 1998, Sauer et al. 1998, Sauer et al. 1999, Eban-Rothschild and Bloch 2008, Klein et al. 2008), while the older castes (food storers and foragers) sleep primarily outside cells (Klein et al. 2008) and foragers sleep with a circadian periodicity (Kaiser and Steiner-Kaiser 1983, Kaiser 1988, Sauer et al. 2003, Eban-Rothschild and Bloch 2008, Klein et al. 2008). The hive consists of wax comb in the center, where the brood and the younger workers that care for them usually reside. Hive architecture, including brood comb placement, could introduce implications for caste-dependent sleep positioning.

Mapping sleep positions of individual honey bees within the hive offers an opportunity to address societal impacts on sleep and sleep's possible functions in a social setting. Mapping sleep in insects has previously been limited to recording fruit flies' stasis within test tubes (Hendricks et al. 2000) and the positions of three fire ant queens in an artificial chamber (Cassill et al. 2009), in contrast with the far more extensive

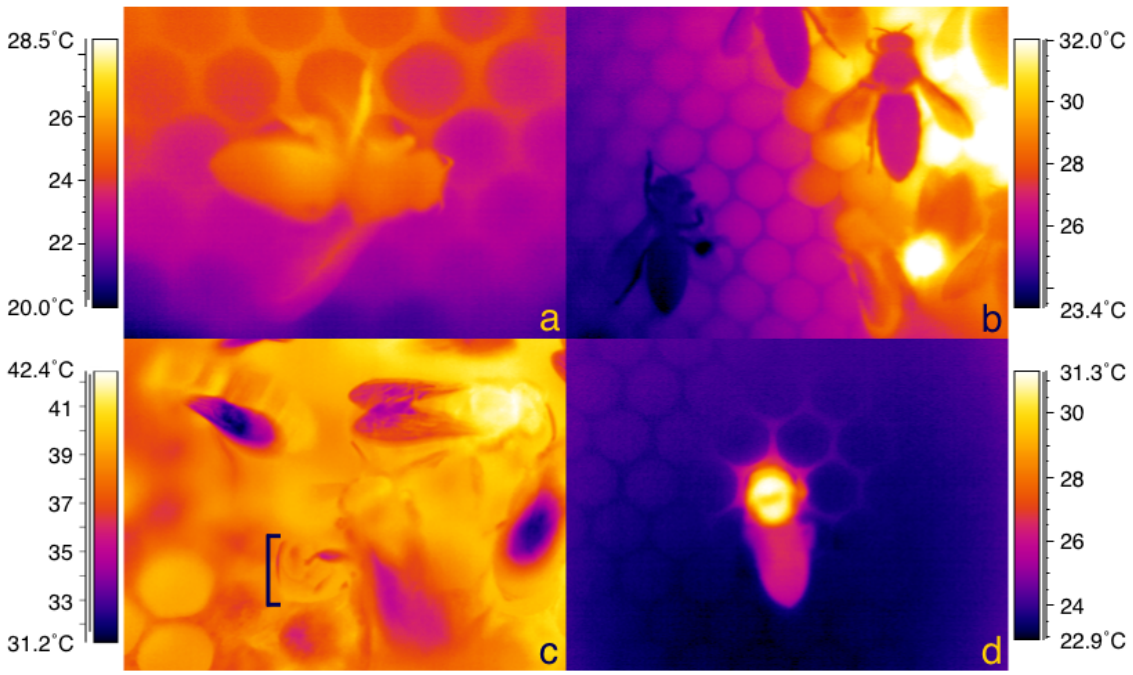

Fig. 11. Infrared images exposing thermal variation across honey bees. (a) A sleeping bee outside of a cell is relatively immobile, hangs in the direction of gravity, and discontinuously ventilates her abdomen (metasoma). This bee is exhibiting deep sleep, with extended bouts of antennal immobility. (b) Sleeping bees display little change in surface temperature across their bodies and are affected by surrounding temperature, as with the bee on the edge of the hot brood comb (upper right) and the inverted bee, hanging on the plastic window a short distance from the brood comb (lower left). For comparison, note the mobile bee at lower right with the hot thorax and cooler abdomen within the same area of the brood comb as the sleeping bee. (c) Bees also sleep inside cells, primarily when they are young adults. Here, framed by the bracket, is the abdomen of a sleeping bee slightly protruding from a cell (leg of neighboring bee resting on the distal tip of her abdomen), distinguished as sleeping by her discontinuously ventilating posterior. (d) This lone bee is far hotter than her surroundings and her thoracic heat temporarily increases the surface temperature of the wax in her immediate vicinity. All images were taken with FLIR thermal cameras by BAK.

literature devoted to the study of vertebrate sleep sites. Tracking sleep across individual vertebrates has offered insight as to some functional implications of sleeping socially, especially with respect to vigilance and predator avoidance. Geese, according to Vavilov (1916) “divide the watches” when they sleep and “wake the whole flock with a loud cry of warning” should they hear anything suspicious. Mallard ducks sleep unihemispherically when positioned at the edge of a group, responsive to external stimuli by having one eye open and head turned away from the center of the paddle (Rattenborg

et al. 1999). Non-human primates select their sleep sites based largely on safety-related factors (reviewed in Anderson 1998, Anderson 2000) and primate sleep sites have been mapped with respect to landmark features important to their safety (Reichard 1998, Di Bitetti et al. 2000, Fan and Jiang 2008, Schreier and Swedell 2008, Phoonjampa et al. 2010) as well as with respect to hygiene and comfort, including avoidance of snowfall (Wada et al. 2007).

Investigating sleep patterns in a society could benefit from a visualization technique that distinguishes sleep from non-sleep behaviors. One feature of sleep shared by mammals and honey bees alike is a telltale decrease in body temperature. In mammals, for example, the suppression of thermoregulation is fundamental to paradoxical (R.E.M.) sleep, possibly more so than muscle atonia (Morrison and Amini-Sereshki 1988). Honey bees of unknown caste have been reported to sleep at ambient temperature at the edge of their hive (Kaiser 1988) or inside cells (Kleinhenz et al. 2003) and foragers have been reported to sleep at ambient temperature when isolated (Kaiser 1988, Kaiser et al. 2002, Schmolz et al. 2002) or in unreported locations of the hive (Schmolz et al. 2002). If honey bees of different worker castes sleep in different areas of the hive, is temperature, alone or in combination with hive landmark data, a good predictor for identifying and distinguishing sleeping honey bees?

We[2] hypothesized that ontogenetic changes and caste-dependent demands of aging honey bees would result in predictable thermal and behavioral patterns across a hive. Because the different worker castes engage in tasks that have some spatial component, we hypothesized that worker bees would exhibit caste-dependent sleep site fidelity, which would depend in part on distance from the bustling brood comb. Sleeping exposed to an incessantly working mass of siblings cleaning cells and tending the brood could result in frequent disturbance and sleep fragmentation. Following Kaiser's (1988) observations of unknown caste members sleeping near the edge of the comb and Klein et al's (2008) observations of a single forager over 24 h, we predicted that foragers sleep closer to the edge of the hive and away from uncapped brood. In contrast, we predicted that younger castes sleep inside cells within the brood comb area. We also predicted that,

[2] This research was a collaborative effort and my co-authors are listed in the Acknowledgements.

like foragers, bees belonging to other castes would experience a decrease in body temperature to ambient. We applied remote sensing thermography with the ambition of mapping caste-dependent sleep by individually marking newly eclosed honey bees and observing them at different stages of their adult lives.

METHODS

We studied the sleep behavior, location, and surface temperatures associated with Carniolan worker honey bees (*Apis mellifera carnica*) shortly after eclosion and during subsequent periods of their adult lives as the bees changed castes. Studies were conducted on two separate colonies in separate years and differences between the studies of Colony 1 (2006) and Colony 2 (2008) are noted throughout. First, we installed a two-frame observation hive (Seeley 1995) in a temperature-controlled room at the bee research facility of the University of Würzburg (Würzburg, Germany, 49º46'47"N, 9º58'31"E), and allowed the hive of bees unrestricted access to the outdoors, where bees freely foraged during the day. I introduced 49 recently eclosed, individually marked worker bees to Colony 1 on 2 June 2006 and to Colony 2 on 14 August 2008. The bees had been extracted within hours of eclosing from a brood comb placed in a 35°C incubator (Colony 1), or had been extracted directly from six different hives (Colony 2). I individually marked the dorsal thorax (mesosoma) and dorsal and ventral abdomen (metasoma) of the bees using either model paints (Games Workshop, England; Colony 1) or oil-based markers (Sharpie; Colony 2), chosen because neither notably dampened surface temperature readings in prior tests. Further preparations for surface temperature recordings included replacing the observation hive's glass windows with transparent polypropylene giftwrap (pbs-factory, Artikel 00347) and adjusting thermal camera settings (emissivity of honey bees 0.97-1.0, transmissivity of polypropylene 0.89). I mounted a thermal camera (FLIR S40 for Colony 1, FLIR SC660 for Colony 2; accuracy ± 1°C or 1% of reading) on an adjustable, rolling monopod and recorded data for each marked bee, following a consistent sequence of hive regions demarcated by string, which was visible relative to the hive when thermally photographed. I lined the hive and feet of a chair used to sit and observe bees with dense foam to reduce substrate-borne vibrations. I also eliminated all ambient light. The hives were perpetually lit on each side with a

desk lamp (Colony 1: 25W, 230V; Colony 2: Megaman, Compact 2000HPF 30W, 4000K) covered with red acetate filters (Colony 2: #27 Medium Red, transparency = 4%, peak at 670 nm, Supergel by Rosco, Stamford, CT). Closer examination of behaviors was facilitated with a headlamp, also covered with the same red filters, selected because honey bees are reported to be insensitive to frequencies beyond 600 nm (von Frisch et al. 1977) or 650 nm (Dustmann and Geffcken 2000). Although a preliminary test showed that bees could detect the filtered light sources, their behavior did not noticeably change if the bees were exposed to gradual changes in light intensity.

Fifteen hours and 20 h after I collected bees for Colony 1 and 2 (3 h and 8.5 h after introduction into the hive, respectively), the bees were integrated into the hive, with no signs of aggression by other bees and no abnormal grooming. M. Stiegler and I systematically scanned for marked bees one section of the hive at a time, surveying bees every hour. As cell cleaners aged and became foragers, we recorded data for 24 consecutive hours during each of these two caste periods for Colony 1 (2 and 24 June 2006), and as cell cleaners aged and became nurse bees, then food storers, we recorded data for 6 daytime hours and 6 nighttime hours during each of these three caste periods for Colony 2 (10:00–16:00 h and 22:00–04:00 h; 15, 18, and 26 August 2008). We began monitoring cell cleaners on the 1st day after they eclosed, nurse bees on the 4th day, food storers on the 12th day and foragers on the 23rd day (Fig. 12). We selected dates based on typical age-based caste determination in workers (Sakagami 1953, Seeley 1985).

We recorded thermal images of marked bees by pointing at each bee with soft forceps (marked with a pointer on one end to distinguish orientation of the head) as the thermal camera automatically recorded images every second. We verbally recorded each bee's behavior and identity (Olympus VN-4100PC Digital Voice Recorder, or audio track of Sony Handycam DCR-HC65); identity was confirmed with a dim LED light. Due to temporary camera malfunction, we were unable to report data for nurse bees or food storers in Colony 1. To supplement the declining number of marked workers as Colony 1's bees became foragers, we selected and marked ten additional foragers of unknown age belonging to the same colony.

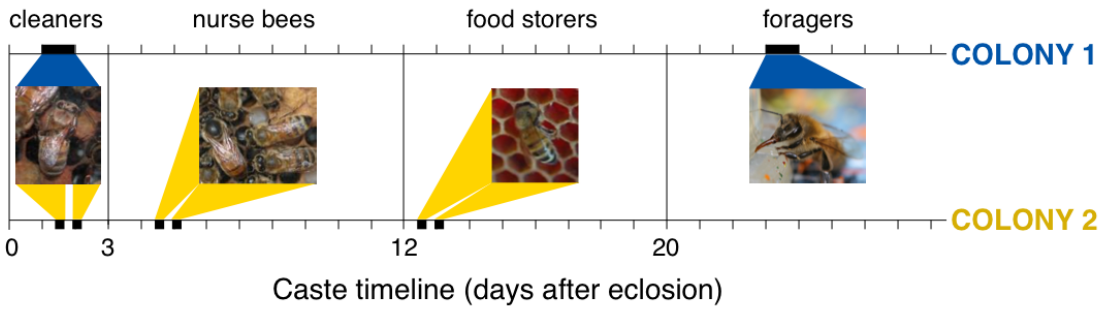

Fig. 12. Timeline of data collection (black bars on timelines) for both Colony 1 and Colony 2. We scheduled census times to fall within periods distinguishing the age-based worker castes. The beginning of the timeline represents eclosion, or the start of adulthood.

We transcribed bee caste, individual identity, and behavior data from voice-recorded notes. Behavior included different sleep states, distinguished from wakeful activity by a bee's relaxed immobility and discontinuously ventilating metasoma. We examined each bee for 3-5 sec to determine her behavior. If she were potentially asleep inside or outside a cell, we examined her for a minimum of ten seconds to determine if she were discontinuously ventilating. If outside a cell, we reported antennae as immobile (deep sleep) (Fig. 11a,b), or exhibiting swaying motions or minute twitches (light sleep).

We transcribed a bee's location, or calculated it from thermal images. Infrared images were relayed to a computer, stored on an external hard drive, and analyzed using camera-specific software (FLIR Systems ThermaCAM Researcher Professional software version 2.9). We recorded the average surface temperature of a bee's mesosoma (T_{bee}) and the average surface temperature of her surroundings (T_{surr}), taken as the average temperature within a circle with the radius of one bee body length (Fig. 13). We report the difference of the two to indicate the temperature of the bee relative to the surface temperature of her surroundings ($T_{difference}$). T_{surr} included the surface temperature of wax comb with a range of contents, wood from the hive frames, or bees. We also mapped the contents of comb cells for Colony 1 within 24 h of each census (5 and 25 June 2006) so that we could analyze bee behavior and temperature with respect to placement in the hive, particularly with respect to uncapped brood. We manually labeled cell contents on hive windows, removed and scanned these windows, and colored the discreet comb contents with different colors in Adobe Photoshop v.7.0.

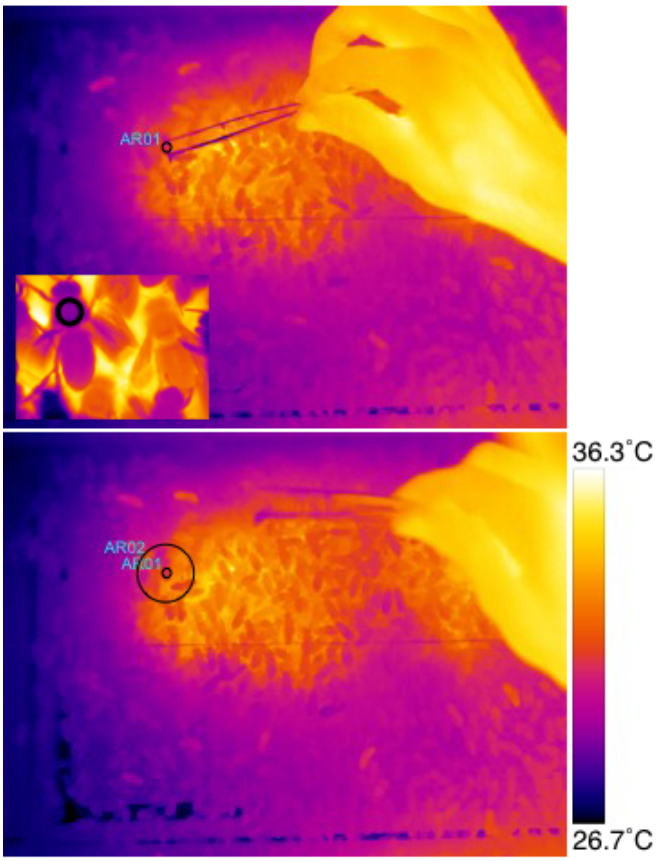

Fig. 13. Collection of temperature data from a sequence of infrared images. The forceps were held open to encompass the heat-generating midsection of the bee (top) and camera software computed the average temperature within the selected region, represented by a circle in the inset image. For recording the surface temperature surrounding a bee (T_{surr}), forceps were removed (bottom) so as not to influence the measurement, taken as the average temperature within the larger circle (radius = bee body length). $T_{difference}$ is the difference of the average temperature within the large circle (T_{surr}) from the average temperature in the smaller circle (T_{bee}).

Over the course of the study, Colony 1 grew from 1500 to 2800 individuals. Colony 2 housed ca. 2000 bees. Room temperature did not vary greatly during study recording sessions (2006: 25.9°C, range = 24.7–26.6°C; 2008: 28.2°C, range = 24.6–28.4°C). The sun rose ca. 05:10–05:54 h and set ca. 20:45–21:30 h (CEST). For purposes of this study, daytime is defined as 06:00–22:00 h and nighttime as 22:00–06:00 h (CEST). No bees were observed to prematurely forage and several observations of cell contents confirmed task-based caste identity for nurse bees and food storers.

Analysis

All analyses are based on a total of 84 h of audio data and 78 h of thermal data (cell cleaners: 32 h, nurse bees: 12 h, food storers: 10 h, foragers: 24 h). Data include bee identity, behavior, location relative to the hive's edge (x, y coordinates), location relative to cell contents, and to T_{bee} and T_{surr}. Analyses computing placement in the hive were conducted using Python (version 2.6, http://www.python.org). Results from tests using continuous response variables and behavior as a categorical, independent variable are products of linear mixed-effects models, programmed in R (R Development Core Team 2005), with bee group (caste), T_{surr}, $T_{difference}$, and position in the hive as fixed effects, and individual bee identity as a random factor (i.e., observations were nested within bee). Linear mixed-effects models were fit using the lmer function in the lme4 package (Bates and Sarkar 2006). I used the multcomp package to perform likelihood-ratio tests to distinguish between competing models (Hothorn et al. 2008); because of the complicated and unbalanced nature of the data (e.g. missing data, correlated covariates), I could not run standard likelihood-ratio tests (Pinheiro and Bates 2002). I performed binary logistic regression using STATISTICA (ver. 7, StatSoft, Inc., 1984-2004) with T_{surr} as continuous predictor and behavior as the dependent variable. Means ± standard error means (s.e.m.) reported throughout the text and figure legends are calculated from averaged values, one value per bee, using JMP (JMP, version 8.0, SAS Institute Inc., 2008). I set alpha at 0.05 and reported two-tailed *P*-values for all tests.

RESULTS

Honey Honey bees belonging to each worker caste exhibited sleep. Cell cleaners slept inside cells, while each subsequent age caste slept less and less inside cells, with foragers having slept exclusively outside of cells (Table 2). The only caste to clearly exhibit day-night periodicity with regard to their sleep was the foraging caste, with more total sleep at night ($z = -6.05$, $P = 2.82 \times 10^{-9}$) (Fig. 14) and more deep sleep at night than during the day ($z = -2.32$, $P = 0.0377$; $n = 199$ observations, 32 bees).

Sleep and proximity to the edge of hive

Individual bees, irrespective of worker caste, did not display sleep site fidelity specific to a single location in the hive and could be found sleeping in many different areas over the course of a 24 h period (Fig. 15). The different castes slept in different areas of the hive relative to position of brood comb and surrounding temperature (T_{surr}). Our first objective was to calculate a bee's position relative to the edge of the hive and correlate this distance to a bee's behavior.

Worker bees could position themselves anywhere between the edge of the comb (0 cm) to the very center of the comb (22.25 cm from the nearest edge), where brood are typically tended. Cell cleaners in Colony 2 slept inside cells at the same distance from the edge as when awake ($z = 1.41$, $P = 0.1960$; $n = 158$ observations, 44 bees), but cell cleaners in Colony 1 slept inside cells closer to the edge of the hive than when awake ($z = 2.10$, $P = 0.0497$; $n = 413$ observations, 47 bees) (Fig. 16a). Like cell cleaners in Colony 2, nurse bees slept inside cells at the same distance from the edge as when they were awake. Unlike cell cleaners, nurse bees slept outside of cells as well; nurse bees exhibited light sleep at the same distance they spent time awake or sleeping inside cells, but exhibited deep sleep closer to the edge of the hive ($z = 3.94$, $P = 0.0001$; $n = 469$ observations, 47 bees) (Fig. 17). When nurse bees became food storers, they continued their practice of sleeping outside cells closer to the edge of the hive than when sleeping inside cells or when awake, but for food storers both light and deep sleep were exhibited closer to the edge ($z = 4.43$, $P = 1.75 \times 10^{-5}$; $n = 332$ observations, 43 bees) (Fig. 17). Foragers were not closer to the hive's edge when asleep than when awake, a possible consequence of activity along the hive edge leading to the entrance during the foraging day by foragers, as well as occasional sleep along the frames' edges running through the center of the hive (Fig. 16d). Food storers and foragers spent more time both asleep and awake closer to the edge of the hive than younger castes (asleep: $F_{3,158} = 27.87$, $P < 0.0001$; awake: $F_{3,204} = 19.77$, $P < 0.0001$, ANOVA of pooled data). No caste spent time closer to the edge with respect to day vs. night.

Table 2. Percent and total number of observations bees of different worker castes engaged in wake and sleep. Percent of sleep devoted to sleep inside cells decreased with age and caste (99.5% for cell cleaners, 59.5% for nurse bees, 18.8% for food storers, and 0% for foragers). All data for each bee are included.

		Worker castes			
		Cell cleaners	**Nurse bees**	**Food storers**	**Foragers**
Observations of behavior	Awake	78.0%, 697	67.3%, 335	60.0%, 216	73.4%, 146
	Light sleep	0	6.4%, 32	8.6%, 31	12.1%, 24
	Deep sleep	1	6.8%, 34	23.9%, 86	14.6%, 29
	Sleep inside cells	21.9%, 196	19.5%, 97	7.5%, 27	0
	Total	894, *n* = 95 bees	498, *n* = 47 bees	360, *n* = 43 bees	199, *n* = 32 bees

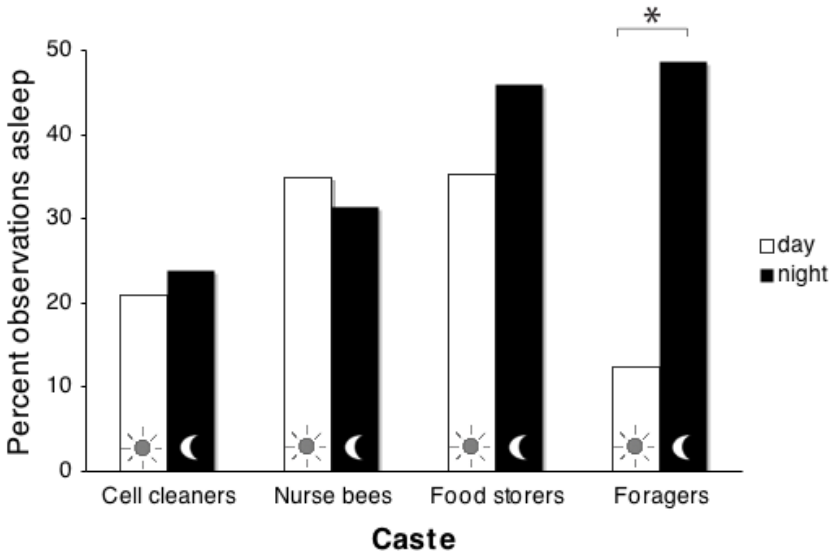

Fig. 14. Proportion of observations bees of different worker castes were asleep during the day and night. Younger castes slept with no distinction between day and night. The clearest statistical distinction (signified with an asterisk) appeared in foragers, with more time spent asleep during the night.

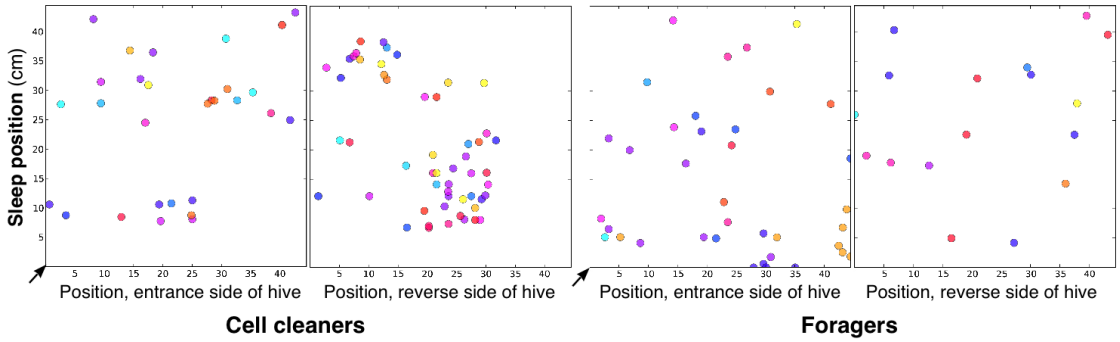

Fig. 15. Position of sleeping cell cleaners and foragers on each side of Colony 1's observation hive over the course of 24 h. Individual bees are represented by unique colors within each caste (n = 14, 27, 11 and 8 bees, from left to right). An individual bee can be seen sleeping in many areas of the hive, rejecting the hypothesis that individuals practice sleep site fidelity specific to a single location within the hive. Individual nurse bees and food storers also sleep in different locations over time. The single hive entrance/exit is indicated by an arrow at lower left.

Sleep position relative to surrounding temperature

Temperatures, particularly away from brood comb, fluctuate in regions of the hive, so we investigated the thermal position of bees with respect to day vs. night, as well as with respect to behavior. Our objective was to determine if differing thermal environments within the hive could serve as a predictor of a bee's behavior. We performed a binary logistic regression analysis to test the probability of predicting a bee's behavior by her T_{surr}.

Cell cleaners slept inside cells in warmer regions of the hive than when awake (33.62 ± 0.13ºC vs. 33.08 ± 0.09ºC, n = 42 & 83 averaged values; χ^2 = 13.69, P = 0.0002). Nurse bees did not spend more time asleep in warmer or colder regions, either inside or outside cells (31.69 ± 0.37ºC vs. awake 31.62 ± 0.27ºC, n = 23 & 42 averaged values; χ^2 = 0.14, P = 0.7129). Food storers slept in colder regions than when awake (χ^2 = 10.08, P = 0.0015), but only when sleeping outside cells (deep and light sleep, 30.68 ± 0.16ºC vs. awake 31.31 ± 0.12ºC), not when sleeping inside cells (31.10 ± 0.27ºC). Foragers slept outside cells in colder regions of the hive than when awake (32.55 ± 0.17ºC vs. awake 33.65 ± 0.15ºC, n = 25 & 32 averaged values; χ^2 = 28.92, P < 0.00001) (Fig. 18).

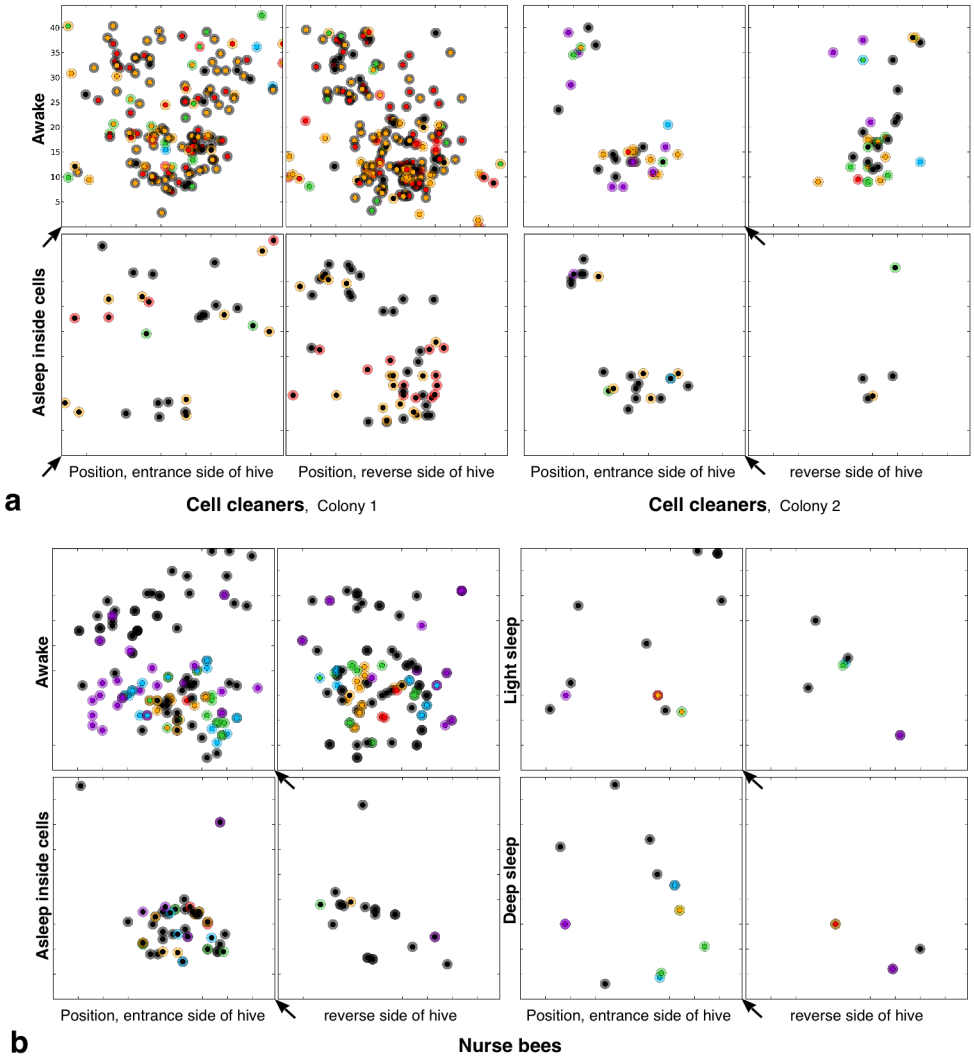

Fig. 16. Position of honey bees with respect to their caste, behavior, and temperatures T_{bee} and T_{surr}. Concentric circles represent T_{bee} (inner circle) and T_{surr} (outer halo) for each bee observation. Temperatures were retroactively binned for graphical purposes according to distributions revealing temperature differences that correlated with behavior (≤31ºC = violet, >31-32ºC = cyan, >32-33ºC = green, >33-34ºC = yellow, >34ºC = red, black = no data). Hive entrance/exit is indicated by an arrow, and was restricted to one side of the hive. All bee data are included in these graphs, but we treated bee as a random factor in mixed effects analyses. **(a)** Cell cleaners slept in the same areas of the hive where they spent time awake (see text for possible exception), and spent time sleeping in warmer areas (i.e., higher T_{surr}) than when awake. No T_{bee} data were available for sleeping bees because cell cleaners slept exclusively inside cells. **(b)** Nurse bees exhibited deep sleep outside cells closer to the edge of the hive than when asleep inside cells or awake.

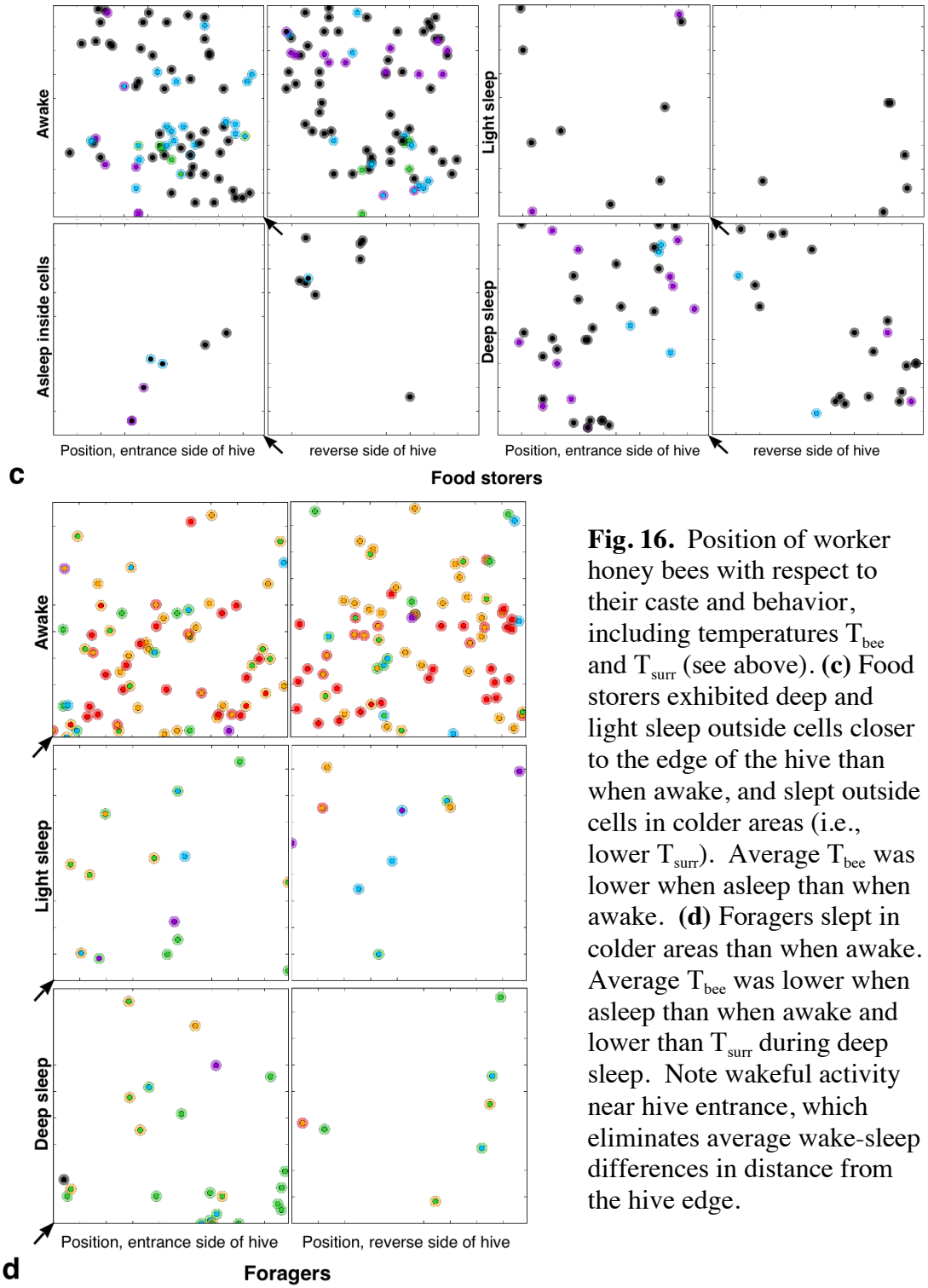

Fig. 16. Position of worker honey bees with respect to their caste and behavior, including temperatures T_{bee} and T_{surr} (see above). **(c)** Food storers exhibited deep and light sleep outside cells closer to the edge of the hive than when awake, and slept outside cells in colder areas (i.e., lower T_{surr}). Average T_{bee} was lower when asleep than when awake. **(d)** Foragers slept in colder areas than when awake. Average T_{bee} was lower when asleep than when awake and lower than T_{surr} during deep sleep. Note wakeful activity near hive entrance, which eliminates average wake-sleep differences in distance from the hive edge.

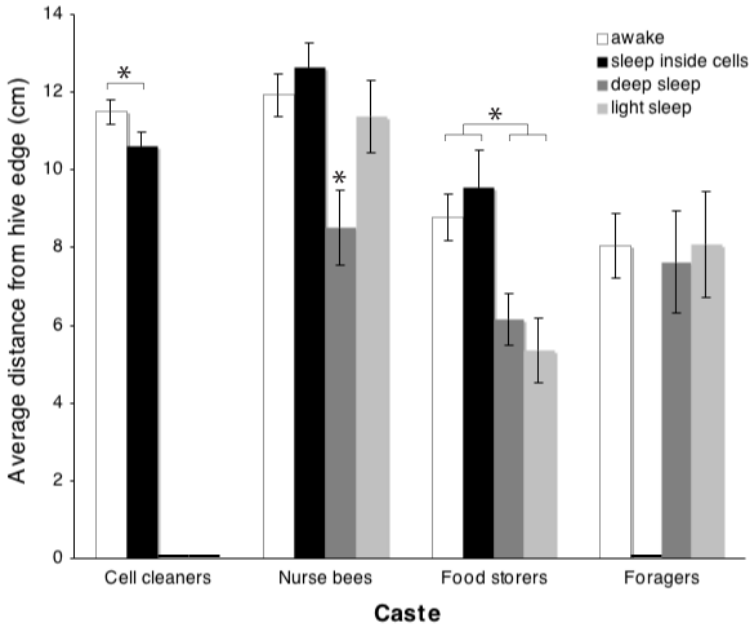

Fig. 17. Distance from the hive's edge with respect to behavior and caste. Younger castes (cell cleaners and nurse bees) slept closer to the center of the hive (i.e., farther from the edge) than older castes (food storers and foragers). Bees slept outside cells closer to the edge of the hive than when they slept inside cells or were awake, except in the case of foragers, which were active during the day near the hive entrance. Cell cleaners never slept outside cells and foragers never slept inside cells. These data represent averages for castes calculated from average values per bee (± s.e.m.). Asterisks signify statistically significant differences.

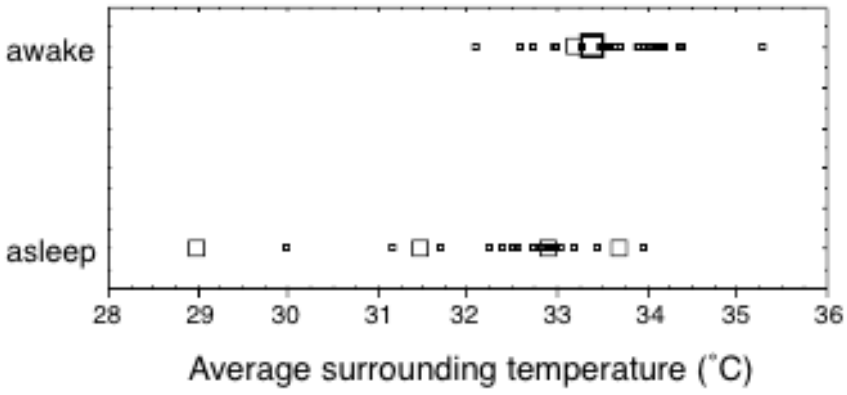

Fig. 18. Relationship showing the average temperature surrounding foragers as a continous predictor of foragers' behavior. When T_{surr} was lower, foragers tended to be asleep ($\chi^2 = 28.92$, $P < 0.00001$, $n = 25$ and 32, one mean value per bee for each behavior). Larger squares signify larger sample (largest $n = 12$ bees).

Applying the same binary logistic regression analysis, we examined Tsurr's ability to predict if a bee was observed during the day or night. Cell cleaners and nurse bees spent more time with warmer surroundings during the night than during the day (cell cleaners: $\chi^2 = 16.39$, $P = 0.00005$; nurse bees: 32.20 ± 0.21ºC vs. day 28.97 ± 0.32ºC, $\chi^2 = 40.28$, $P < 0.00001$). Food storers, however, did not spend more time in colder or warmer regions of the hive ($\chi^2 = 0.66$, $P = 0.4179$) and foragers spent more time in colder regions of the hive at night than during the day (32.76 ± 0.14ºC vs. 33.85 ± 0.14ºC, $n = 24$ & 27 averaged values; ($\chi^2 = 26.43$, $P < 0.00001$).

Sleep temperature

We examined the average temperature of the bees' mesosomas (T_{bee}) and took the difference of T_{surr} from T_{bee} as a measure of a bee's temperature relative to her surroundings ($T_{difference}$) to test if variation in T_{bee} or $T_{difference}$ is explained by a bee's behavior. All data were extracted from bees when they were exposed, and not inside comb cells.

Nurse bees occasionally slept outside of cells and T_{bee} did not differ between sleeping and awake bees. Food storers often slept outside of cells and T_{bee} was lower when asleep (notably when exhibiting deep sleep) than when awake (30.71 ± 0.16ºC vs. 31.42 ± 0.12ºC; deep: $z = -3.24$, $P = 0.0035$; light: $z = -2.27$, $P = 0.0651$; $n = 83$ observations, 36 bees). Foragers always slept outside of cells and T_{bee} was also lower when asleep than when awake. This reduced temperature while asleep was true for foragers exhibiting deep sleep (32.29 ± 0.23ºC vs. 33.43 ± 0.15ºC, $z = -3.01$, $P = 0.0099$) or light sleep (31.84 ± 0.24ºC, $z = -5.59$, $P < 0.001$) (Fig. 19a).

Day and night had an effect on some of the bees' T_{bee}. Nurse bees were warmer at night than during the day (32.11 ± 0.22ºC vs. 28.92 ± 0.36ºC, $z = 5.55$, $P < 0.001$), food storers' T_{bee} did not differ between day and night (31.24 ± 0.12ºC vs. 31.03 ± 0.15ºC, $z = -0.07$, $P = 0.9999$; $n = 83$ obs., 36 bees), and foragers' T_{bee} was lower when asleep than when awake during the day and night ($z = -6.03$, $P < 0.001$; $n = 199$ obs., 32 bees) (Fig. 19b). T_{bee} was lower in sleeping nurse bees during the day vs. the night ($z = 2.42$, $P = 0.0214$; $n = 22$ obs., 9 bees), but not significantly different between day and night for

sleeping food storers ($z = 0.48$, $P = 0.8170$; $n = 22$ obs., 18 bees) or for sleeping foragers ($z = -1.44$, $P = 0.2420$; $n = 53$ observations of 16 bees) (Fig. 20).

The only caste for which $T_{difference}$ differed between sleeping and awake bees was the foragers. A sleeping forager's $T_{difference}$ was lower than an awake forager's (-0.48 ± 0.07ºC vs. -0.21 ± 0.06ºC), particularly during deep sleep (-0.55 ± 0.09ºC, $z = -3.56$, $P = 0.0011$) (Fig. 19c). Examples of $T_{difference}$ in which $T_{bee} < T_{surr}$ are visible in Fig. 16d.

All bees outside of cells were colder when situated closer to the edge of the hive, although foragers were colder specifically at night when closer to the edge of the hive (T~day/night*proximity to edge; $z = 2.73$, $P = 0.0200$; $n = 199$ observations, 32 bees); proximity to edge did not independently affect foragers' T, but foragers were often active and hot near the hive entrance (Fig. 16d). We could not examine sleep by cell cleaners with respect to their T_{bee} or $T_{difference}$ because cell cleaners slept inside cells, but awake cell cleaners' T and $T_{difference}$ did not differ day vs. night (Fig. 20).

Sleep relative to position of brood comb

We analyzed comb cell contents with respect to position of cell cleaners and foragers to test if variation in the proportion of specific cell contents is explained by a bee's behavior. Cell cleaners were more likely to be asleep than to be awake as the proportion of cells in their vicinity increasingly consisted of uncapped brood (surrounded by 32.27 ± 0.03% uncapped brood vs. awake: 22.56 ± 0.03% uncapped brood, $t_{83} = 2.14$, $P = 0.0356$; with trend in mixed effects model: $z = -1.79$, $P = 0.0738$; $n = 413$ observations, 47 bees). When cell cleaners became foragers, they were more likely to be awake than to be engaged in deep sleep as the proportion of cells in their vicinity increasingly consisted of uncapped brood (surrounded by 23.96 ± 0.08% uncapped brood vs. awake: 43.11 ± 0.05% uncapped brood, $z = 2.45$, $P = 0.0267$; $n = 199$ observations, 32 bees) (Fig. 21). Cell cleaners outside of cells did not differ with respect to their T_{bee} as they were surrounded by an increasing proportion of uncapped brood. Foragers' T_{bee} increased as they were surrounded by an increasing proportion of uncapped brood ($z = 2.88$, $P = 0.0075$; $n = 199$ observations, 32 bees). Neither cell cleaners' nor foragers' T_{bee} differed as they were surrounded by a majority of uncapped brood relative to a majority of any other single cell content (e.g., honey).

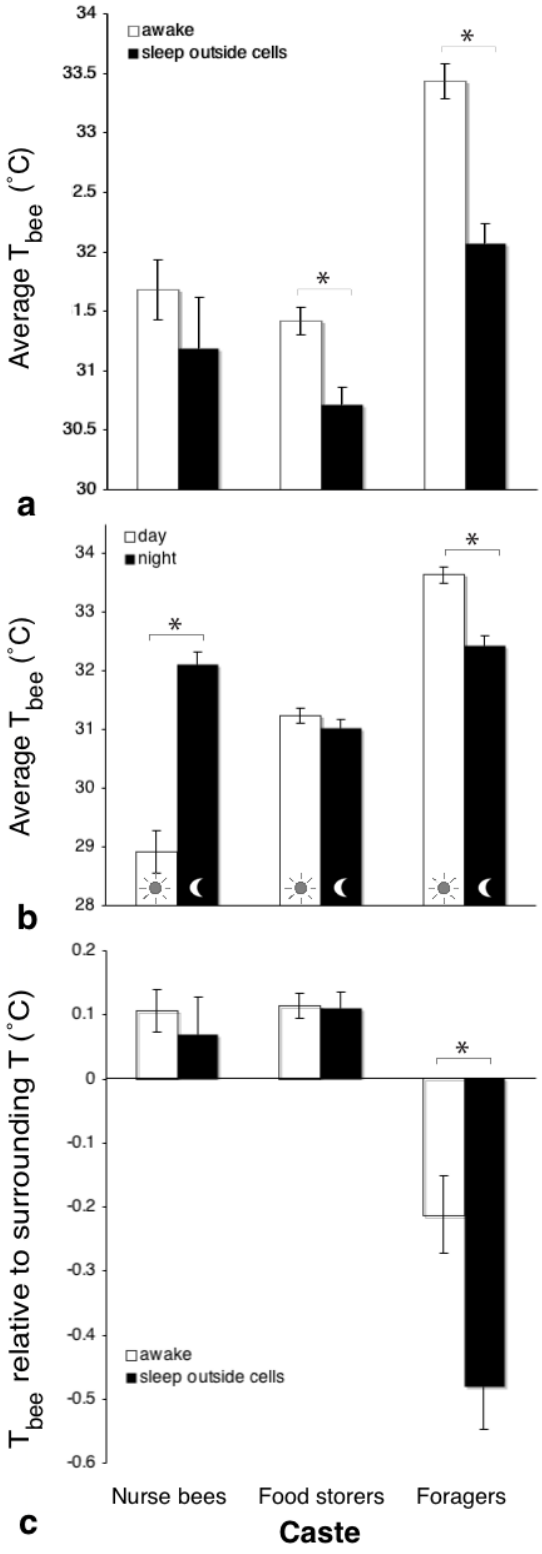

Fig. 19. Average temperature of bees' mesosomas (T_{bee}) with regard to caste, behavior, day vs. night, and relative to surface temperature of surroundings (T_{surr}). All measures were taken from bees outside of cells. Because cell cleaners slept exclusively inside cells, cell cleaners are excluded. Data represent averages for castes calculated from average values per bee (± s.e.m.). Asterisks signify statistically significant differences. (a) Average T_{bee} was lower when asleep than when awake, except in the case of nurse bees. (b) Average T_{bee} was higher during the night for nurse bees and lower during the night for foragers. (c) Foragers, asleep and awake, exhibited lower T_{bee} relative to T_{surr} ($T_{difference}$) and a significantly lower $T_{difference}$ when asleep than when awake.

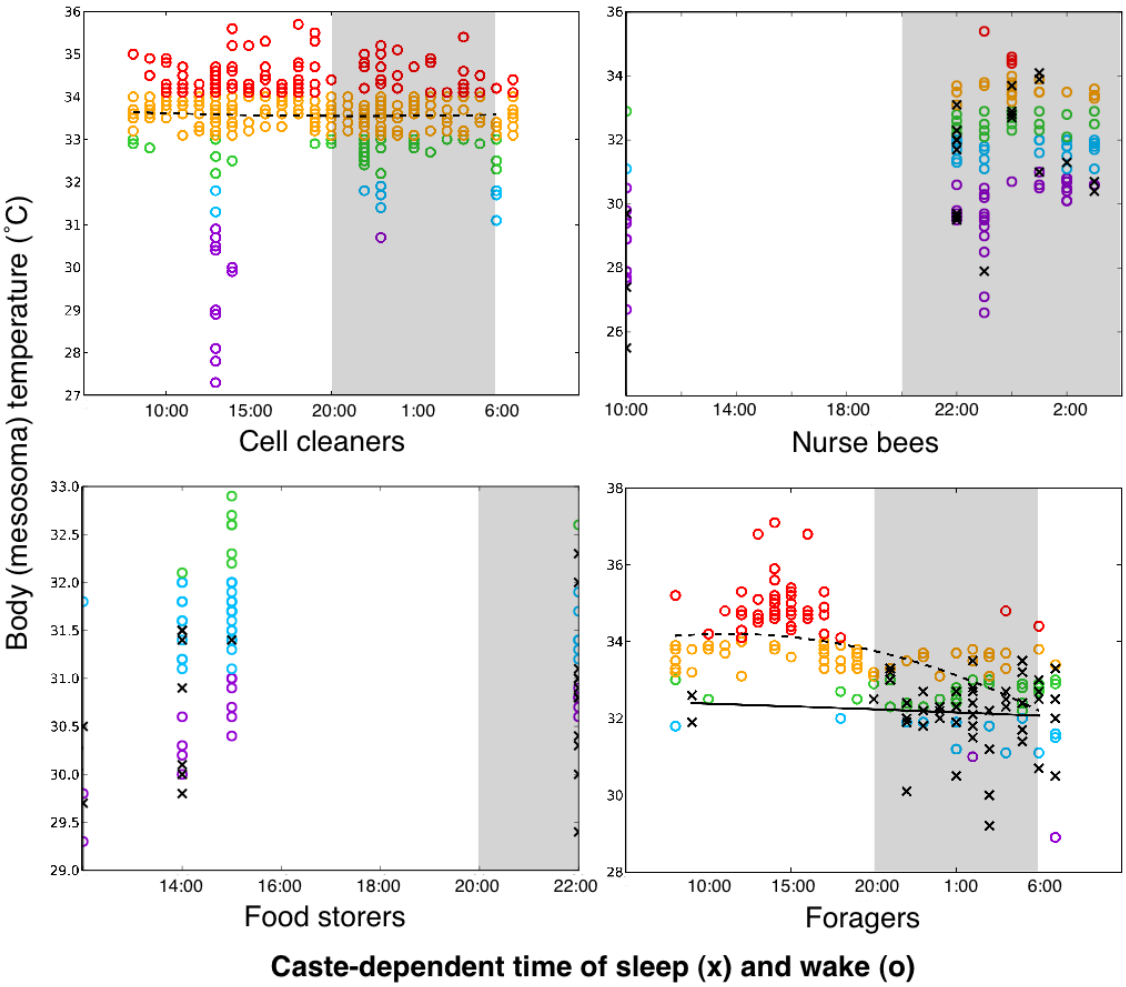

Fig. 20. T_{bee} over the course of 24 h with respect to bees' caste and behavior. Awake cell cleaners' T_{bee} did not change over time. Nurse bees were warmer at night, but did not differ with respect to wake or sleep. Food storers and foragers were cooler at night than during the day, but their T_{bee} while sleeping did not significantly change over time. Average T_{bee} is reported per bee per census period and, although all bee data are included in these graphs, we treated bee as a random factor in mixed effects analyses. Colors signify temperature bins (≤31ºC = violet, >31-32ºC = cyan, >32-33ºC = green, >33-34ºC = yellow, >34ºC = red). An observation of an awake bee is represented by **o** with dashed lines fitting the data; an observation of a sleeping bee is represented by **x** with solid lines fitting the data. All measures were taken from bees outside of cells. No data exist for cell cleaners sleeping outside cells, hence the absence of **x**'s in the cell cleaner graph. Note data on edges of graphs. Gray backdrop represents nighttime.

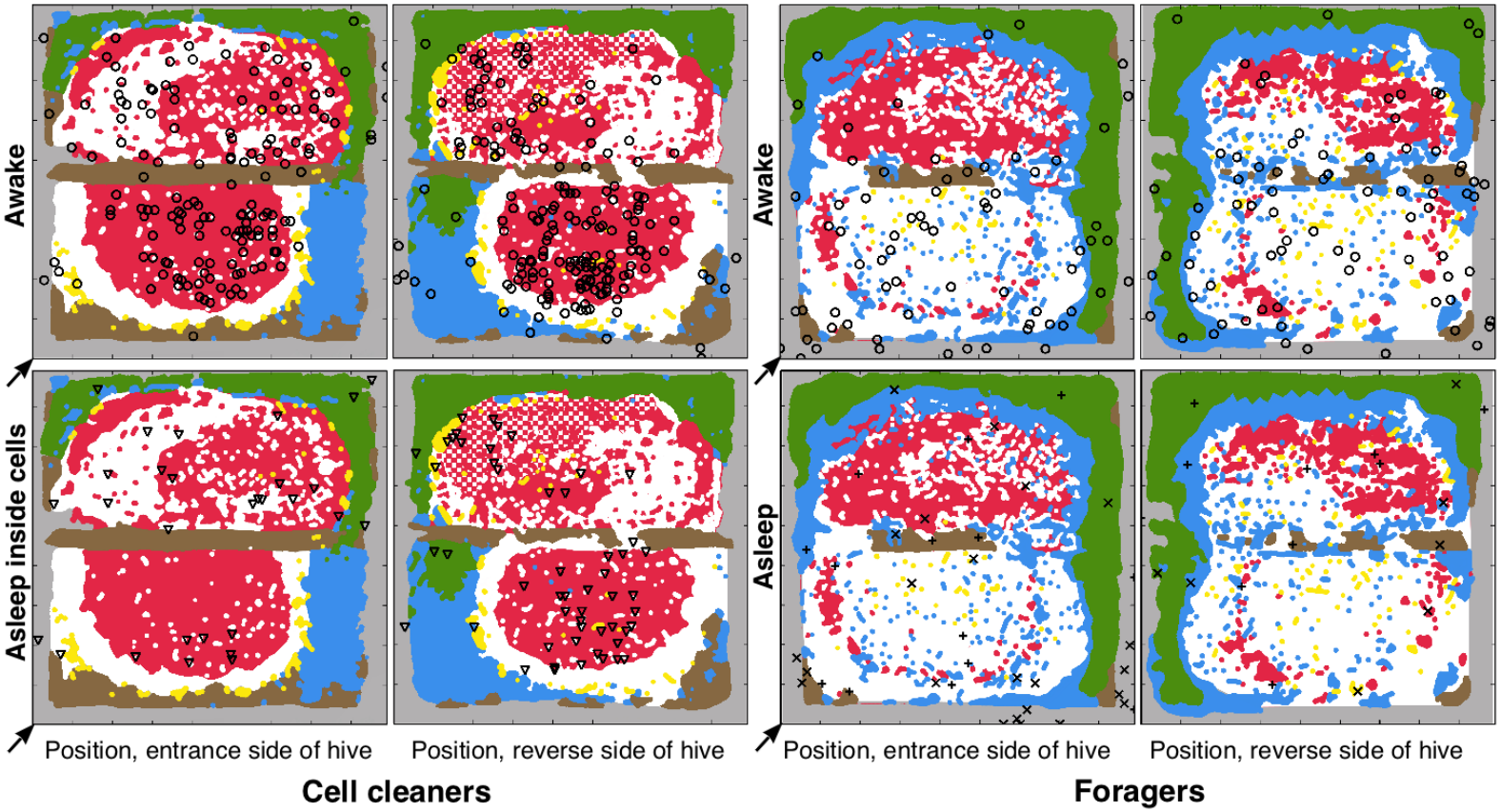

Fig. 21. Caste-dependent maps of behavior with regard to comb cell contents. Colony 1's hive changed as the bees aged, resulting in a spatial organization of bees with respect to comb cell contents that correlated with their behavior. Cell cleaners were awake (circles) and asleep (triangles) primarily in the brood comb area, spending more time asleep than awake with uncapped brood in their midst. Foragers spent more time awake (circles) than asleep (**x** = deep sleep, **+** = light sleep) near uncapped brood comb. White = uncapped brood, red = capped brood, yellow = pollen cells, blue = uncapped nectar, green = capped honey, brown = empty comb, gray = no comb. Empty comb (brown) is underrepresented, as evidenced by the lack of brown spots representing single cells used as sleep sites by cell cleaners.

DISCUSSION

The observed caste-dependent patterns of sleep were consistent with previous studies of honey bees, including sleep inside and outside of cells (Klein et al. 2008), day-night periodicity of sleep in foragers (Kaiser 1988, Sauer et al. 2003, Klein et al. 2008) and the absence of periodicity in the younger castes (cell cleaners and nurse bees) (Sauer et al. 1998, Sauer et al. 1999, Eban-Rothschild and Bloch 2008, Klein et al. 2008). Food storers did not exhibit day-night periodicity in this study, but have previously been reported to exhibit either circadian sleep (Klein et al. 2008) or, in the case of a single subject, ultradian periodicity (12 h sleep-wake cycles) (Sauer et al. 1998). This transitional age caste appears to be inconsistently periodic, a possible product of changing colony demands driven by factors such as foragers' unloading rates.

Sleep locations, although spatially variable within individual bees of each caste, differed among the worker castes due to the frequency with which caste members slept inside or outside of cells. All worker castes that spent time asleep inside cells did so at the same distance from the edge of the hive as when they were awake, but slept outside cells closer to the edge of the hive, with the exception of foragers and cell cleaners in Colony 1. Foragers appear to sleep, and especially to exhibit deep sleep, on the periphery of the hive (Fig. 21), but forager activity during the day along the bottom edge of the hive leading to the hive entrance probably eliminated a statistical distinction between sleep and wake with respect to proximity to the hive's edge. Additionally, several sleep sites fell along the wooden edges of the combs' frames, which run through the center of the hive (Fig. 16d) and were statistically treated as distant from the hive's edge. Although cell cleaners in Colony 1 slept closer to the edge than when awake, they were primarily on uncapped brood, as pictured in Fig. 21, and certainly within the brood comb, as evidenced by the expansive heat of the brood comb in the top right image of Fig. 22a. Overall, variation in distance from the hive edge is explained in part by behavior. Were proximity to the hive edge to be used to predict probability of sleep, forager activity near the hive entrance should be accounted for and hive areas without comb (i.e., frames) should be treated as edges.

Temperature of a bee, her surroundings, and the difference of the two can serve as helpful correlates of sleep behavior. First, we begin with the average surface temperature

of a bee's surroundings (T_{surr}). A bee can impact T_{surr}, as best demonstrated by the actions of heater bees (Bujok et al. 2002, Kleinhenz et al. 2003). When not heating, however, the effect on T_{surr} is less dramatic (Fig. 11d) or insignificant (Fig. 11a-c), and is more appropriately treated as the independent variable and behavior as the dependent variable. We tested the probability of predicting a bee's behavior by her T_{surr} and found that sleep inside cells occurred either in areas as warm as when they were awake (nurse bees and food storers) or even warmer areas than when awake (cell cleaners). Sleep outside of cells occurred in colder regions than when awake (food storers and foragers), except in nurse bees, although this was likely due to the small sample size of nurse bees sleeping outside cells (non-black halos in graphs of light and deep sleep, Fig. 16b). Worker castes exhibited different patterns with respect to T_{surr} at night versus during the day, giving temporal context possible importance when inferring bee behavior using T_{surr}.

Surrounding surface temperature may not perfectly reflect the ambient temperature experienced by a bee, considering that each bee moving in her vicinity has the potential to cause ephemeral changes in T_{surr} and the air temperature can differ from the surface temperature of wax and bees in her midst. This may account for the difference between our measure of T_{surr} encircling the average sleeping forager (32.6 ± 0.2ºC) and the preferred ambient temperature of sleep, as reported by Kaiser et al. (2002) in isolated foragers (23–26˚C, with extremes of 21 and 29˚C) and Schmolz et al. (2002) in isolated foragers (28˚C, range: 26–29˚C) and foragers within a hive (27.9˚C, range: 23.8–30.8˚C). On the other hand, our average measure of a forager's T_{bee} during deep sleep (32.29 ± 0.23ºC) was congruent with measurements of resting bees inside cells by Kleinhenz et al. (2003) (32.7 ± 0.1–33.4 ± 0.3˚C).

We tested if variation in T_{bee} or $T_{difference}$ is explained by a bee's behavior and found that T_{bee} is lower for sleeping food storers and foragers and this lower T_{bee} is statistically indistinguishable between day and night. A relatively static lower sleeping temperature for the older castes is a consequence of sleeping closer to the colder periphery of the hive, although this correlation was confounded in foragers by their activity near the hive entrance. T_{bee} of sleeping nurse bees may not have differed from their wakeful T_{bee} due to either insufficient sample size of nurse bees sleeping outside cells, or because sleep bouts are shorter in nurse bees than in older workers (Klein et al.

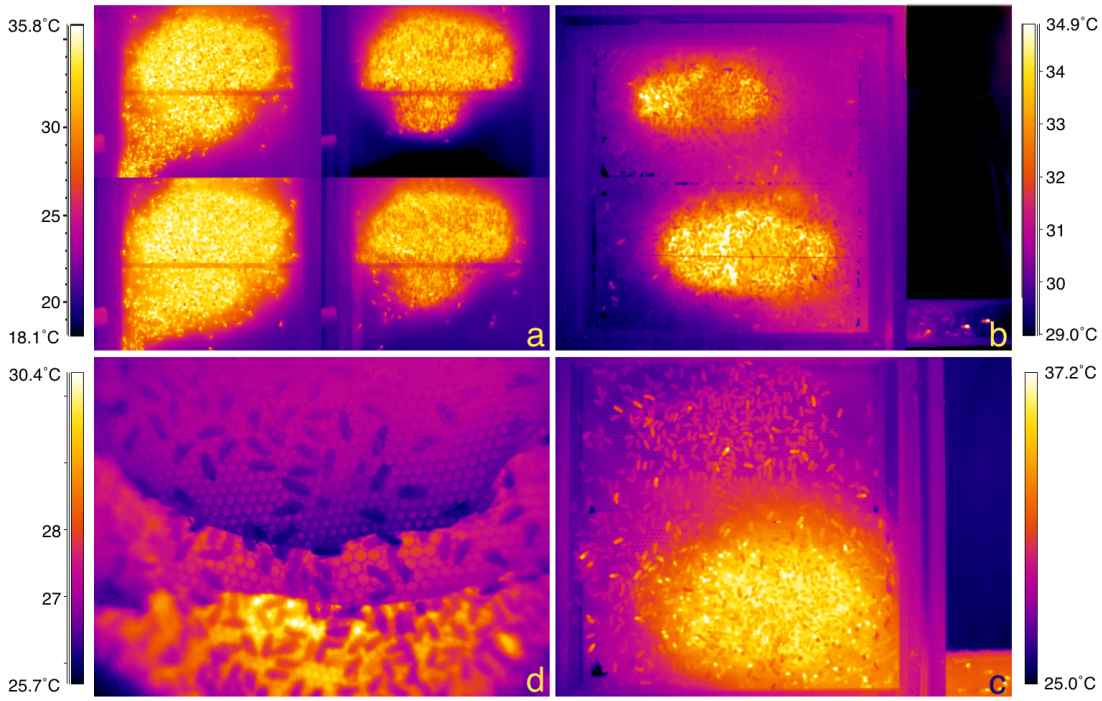

Fig. 22. Infrared images revealing thermal activity across honey bee hives. (a) Sequence of colony-scale changes across the entrance side of Colony 1. In clockwise order from the upper left corner, 17:00, 04:00, 09:00 and 15:00 h. Hive entrance/exit is in the lower left corner of the hive (tube on left of hive frame in each image of sequence). (b) Colony 2, with hive entrance/exit tunnel at lower right. (c) Colony with single brood comb in lower frame. (d) Exposed hive composed of parallel sheets of comb, set up by D. Ahrens-Lagast to induce bees' to construct a more natural nest architecture. All images were taken with FLIR thermal cameras by BAK.

2008), not allowing for T_{bee} to significantly decrease. A sleeping forager's $T_{difference}$ was lower than an awake forager's and this difference could serve as a predictable guide for thermally distinguishing sleeping foragers. The $T_{difference}$ was less than zero (i.e., $T_{bee} < T_{surr}$), confirming that T_{surr} does not match the ambient T experienced by a bee.

Brood comb is warmer than other regions of the hive and is typically the most active area of the hive, setting the stage for brood comb location as a factor impacting sleep positioning. The proportion of uncapped brood comb in the vicinity of cell cleaners and foragers correlated with behavior and we would expect nurse bees to exhibit the same pattern as cell cleaners, with more brood nearby as they sleep. The pattern of sleep with respect to brood comb depends, of course, on the hive's distribution of brood. Brood comb is often centrally located, but can vary across observation hives (Fig. 22a-c).

Observation hives are typically two-sided, but they are not as three-dimensional as natural hives, which consist of a series of parallel combs (Fig. 22d). The organization of brood comb and of thermal microclimates in an observation hive will differ from that of the three-dimensional hive box (Szabo 1985, Humphrey and Dykes 2008) or architecture of a feral colony. Sleep within more three-dimensional hives may follow the same rules of T_{surr}, brood comb positioning, and distance from the edge as we report here, or it may translate into a more complicated set of determinants (e.g., comb position relative to central comb, or shape and volume of cavity housing hive). Finding a location to sleep could vary for a bee depending on if she is in an observation hive, a hive box with multiple frames, a natural hive, or a perched swarm, which features its own thermal dynamics (Heinrich 1981). Sleep positioning could differ across honey bee species, some of which produce exposed comb, exist in hotter climates, and differ in colony demography and individual energetics (Dyer and Seeley 1987). Even honey bee subspecies show different behavioral traits and metabolic rates (W-Worswick 1987), which could contribute to sleep positioning by its individuals.

Why do bees sleep inside cells, why does this differ temporally among castes, and why is there a spatial distinction between sleep inside and outside cells within the same caste of bees? Caste-dependent sleep patterns could emerge by a variety of mechanisms. Sleep outside cells exposes bees to the arousing interactions of wakeful, mobile siblings (Klein et al. 2008 supplementary video) and the greater density of bees found in the brood comb area would mean more frequent arousals and more fragmented sleep. By sleeping closer to the edge of the hive, exposed bees may increase their sleep. Klein et al. (2008) reported increasing durations of uninterrupted sleep outside cells for bees as they aged/changed castes, which is consistent with this hypothesis.

Sleep inside cells may constitute an adaptive response to avoiding sleep fragmentation within the brood comb area. Younger bees sleep more frequently inside cells than older bees and this could conceivably be the result of differential cell vacancy rates, with empty cells less frequently available in the brood comb, and more readily inhabited by bees already working in the brood comb area and working within actual comb cells. Older bees may sleep less (food storers) or not at all (foragers) inside cells closer to the edge of the hive because they do not face the same degree of disturbance as

they would if exposed in the brood comb. Sleep away from the brood comb may be a result of either learned or instinctual avoidance, or unlearned displacement due to repeated disturbance in the brood comb. Empty cells often dominate along the periphery of the comb, ruling out the possibility that sleep inside cells in the brood comb area by younger bees is due to a greater availability of empty cells toward the center of the comb.

Caste-dependent sleep patterns may be the consequence of selection pressures for sleeping in warmer or cooler areas. By sleeping in colder areas, food storers and foragers may conserve energy and by sleeping in warmer areas, cell cleaners may increase neural development or facilitate consolidation of memories. Schmolz et al. (2002) reported that foragers sleep ectothermically and hypothesized that foragers select cool, but not maximally cool regions to sleep for the purpose of conserving energy while still promoting regenerative processes during sleep. Stabentheiner et al. (2010) reported that ectothermy is most common in the youngest bees (0 to ~2 d) and proposed that visitation to warm cells within the brood comb serves to increase flight muscle development. Non-mutually exclusive additional explanations could include reduction of pathogen spread by segregation of sleeping castes, or an increased protection of younger, less expendable bees at the center of the hive. Alternatively, an awake bee may fall asleep without changing location simply by default. Cell cleaners and nurse bees may sleep inside cells in the same area of the hive as when awake for no other reason than they happened to be there. Foragers, likewise, may sleep near cooler edges of the hive because they were awake along the edges, or traveled to edges for non-sleep-related reasons (e.g., to decrease their temperature after flight activity).

Some of the variables that have great potential in affecting the timing and extent of caste-dependent sleep patterns include the size, density and demographics of a colony, timing of resource availability, colony demands, architecture of the hive, season and weather, and honey bees' genetic predisposition. Colony size, social interactions (Huang and Robinson 1996), weather (Riessberger and Crailsheim 1997), resource availability (Schulz et al. 2002) and the timing of resource availability (Klein and Seeley 2007) affect foraging and hive activity. Colony density, ambient temperature (Kronenberg and Heller 1982), and nest architecture (Szabo 1985) greatly determine the temperature and thermal flow within the hive, and seasonal change (Fahrenholz et al. 1989, Bloch et al. 2006),

genetic stock of a colony (Calderone and Page 1989) and gene-environment interactions (Toma et al. 2000) undoubtedly each play a role in shaping bees' sleep behavior.

Our research objective was to produce "sleep maps," which could serve as spatial and temporal guides to the organization of sleep within a colony. Many factors contribute to the organization of sleep in honey bees, but certain correlations of behavior with temperature, presence of brood, and distance from the edge of the hive can offer the framework for testing mechanisms and functions relating to patterns of sleep behavior. By mapping sleep, we can better appreciate the plasticity and test the task-specific functionality of sleep in colonies of honey bees. Thermal and behavioral maps of honey bees may serve as a new means of systematically visualizing sleep within a society.

CHAPTER 3

Change in work schedule induces change in sleep schedule in honey bee foragers

ABSTRACT. Shift work tests humans' capacity to be flexible when scheduling both work and sleep. Foraging honey bees shift their work schedules in response to resource availability and they exhibit a human-like pattern of sleeping primarily at night. No previous study, however, has tested the plasticity of the timing of sleep by foragers with regard to their shifting work schedules, despite the importance of colony-level plasticity in the face of a changing environment. I hypothesized that sleep schedules in honey bees are not fixed and instead vary depending on the timing of resource availability. I trained individually marked bees to visit a food source early in the morning or late in the afternoon, then monitored the bees' behavior for 24 h after each training session to compare sleep behavior. Following AM-training, foraging honey bees slept more during the afternoon than during the morning, but when trained to a food source in the late afternoon, the reverse was true: the same bees consequently slept more in the morning than in the late afternoon. Although total amount of time devoted to any behavior, including sleep, did not change with respect to resource availability, the *timing* of sleep changed. Thus, plasticity in timing of foraging was matched by plasticity in timing of sleep. The correlation between the schedules of foraging and sleeping demonstrates, for the first time, temporal plasticity of sleep under ecologically realistic conditions in an invertebrate.

INTRODUCTION

Scheduling sleep can be a challenge, particularly when external factors, such as employment and social demands impinge upon otherwise circadian, consolidated sleep. Humans often institute "shift work," an employment practice that attempts to make full use of each day's time by requiring employees to accommodate shifting work schedules. Extreme changes in circadian cycles of work/sleep can cause varied health and performance problems (reviewed by Rajaratnam and Arendt 2001, Garbarino et al. 2002, Arendt 2010) ranging from cancers and gastrointestinal dysfunction (Costa 1996) to

degraded ability to detect simulated threats (weapons) as a luggage screener (Basner et al. 2008). Extreme shifting of work schedules requires shifting of sleep schedules, whereupon planned napping can aid in performance (Smith-Coggins et al. 2006), as it can even under stable daytime work schedules (reviewed by Dhand and Sohal 2006). The ability to respond flexibly to changes in work schedules by changing sleep schedules exists in humans, but is not well established in non-human animals under relatively normal working conditions. To probe the evolutionary breadth of sleep flexibility requires an understanding of what defines sleep and requires tests of non-humans' capacity to accommodate changes in the timing of wake-state activities with changes in the timing of sleep. Our aim was to test the flexibility of timing of sleep in foraging honey bees, following a change in timing of work.

Defining sleep behaviorally requires identifying a suite of behavioral traits indicative of sleep. A sleeping organism tends to be quiescent, exhibits a posture that is stereotypical or species-specific, and is in a state that is easily reversed (i.e., can be woken), but only with increased external stimulation (Piéron 1913, Flanigan 1973). According to Tobler (1985), a sleeping organism also experiences a sleep rebound (i.e., more sleep, or deeper sleep) after suffering a sleep deficit. Behavioral sleep has been defined in honey bees (*Apis mellifera*) (Kaiser 1988, Sauer et al. 2004), the fruit fly *Drosophila melanogaster* (Hendricks et al. 2000, Shaw et al. 2000) and other invertebrates (Tobler 1983, Tobler and Stalder 1988, Tobler and Neuner-Jehle 1992, Klein 2003, Duntley and Morrissey 2004, Ramón et al. 2004, Brown et al. 2006). One feature that invariably accompanies other sleep indicators in honey bees is discontinuous ventilation (Sauer et al. 2003). Sleeping bees ventilate their bodies by performing several dorso-ventral pulses of the abdomen followed by extended pauses of immobility (Kaiser et al. 1996, Kleinhenz et al. 2003, Sauer et al. 2003). This behavior can be identified when bees are inside or outside of comb cells. When honey bees discontinuously ventilate and they exhibit extended bouts during which their antennae are immobile, this correlates with increased response thresholds and has been referred to as a deep sleep state (Kaiser 1988). Foragers appear to exhibit sleep stages (Eban-Rothschild and Bloch 2008) and sleep intensity changes through the night (Sauer et al. 2003).

If sleep is important for honey bees, as is suggested by the results of sleep deprivation studies (Sauer et al. 2004; Hussaini et al. 2010; Klein, Chapter 4), then the diurnally-active foragers in a colony should sleep at night. Like humans, honey bees do so (Kaiser 1988, Sauer et al. 2003, Eban-Rothschild and Bloch 2008, Klein et al. 2008). Foragers have strong circadian tendencies (Lindauer 1952, Spangler 1972, Kaiser and Steiner-Kaiser 1983, Moore 1998, Toma et al. 2000, Moore 2001), but they can often appear inactive during the day (Anderson 2001). Foraging need not be a day-long pursuit, and is often driven by a stunningly precise anticipatory Zeitgedächtnis, or time sense (Koltermann 1971, Moore et al. 1989). Unlike their younger siblings (Moore et al. 1998), foragers can perform tasks in a manner that resembles shift work, leaving time in the day available for rest. Foragers can also be entrained to feeding cycles other than 24-h cycles, giving further evidence of their working flexibility (Frisch and Aschoff 1987). While the flexibility of foraging activity is well understood to depend on factors that include resource demand and availability (reviewed in Seeley 1995), nothing is known about how foragers change their sleep schedules in response to work schedules.

Given the capacity of honey bees to be temporally flexible in their foraging and their need for sleep, I hypothesized that honey bees can adjust their sleep schedules in relation to the timing of resource availability. If the timing of work affects the timing of sleep, then I predict that foragers trained to exploit an early morning resource will exhibit an earlier onset of sleep signs than foragers trained to exploit a late afternoon resource. Likewise, I predict that foragers trained to exploit a late afternoon/early evening resource will exhibit a later arousal in the morning (i.e., will "sleep in"). If, however, honey bees cannot adjust their sleep schedules in relation to when forage is available, then I predict that foragers will show the same timing of sleep if they are trained to forage early or late in the day.

METHODS

We[3] transported two colonies of European honey bees (Hive 1: *Apis mellifera carnica*, Hive 2: *A. mellifera ligustica* queen with mixed offspring; queen breeder: C. F. Koehnen and Sons, Inc., Glenn, CA, USA) from Liddell Field Station (Cornell

University, Ithaca, NY) to Cranberry Lake Biological Station in the Adirondack State Park (NY, USA, 44º09”N, 74º48”W). This region of the Adirondacks is otherwise devoid of honey bees and produces few natural food sources for honey bees. We placed each colony (ca. 1500 bees) inside a two-frame observation hive, suspended from the ceiling of a wooden hut (Seeley 1995) (Fig. 23), on 15 July and 9 August 2006. A queen excluder divided the top and bottom frames of each colony, restricting a queen's access to the top frame and eliminating any threat of absconding. To prepare for the study, we began training foragers to a sucrose water solution the morning after setting up each hive (Hive 1 on 16 July and Hive 2 on 10 August) and continued training bees through the afternoon. We individually marked foragers with dry pigments mixed in shellac, painting color combinations unique to each bee on the dorsal side of the thorax (mesosoma) and abdomen (metasoma) (Fig. 23). We trained the marked bees on the first day of the study, but we limited availability of the food source to the early morning (06:45 – 09:00 h for Hive 1 and 06:45 – 10:00 h for Hive 2); we refer to this as the AM-training day. On the second day, we repeated the early morning feeder schedule and recorded visits to the feeder by marked bees; we refer to this as the AM-testing day. Beginning at 06:00 h on the AM-testing day, I recorded each bee's behavior within the hive every 30 min for 24 h, scanning both sides of the observation hive in a consistent manner using a grid superimposed on the glass windows as a visual guide. On days three and four, we blocked the hive entrance with steel screening until 15:30 h, restricting the period during which food was made available to bees to the late afternoon (16:00 – 19:00 h) so that we would eliminate the risk of losing the AM-trained bees to alternate, natural food sources in the morning. I reexamined behaviors exhibited by the same individual bees across a second 24-h period, beginning at 16:00 h on day four. We refer to day three as the PM-training day and day four as the PM-testing day (Fig. 24).

We monitored how many marked bees attempted to leave Hive 1 on the AM-testing and PM-testing mornings. Attempts to leave the hive were defined as records of marked bees entering the hive entrance tunnel and approaching the screened exit. We could not make a comparable count for Hive 2 because bees filled the entrance tunnel in the mornings.

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[3] This research was a collaborative effort and my co-authors are listed in the Acknowledgements.

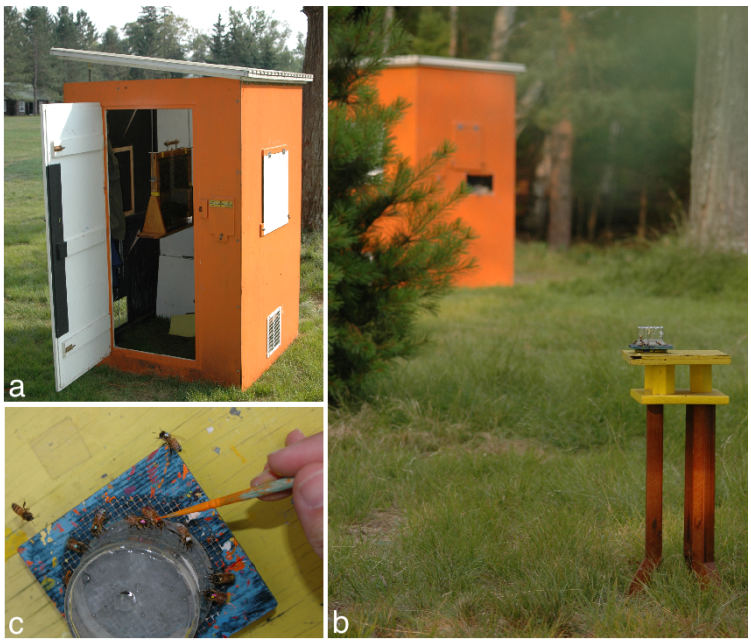

Fig. 23. Training and marking of sleeping subjects. Honey bees were (a) housed in an observation hive suspended from the ceiling of a hut constructed by T.D.S., (b) trained to a sucrose solution, and (c) individually marked for study.

For each colony, we trained the foragers to a feeder 18 m from the study hut and offered a 2.5 molar sucrose solution, scented with anise. We squirted ca. 1-3 mL of the scented solution into the top of the hive and into the entrance tunnel every day of the study at the beginning of the resource availability period (09:00 h or 16:00 h). We used ambient light to observe bees within the hut during the day, and red-filtered lights (headlamp and bulb within desklamp), chosen because of the decreased sensitivity to red perceived by honey bees (von Frisch 1967, Dustmann and Geffcken 2000). We defined "night" as 22:00 h–06:00 h, roughly between sunset and sunrise.

Although we recorded 17 distinct behaviors in the hive, we limited most of our analyses to "awake" versus three different forms of sleep. Awake included body turning/lifting, grooming, locomotion, trophallaxis, tremble dancing, waggle dancing, activity inside comb cells, continuous ventilation inside comb cell (> 5 sec), established departure from the hive, and states of immobility that do not constitute sleep (e.g., not discontinuously ventilating while either masticating, being groomed, engaging in trophallaxis, or while in an alert stance). Our operational definition for sleep inside cells was discontinuous ventilation, with pauses of at least ten seconds between respiratory

pulses of the visible abdomen. Our operational definition for sleep outside cells was discontinuous ventilation for at least ten seconds, combined with relative immobility and antennae either immobile (deep sleep), or exhibiting swaying motions or minute twitches (light sleep) (Klein et al. 2008).

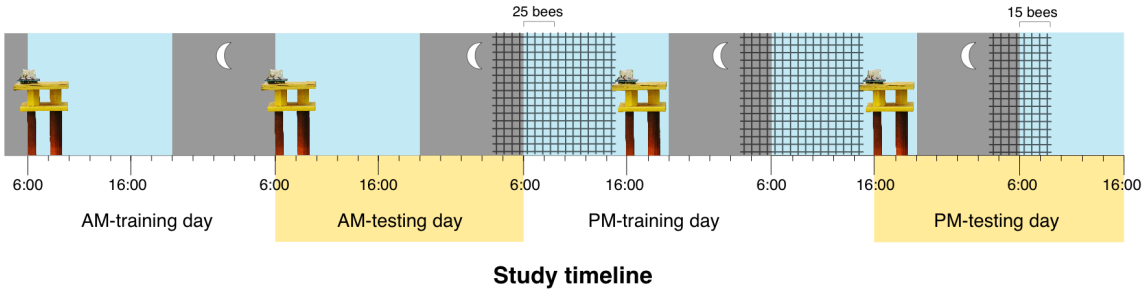

Fig. 24. Schedule of training and resource availability. The study timeline begins after a day of training foragers to a feeder and individually marking them. Foragers were trained to a food resource during the morning (AM-training day), monitored within the hive for 24 h (AM-testing day), then the same bees were trained to the food resource during the afternoon (PM-training day) and monitored within the hive for another 24 h (PM-testing day). Steel screen blocked the entrance on the PM-training and PM-testing days until 15:30 h so that we would eliminate the risk of losing the AM-trained bees to alternate, natural food sources in the morning. Nights are represented by gray areas with crescent moons, the feeder image encompasses the resource availability periods, and the yellow blocks below the timeline highlight 24 h census periods. Twenty-five AM-trained bees and 15 PM-trained bees attempted to depart Hive 1 between 06:00—09:00 h, noted above with bracketed numbers.

Our data on the percentage of sleeping foragers in each census period were not normally distributed, even after arcsine square root transformation, so we applied the non-parametric alternative to the t-test, Wilcoxon signed-rank, to compare the percentage of observations AM-trained bees were asleep on the AM-testing day during the morning versus during the afternoon (AM and PM periods defined above). We then applied the same signed-rank test to compare the percentage of observations PM-trained bees were asleep on the PM-testing day during the afternoon versus the morning. We performed a paired-samples *t* test (Lehman et al. 2005) to confirm the signed-rank test results. To further test for an overall treatment effect, we performed separate chi-square tests on each treatment and summed the chi-squared statistics (if g equals the number of chi-squared

tests, their sum is approximately normal with mean 0 and standard deviation $\sqrt{g}$). The relation can be used to calculate a z-score for the overall model:

$$Z = \sum_{i=1}^{g} {}_i \frac{\sqrt{X_i^2}}{\sqrt{g}}$$

Some bees were both AM- and PM-trained, so to address the assumption of independence of data, we performed a simulation that randomly sub-sampled our data, by randomly selecting half of the bees trained during both periods as AM-trained and the other half as PM-trained for each iteration. The simulation sampled bees without replacement (i.e., each bee appeared once per iteration) for 1000 iterations. To test total observations devoted to individual wakeful and sleep behaviors during the AM-testing and PM-testing periods, we ran a paired-samples *t* test. For every test we used the mean value of percentage observations sleeping per bee per treatment period. We eliminated bees from our sample that were new visitors to the feeder the day prior to testing and eliminated bees known to be dancing for other resources on the day during which they danced ($n = 2$ bees). Also excluded from analyses were AM- or PM-testing periods for which few to no behavioral data were recorded for a bee ($\leq$ 3 census periods, or $\leq$ 6.25% of 48 census periods). The simulation was programmed in R (R Development Core Team 2005) and all other analyses were performed with the JMP (version 8.0; SAS Institute Inc.) computer package. We report summary statistics of continuous variables as means ± standard error (s.e.m.). We set alpha at 0.05 for all tests and all tests were two-tailed.

RESULTS

Foragers trained to a feeder in the morning (06:00–09:00 or 10:00 h) or in the afternoon (16:00–19:00 h) responded by sleeping, and engaging in deep sleep, at different times of the day (Fig. 25). Foragers trained to a feeder in the morning subsequently slept more during the afternoon than during the morning of the AM-testing day (Hive 1: $P = 0.0384$, $n = 15$ bees; Hive 2: $P < 0.0001$, $n = 43$ bees, Wilcoxon signed-rank test) (Fig. 26). This tendency to sleep in the afternoon when trained to a morning resource occurred whether sampling only bees for which we had data for both the AM- and PM-testing periods, or after including bees for which we had data for only the AM- or PM-testing

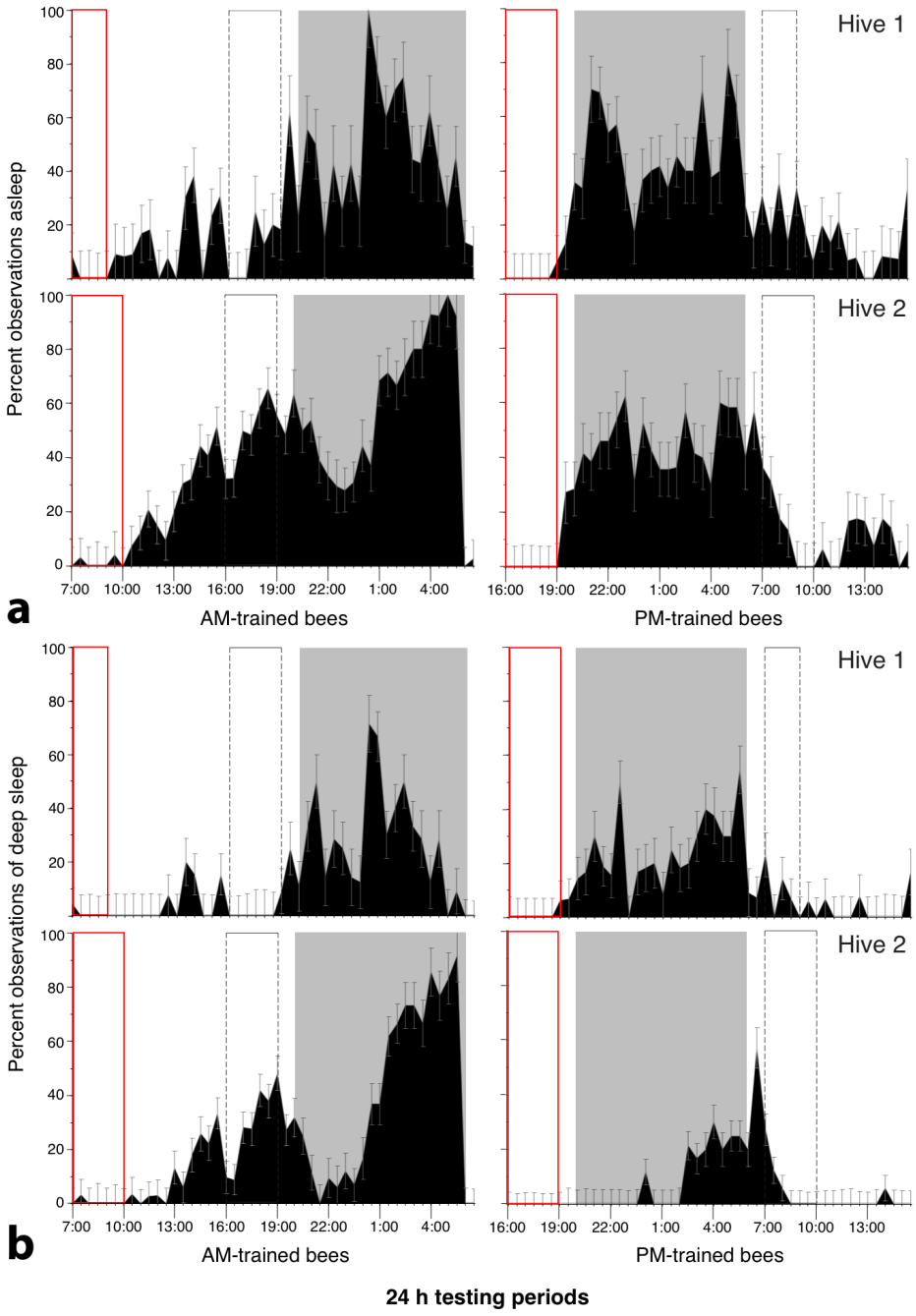

Fig. 25. Percent of observations AM-trained and PM-trained bees were (**a**) asleep and, more specifically, exhibited (**b**) deep sleep outside of cells. Hive 1 bees and Hive 2 bees both experienced daytime sleep, but AM-trained bees slept more in the afternoon (e.g., during the PM-training time slot; dashed outline) than when a food resource was made available in the morning (red outline). Likewise, PM-trained bees slept more in the morning (e.g., during the AM-training time slot; dashed outline) than when a food resource was made available in the afternoon (red outline). Data are average percentiles across bees each 30 min for 24 h. The gray backdrop signifies nighttime and error bars indicate s.e.m.

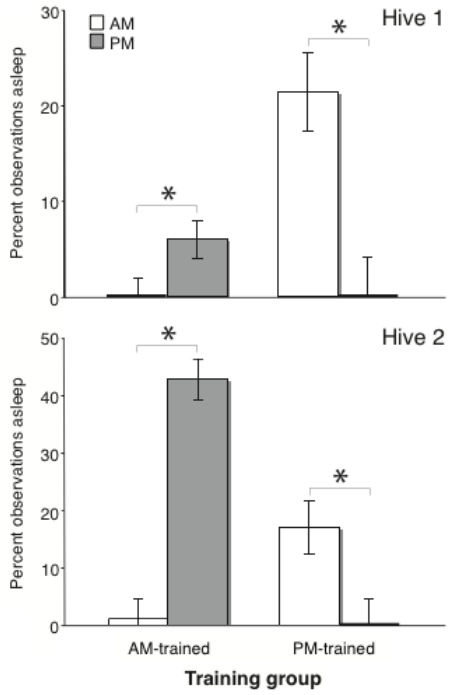

Fig. 26. Percent observations foragers in Hive 1 and Hive 2 were asleep in the morning (AM, 06:00—09:00 h, or 10:00 h for Hive 2) or in the afternoon (PM, 16:00—19:00 h), depending on whether they were recently trained to a morning food resource (AM-trained) or to a late afternoon food resource (PM-trained). AM-trained bees slept more during the afternoon than during the morning. PM-trained bees slept more during the morning than during the afternoon. Values were averaged for each bee per AM or PM period. Error bars indicate s.e.m. and asterices signify statistically significant differences.

periods (Hive 1: same as above; Hive 2: $P < 0.0001$, $n = 46$ & 45 bees, Wilcoxon signed-rank test).

Likewise, foragers trained to a feeder in the afternoon subsequently slept more during the morning than during the afternoon of the PM-testing day (Hive 1: $P = 0.0007$, $n = 16$ bees; Hive 2: $P = 0.0098$, $n = 21$ bees, Wilcoxon signed-rank test) (Fig. 26). As with the AM-testing, these results are nearly identical after including the few bees for which we only had data during the AM- or PM-testing periods. Each result above is confirmed by a paired-samples t test ($P = 0.0503$, < 0.0001, 0.0022, 0.0165, respectively) and the overall treatment effect is confirmed with a chi-square test (AM: $Z = 5.89$ $P < 0.00001$, PM; $Z = 3.88$, $P = 0.0001$, overall $Z = 6.91$, $P < 0.000001$). A simulation testing the likelihood the above results were due to repeated measures on the same bee

yielded the same conclusions as above in a majority of iterations for three of the treatment groups (97%, 98%, and 100% of the iterations), but a minority in the fourth group (16% of the iterations). However, for all four treatment groups, failure to replicate the results during the simulations was due to insufficient sample size, with 14.5 bees serving as the critical minimum. Of the total observations asleep during morning and afternoon periods, approximately half were devoted to deep sleep and a small percentage to sleep inside cells (Table 3).

Table 3. Percent observations bees trained to a morning food resource (AM-training) and an afternoon food resource (PM-training) spent asleep (total sleep), either outside of cells in deep sleep, non-deep sleep (not pictured), or inside cells. Gray cells highlight periods during which daytime sleep occurred in the absence of a food resource. White cells highlight periods during which sleep occurred in the presence of resource availability.

		Hive 1 (% observations)		**Hive 2** (% observations)	
		Morning	Afternoon	Morning	Afternoon
Total sleep	AM-training	0	6.0 ± 2.0	1.0 ± 3.6	42.9 ± 3.6
	PM-training	21.4 ± 4.1	0	17.0 ± 4.6	0
Deep sleep	AM-training	0	0	0.6 ± 3.0	21.6 ± 3.0
	PM-training	9.9 ± 3.7	0	5.2 ± 2.2	0
Sleep inside cells	AM-training	0	0	0	8.3 ± 2.4
	PM-training	0	0	2.4 ± 1.7	0

Assessing morning activity with respect to AM-trained versus PM-trained bees, we counted the number of individually marked bees that attempted to leave the hive by 09:00 h. The earliest foraging attempts were made by AM-trained bees in both hives. By 09:00 h, 25 of the AM-trained bees attempted to depart Hive 1, but only 15 of the PM-trained bees attempted the same (Fig. 24).

Did total time devoted to sleep, or any other behavior, change with respect to the schedule of resource availability? The net percentage of observations made over a 24-h period did not significantly differ with respect to any behavior after bees had been trained in the morning versus after the same bees had been trained in the late afternoon (Fig. 27). The only exceptions to this were deep sleep and sleep inside cells for Hive 2, but the sum of these two sleep measures are statistically indistinguishable between the two testing days. Total sleep at night also did not differ between AM- and PM-trained bees, although

the temporal pattern of sleep did differ: PM-trained bees slept more inside cells during the night than AM-trained bees (Hive 1: P = 0.0086, n = 14 AM- and 17 PM-trained bees; Hive 2: P = 0.0007, n = 46 AM- and 24 PM-trained bees, Wilcoxon signed-rank test).

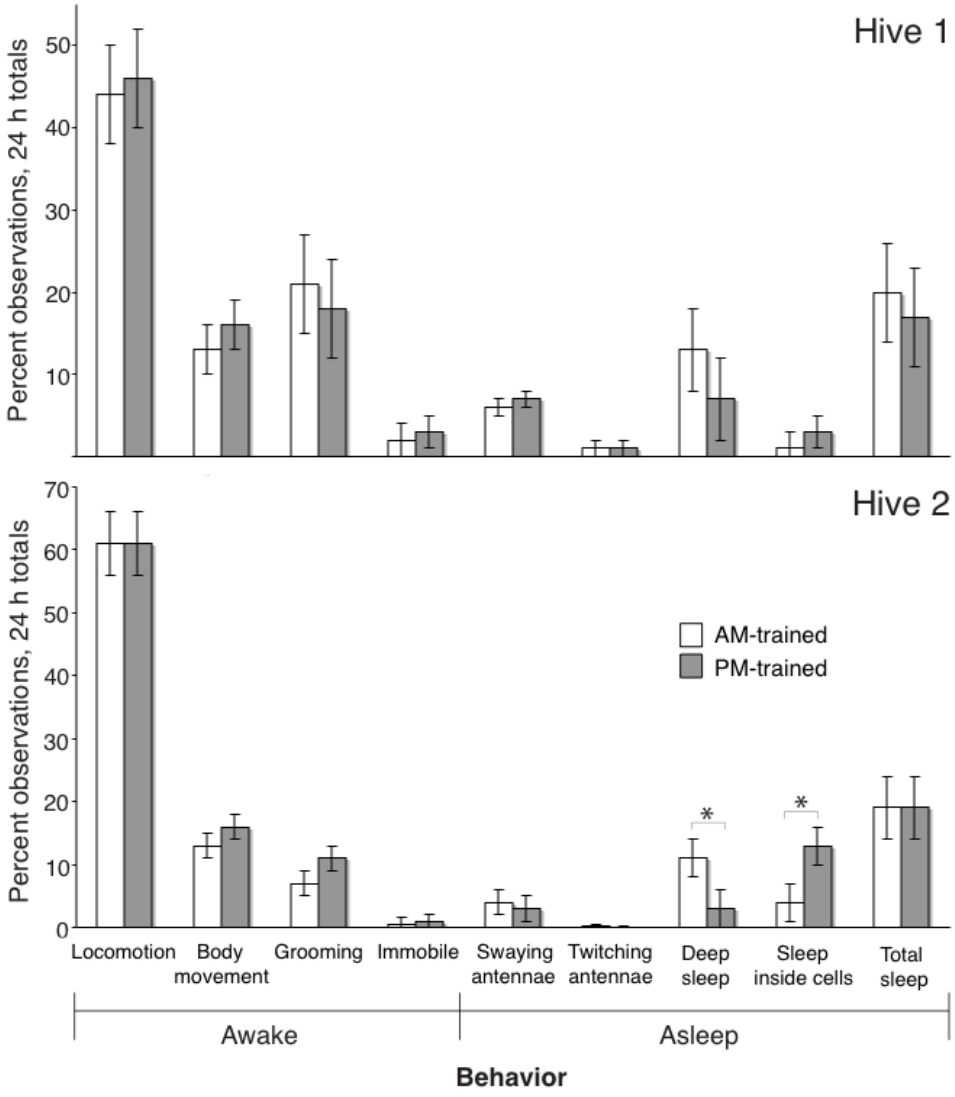

Fig. 27. Percent observations (± s.e.m.) of primary behaviors exhibited across 24 h periods by AM-trained and PM-trained bees in Hive 1 and Hive 2. Bees exhibited each behavior the same total amount, irrespective of when they were trained to a food resource. Exceptions are noted with asterices, but when summed together these two sleep measures are also statistically indistinguishable between the two testing days.

DISCUSSION

Foraging honey bees demonstrated a shift in their sleep schedule following a temporal shift in resource availability. Whether trained in the early morning or in the late afternoon, a forager exhibited no change in total time devoted to any of the recorded behaviors, only a change in the *timing* of some behaviors. Foragers exhibited virtually no sleep during periods of resource availability, but did sleep during other periods of the day (Fig. 25). Previous reports document timing of forager sleep as primarily restricted to the night (Kaiser 1988, Sauer et al. 2003, Eban-Rothschild and Bloch 2008, Klein et al. 2008), but no specificity of what factors may contribute to timing of diurnal sleep by foragers.

The flexibility of diurnal sleep by foragers is likely an adaptive response to changing demands and environmental pressures. Factors that affect daytime forager activity and may influence the timing of daytime sleep include season (Bloch et al. 2006), weather conditions (Riessberger and Crailsheim 1997), hormonal activity (Bloch and Meshi 2007), genetic makeup (Toma et al. 2000), resource availability (as this study suggests) (Schulz et al. 2002), and colony resource needs (Seeley 1995). Colony needs can radically reorchestrate a forager's sleep, including inducing a reversion to nurse bees' non-periodic behavior (Bloch and Robinson 2001). Additionally, competition by other pollinating colonies or species, and intra- or interspecific invasion could potentially impact a forager's daytime sleep.

We limited the influence of several possible sleep-altering variables in order to focus on the potential for resource availability alone to affect sleep schedules. In spite of this, alternate factors may have influenced sleep's timing in the two study colonies. AM-trained bees were prevented from foraging for portions of the days prior to afternoon resource availability. This enforced shift in timing of foraging may have, in itself, induced a change in timing of sleeping. Also, bees aged and weather conditions varied across treatments. These factors appear unlikely candidates to explain the consistent shift we found in timing of sleep by individual bees in both colonies. We would expect the stress induced by changing the timing of the resource availability to reveal itself in other ways, such as increased agitation or activity during the morning periods—the opposite of what we recorded in PM-trained bees. Age seems an unlikely factor to have affected our

results because testing of the foragers was conducted only two days apart, and variation in weather was not consistent enough across the two trials to garner the consistent actions exhibited by foragers in both colonies. Also, total observations of each behavior were remarkably consistent within bees across days (Fig. 27), consonant with the notion that weather conditions did not drive the large scale differences we found between treatments.

The timing of sleep can have profound impacts within the context of a society, especially when that society is dependent on the actions of those requiring sleep. Foragers primarily sleep at night, but we now see that they can also sleep during the day. When their duties are not needed, or external factors such as bad weather block them from foraging, foragers, by default, engage in other behaviors, which evidently include sleep. Shift work in bees may set into motion a complicated array of factors that contribute to scheduling of sleep and our results suggest that resource availability is an effective ecological factor influencing the schedule of honey bee sleep. In a honey bee colony, as in certain human organizations, individuals can shift their sleep schedules to accommodate shifts in their work schedules.

CHAPTER 4

Sleep deprivation impairs precision of waggle dance signaling in honey bees

ABSTRACT. Sleep is essential for basic survival and insufficient sleep leads to a variety of dysfunctions. In humans, one of the most profound consequences of sleep deprivation is imprecise communication. Communication in non-human animals may suffer analogous degradation of precision, perhaps with especially damaging consequences for social animals. However, society-specific functions of sleep have rarely been explored and no function of sleep has been ascribed to a eusocial organism in the context of its society. Here I show that sleep-deprived honey bees (*Apis mellifera*) exhibit reduced precision when encoding direction information to food sources in their waggle dances. The deterioration in their ability to communicate is expected to reduce foraging efficiency of fellow bees. Enabling precise communication may be one key benefit of sleep shared by social organisms.

INTRODUCTION

When deprived of sleep, human speech performance declines, such as in word fluency and intonation (Harrison and Horne 1997). A speaker's voice is sensitive to fatigue (Greeley et al. 2007), with fundamental frequency and word duration both differing in sleep-deprived subjects (Whitmore and Fisher 1996). Speech deterioration is so obvious after sleep deprivation that "rambling, incoherent speech for brief periods" features in a cognitive disorganization scale (Morris et al. 1960). Although there is potential for sleep to impact communication in non-human animals, I am only aware of studies relating to the role of sleep in song learning in zebra finches (Dave and Margoliash 2000, Derégnaucourt et al. 2005).

This study investigates a possible social function of sleep by testing the effect of sleep deprivation on the precision of signaling among European honey bees (*Apis mellifera*). Honey bees regularly inform fellow workers about the distance and direction to desirable locations by performing waggle dances (Fig. 28A), in which the distance to the advertised destination is indicated by the duration of the waggle phase of the dance,

and the destination's direction relative to the sun's azimuth is indicated by the angle of the dance relative to the vertical (i.e., zenith angle) (Fig. 28A) (von Frisch 1967). Deviations in a bee's performance of the waggle dance could result in degraded transfer of information and consequential decline in foraging efficiency for vital resources (Sherman and Visscher 2002, Dornhaus and Chittka 2004).

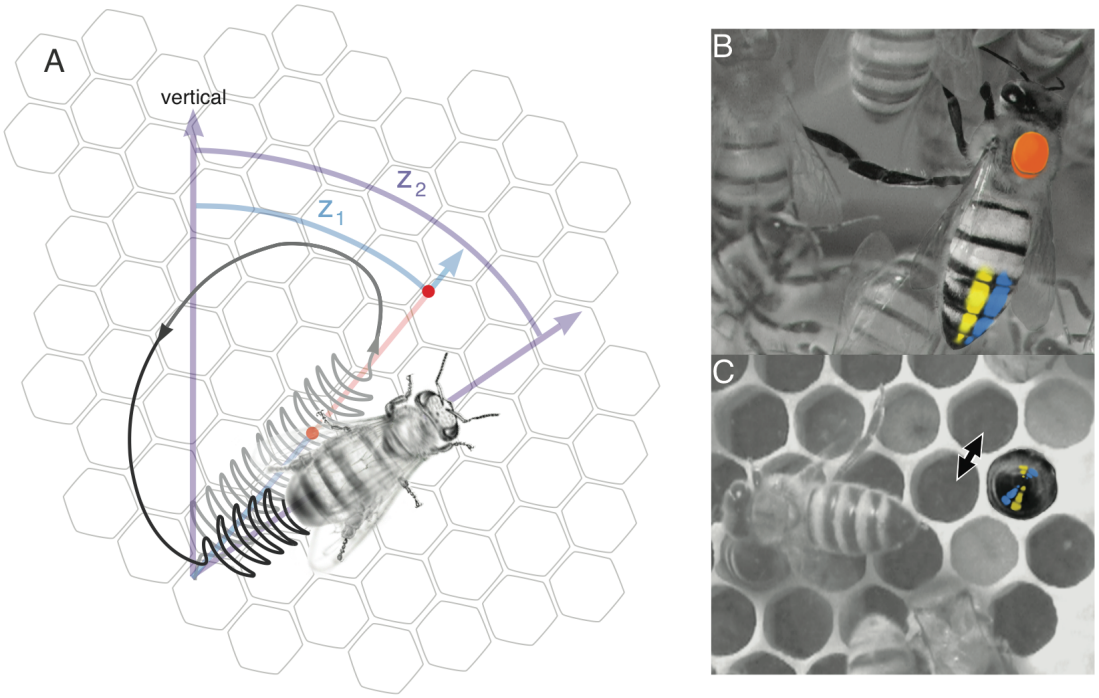

Fig. 28. Dancing and sleeping bees. Foragers were monitored during the day for waggle dances (left) and at night for sleep behavior (right). (A) A dance consists of waggle phases (jagged lines, connected by curved paths), with angle of the waggle phase relative to the vertical (zenith angle, z) corresponding with flight angle to a food source relative to the sun's azimuth. Red dots connected by the line segment illustrate start and end points of a dancer's head during a waggle phase. z_1 & z_2 = two consecutive zenith angles, with standard deviation of z's within a dance serving as a measure of directional precision. (B) An exposed sleeping bee is relatively immobile, with body drooping in the direction of gravity, antennae often bent with scape $\leq$ 90 degrees relative to flagellum, and antennae either immobile (deep sleep), twitching, or swaying. (C) Bees also sleep inside cells. Sleeping bees can be identified by their dorso-ventral discontinuous ventilatory motions, represented by arrows. Bees were individually paint-marked, and bore either a magnetic steel or nonmagnetic copper disk, concealed under orange paint (B). Photographs were modified to single out a sleeping bee and portray markings.

I hypothesized that depriving honey bees of sleep would decrease the precision of their dance's direction and distance information. For directional precision, I predicted that the standard deviation (SD) of a dance's zenith angles would increase after sleep deprivation. For distance precision, I predicted that the SD of a dance's waggle phase durations would increase after sleep deprivation.

Sleep deprivation is the primary means to test sleep's effects on the functioning of an organism. Sleep is internally controlled (Tobler 1985) and when deprived of sleep, an organism is expected to exhibit recovery sleep. Recovery sleep often takes the form of subsequent increases in total sleep, or increases in sleep intensity. Some studies report a period of increased sleep of some form (e.g., sleep stage or intensity) combined with increased activity during "inactive" periods (Nakazawa et al. 1978, Friedman et al. 1979, Tobler 1983, Tobler and Neuner-Jehle 1992, Klein 2003). Studies in which isolated honey bee foragers were sleep-deprived report recovery sleep either during the day following sleep deprivation (Bösebeck 1989), or during the night following sleep deprivation (Sauer et al. 2004).

To establish the effectiveness of sleep deprivation, it is essential to identify features diagnostic of sleep. Honey bees exhibit criteria that define behavioral sleep (Kaiser 1988), including an increased threshold of response to disturbance and a specific posture during easily reversed bouts of relative immobility (Flanigan et al. 1973). A sleep-specific behavior in honeybees is discontinuous ventilation, consisting of several pumping motions of the abdomen, followed by an extended ventilatory pause (Kaiser et al. 1996, Chapman 1998, Kleinhenz et al. 2003, Sauer et al. 2003). Discontinuous ventilation co-occurs with increased antennal immobility and other indicators of sleep (Sauer et al. 2003) and can therefore be used as a proxy for detecting honey bees sleeping either inside or outside comb cells (Klein et al. 2008) (Fig. 28B, C). When exhibiting reduced antennal mobility, bees exhibit increased response thresholds and total antennal immobility is suggestive of a deeper sleep state (Kaiser 1988). I used relative immobility, combined with discontinuous ventilation as our indicators of sleep, and these conditions combined with immobile antennae to identify deep sleep.

Because honey bee foragers sleep primarily at night, we[4] disturbed foragers for one night using a novel experimental approach: magnetic disturbance of a select subset of bees in the hive. We adhered magnetic steel to treatment bees and nonmagnetic copper to control bees. Unlike the automated sleep deprivation devices previously used on isolated, caged honey bees (Bösebeck 1989, Sauer et al. 2004), our magnetic "insominator" (Fig. 29) selectively disrupted uncaged treatment bees while they were in their hive, to maintain normalcy of the social conditions while eliminating any observable disruption of control bees. Treatment effects were compared with results following a daytime disturbance period to test for differences between sleep deprivation and general disturbance. Treatment effects were also compared with the period before sleep deprivation and two days after sleep deprivation (Fig. 30). Magnetic disturbance at night disrupted sleep; disturbance during the day served as a control treatment that did not disrupt sleep.

METHODS

We suspended a two-frame observation hive of approximately 4200 European honey bees (*Apis mellifera ligustica*; queen breeder: C. F. Koehnen and Sons, Inc., Glenn, CA, USA) from the ceiling of a wooden hut (Seeley 1995) on 7 July 2007 at Cranberry Lake Biological Station in the Adirondack State Park (NY, USA, 44º09"N, 74º48"W) and allowed the bees to forage freely for two weeks prior to the experiment. After this two-week period, we trained a group of 50 bees to visit a feeder with sucrose solution 1 km away from their hive. We collected the bees in perforated Ziplock bags at the feeder, individually cooled them in a refrigerator, then marked each bee with a unique combination of colors (Sharpie oil-based pens), repeating marks on the dorsal and ventral sides of the abdomen (metasoma) to facilitate identification of a bee when she was in a cell (Fig. 28C) or dorsally obscured (e.g., while clinging to the glass pane of the hive). We used shellac to adhere a magnetic disk (2.38 mm diameter, 0.25 mm thick) of cold rolled steel punched from shim stock (Lyon Industries, Chicago Inc., South Elgin, IL) to the dorsal mesosoma of 25 of the foragers (Gary 1971) (Fig. 28B). Applying identical handling to control bees, we adhered a piece of nonmagnetic copper of equal dimensions

[4] This research was a collaborative effort and my co-authors are listed in the Acknowledgements.

and mass to the dorsal mesosoma of the remaining 25 foragers. Metal disks were coated with orange paint to obscure bees' status as treatment or control, then bees were fed and caged, to allow shellac to dry before reintroducing them into their hive.

We constructed a manually-operated sleep deprivation device ("insominator," Fig. 29) designed to jostle only bees with magnetic metal. The insominator consisted of two pieces of Plexiglas, each containing three columns of 14 neodymium rare earth magnets (nickel-plated NdFeB, Grade N42, #RX054, K&J Magnetics, Inc.), totaling 42 magnets per side, arranged 1 cm apart within an array, and so that magnetic polarities of the two arrays canceled each other. When viewed laterally, facing Plexiglas pieces were parallel, setting up a consistent magnetic attraction throughout the hive. The magnetic arrays slid along an aluminum rail, permanently affixed below the suspended hive, limiting points of contact between the insominator and the hive to only one felt-lined magnet per side (Fig. 29).

The hive was lit by day (08:30-20:30 h) with 15W incandescence and by night (20:30-08:30 h) with 52W incandescence filtered by red acetate, to minimize bees' vision. The feeder was available at 1 km distance from 21-26 July 2007 between 10:00-14:00 h. Individual bees' visits to the feeder were recorded on a voice recorder (Olympus VN-400PC) and all dances by marked bees were videotaped (Panasonic AGDVC 30). On night one and day one, we recorded normal behavior (sleep and dance, respectively) prior to sleep deprivation. On night two, we operated the insominator for 12 h (20:30-08:30 h). For the next 24 h, we recorded the daytime dances and nighttime sleep behavior, and repeated recordings for an additional 24 h "recovery" period (Fig. 30). A growing number of sleep deprivation studies include a stress control, disturbing subjects during the period when they are primarily active (Hendricks et al. 2000, Shaw et al. 2000, Sauer et al. 2004), so we also operated the insominator 12 h during the day and recorded subsequent sleeping and dancing as a control for disturbance-induced stress (Fig. 30). During disturbance periods we moved the arrays of magnets a minimum of three times each minute across the hive, 3 s per sweep. Late in the night (beginning at 03:00 h), we jostled bees on the anterior side of the hive (Fig. 29) when they were not responding to the insominator by shaking a single magnet in each bee's vicinity for several seconds between insominator sweeps. Audio notes were recorded during the nighttime

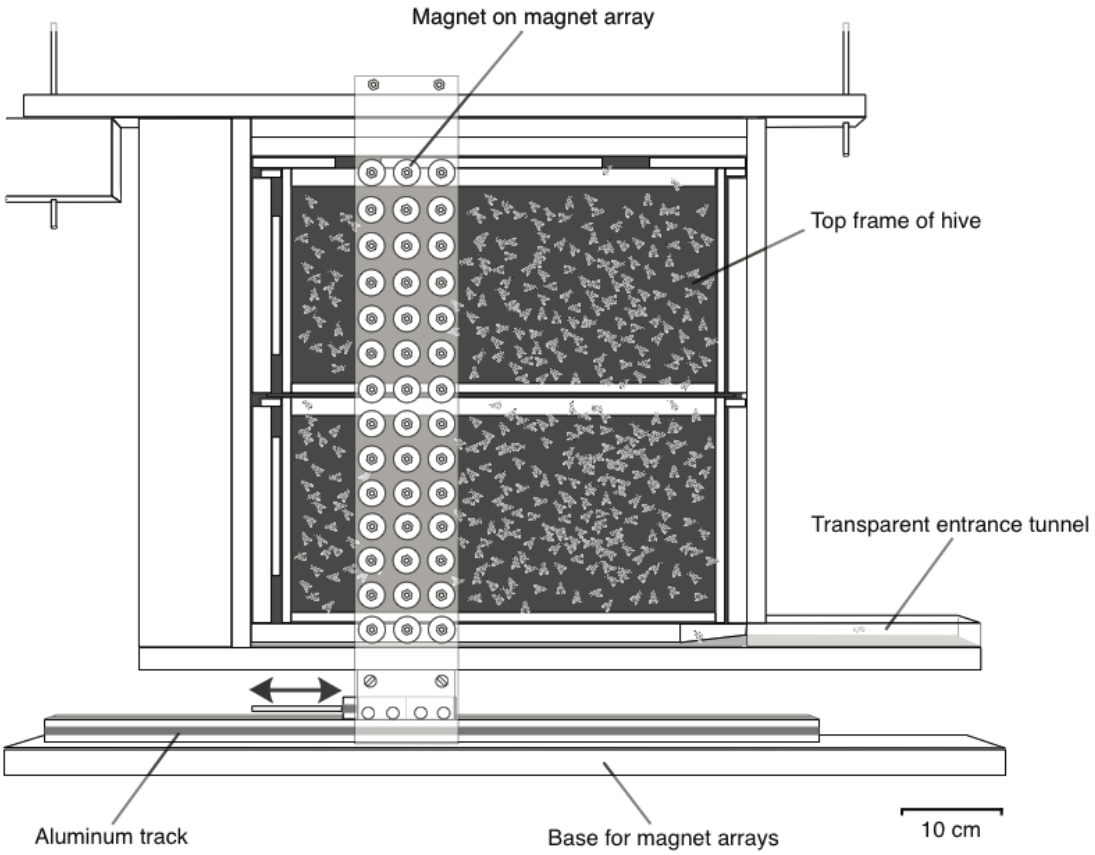

Fig. 29. Anterior view of two-frame observation hive, suspended from above, and insominator, braced from below. Insominator, consisting of two magnetic arrays, track, and base supporting track and arrays, directly contacts the suspended observation hive at only one point on each side of the hive. A felt-lined magnet from each array limits disturbance to the hive as the insominator slides down an aluminum track. Arrows represent directional movement of magnetic arrays, on anterior and posterior sides of the hive. Dark areas on the periphery of the hive represent passageways between anterior and posterior sides of the hive. Magnets jostled treatment bees, but not control bees. Magnet arrays remained to the left or right of the hive between insominator movements.

disturbances and the frequency of both types of disturbances was replicated as closely as possible during the daytime disturbance period, guided by playback of the audio recording. To verify impact and evidence of sleep deprivation, I observed marked bees with a red-filtered headlamp during the nights before and after the night of sleep deprivation, scanning both sides of the hive in a consistent manner across a grid superimposed on the observation hive, and recorded each bee's behavior every 30 min between 20:30-04:00 h. Descriptions of sleep behaviors and more extensive methods for distinguishing different sleep states can be found in previous work (Klein et al. 2008).

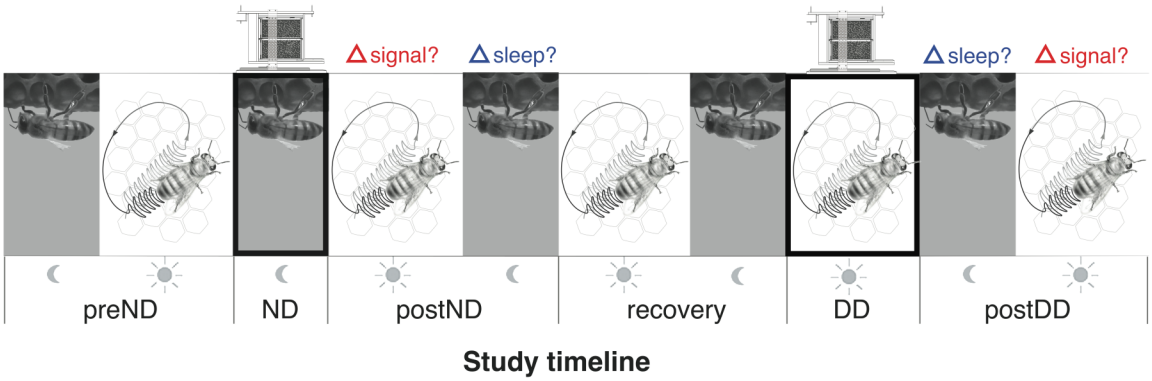

Fig. 30. Schedule of study. Foragers were trained to a feeder, individually marked with magnetic or nonmagnetic metal tags and paint, then reintroduced to the hive. We then examined bee sleep each night (bars with bee hanging upside-down and moon below), and examined bee dances each day (bars with waggle dance and sun below), except when the insominator was in operation: 12 h of nighttime disturbance (ND) and 12 h of daytime disturbance (DD) (insominator pictured above ND & DD). Examinations began with the night and day before ND (preND), continued during the day and night following ND (postND), the second day and night after ND (recovery), and the night and day following DD (postDD). To test for effect of sleep deprivation on sleep (Δ sleep?), we recorded bees' behavior during the night following ND and compared results between treatment and control bees to results during the night following DD. To test for effect of sleep deprivation on signaling (Δ signal?), we examined waggle dances the day after ND and compared results between treatment and control bees with dance results following DD. We also compared postND results with those of preND and with recovery.

Videos of waggle dances were transcribed using QuickTime (v7, http://www.apple.com/quicktime). We measured directional precision of the dance as SD of zenith angles. We measured distance precision as standard deviation of waggle phase durations. The time and place of each waggle phase start and end point was transcribed by playing videos frame by frame beneath a transparent web browser (aeroFox v1.0.5), and using the computer mouse to click on a single point of a dancing bee, viewed through the transparent browser window (Fig. 31). Of the dancer's body regions, the head moves least in the lateral direction (von Frisch 1967), so a point located medially and immediately posterior to the head was selected to establish start and end points of a waggle phase. A. Klein wrote a computer program that calls the JavaScript libraries jQuery (1.2.1) (http://jquery.com/) and Vector Graphics Library (3.0.3) (http://www.walterzorn.com/jsgraphics/jsgraphics_e.htm) to store each pair of browser-based mouse-click coordinates in a MySQL database, and wrote a second program in

Python (v2.6, http://www.python.org) to compute zenith angles and waggle phase durations from the pairs of mouse clicks. Transcription of data was conducted blindly, as transcribers were not privy to the treatment day or treatment vs. control status of each bee (steel and copper tags were obscured by pigmented shellac during marking, Fig. 28B). Inter-observer error for calculating angles was 1.06 ± 0.24% (3.33 ± 0.82 degrees).

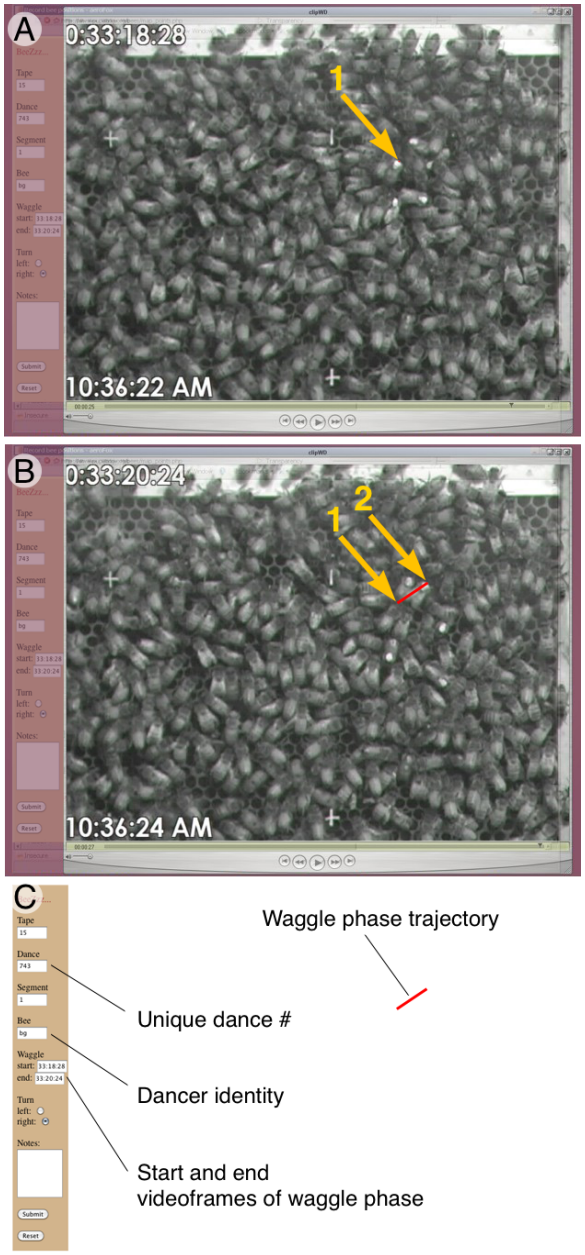

Fig. 31. Transcription of waggle dance direction and distance information. Each dance performed by an individually marked forager was videotaped and a frame-by-frame selection of (1) start and (2) end times of each waggle phase was recorded with a mouse click in a transparent browser window. (A) The first video frame after a dancer has turned and begun her lateral body waggling is marked by a mouse click in the medial, anterior region of the mesosoma; mesosoma was painted and appears in the black and white videos as a large white spot. Frame counter is in upper left corner; time of day is in lower left corner. (B) The final video frame of a dancer's waggling (at the end of her dance, or immediately before turning and beginning another waggle phase) is marked by a second mouse click. (C) The line segment connecting start and end points of a waggle phase is used to calculate zenith angle for direction information. Start and end times (duration) of waggle phase is used for distance information.

We included copper bees in the study as a control for any baseline disturbance caused by the insominator, and for environmental factors specific to the day. We tested bees within the hive to maximize normalcy, increase the likelihood of bees dancing, and to avoid confounds associated with testing isolated bees, which sleep less over consecutive days and experience premature mortality (Kaiser 1988, Sauer et al. 2003, Sauer et al. 2004). Results are products of linear mixed-effects models, programmed in R (R Development Core Team 2005), with bee group (treatment vs. control) and treatment day serving as fixed effects, and bee identity as a random factor (i.e., observations were nested within bee). Linear mixed-effects models were fit using the lmer function in the lme4 package (Bates and Sarkar 2006). The multcomp package was used to perform likelihood ratio tests to distinguish between competing models (Hothorn et al. 2008); because of the complicated (e.g. missing data, correlated covariates) and unbalanced nature of the data, standard likelihood ratio tests could not be performed (Pinheiro and Bates 2002). The resulting continuous, linear predictors are reported as means ± standard error (s.e.m.). To visualize the data (Fig. 32, 33) and for confirmation of mixed-effects model results, we also calculated average values (one per bee) using JMP (JMP, version 8. SAS Institute Inc., 2008). We set alpha at 0.05 for all tests. The sleep deprivation literature overwhelmingly documents decrements in precision and efficiency; because sleep deprivation was not expected to induce greater precision, we report one-tailed *P-values* when testing our predictions relating to impacts of sleep deprivation on dances on the day following nocturnal perturbations. We report two-tailed *P-values* under all other conditions.

RESULTS

Nineteen of the 50 individually marked honey bee foragers survived and retained their metal tags, 11 with magnetic steel and 8 with nonmagnetic copper. One treatment bee and one control bee never danced, so we could not include them in dance analyses. Two treatment bees and two control bees could not be distinguished from each other at the feeder, so we excluded them from the feeder analysis. Weather was stable throughout the study (16.9 ± 1.1ºC, no precipitation) and bees foraged and danced every day.

Directional precision was lower (i.e., SD of zenith angles was greater) for treatment bees than for control bees following a night of sleep deprivation, as compared with the control day following daytime magnetic disturbance ($z = 2.36$, 1-tailed $P = 0.0499$, $n = 545$ observations of 17 bees). As predicted, this difference in directional precision between treatment and control bees was not apparent on any other day when compared with the control day. Results from *t*-tests examining averages of angles, one angle per bee per day, are consistent with this result: treatment bees exhibited greater SDs associated with dance angles than control bees during the day following sleep deprivation (16.49 ± 0.98 vs. 13.78 ± 0.91, $t = 2.02$, 1-tailed $P = 0.0171$, $n =$ 6 treatment & 7 control bees) (Fig. 32), but not on any other day. Directional precision was reduced in waggle phases performed specifically after left turns. Distance precision did not differ between treatment and control bees (SD = 0.36 ± 0.04 vs. 0.32 ± 0.04 s, $t = 0.79$, 1-tailed $P = 0.2234$). Measures unrelated to the waggle dance, such as feeder visitation, visitation rates, tendency to perform waggle dances, or waggle dance rates also did not significantly differ between treatment and control bees.

Total sleep between treatment and control bees did not differ on any night before or after sleep deprivation. However, sleep deprivation had an activating effect during the subsequent circadian sleep period, followed by increased sleep in the middle of the night. The treatment bees slept less than the control bees for the first 3 h ($22.6 \pm 8.3\%$ vs. $58.6 \pm 8.6\%$ of observations, $z = 2.69$, $P = 0.0127$; $n = 60$ observations of 16 bees), but slept more and proportionally more deeply than the control bees during the subsequent 2 h (pooled data: $72.2 \pm 11.0\%$ vs. $30.4 \pm 9.7\%$ observations asleep, $t = 2.85$, $P = 0.0070$, $n =$ 41 observations of 13 bees; mixed-effects: $z = 1.83$, $P = 0.1140$, $n = 34$ observations of 13 bees in deep sleep) (Fig. 33). There was no difference between treatment and control bees during any period on any other night, except for an initial decrease in sleep the night after daytime disturbance in treatment bees.

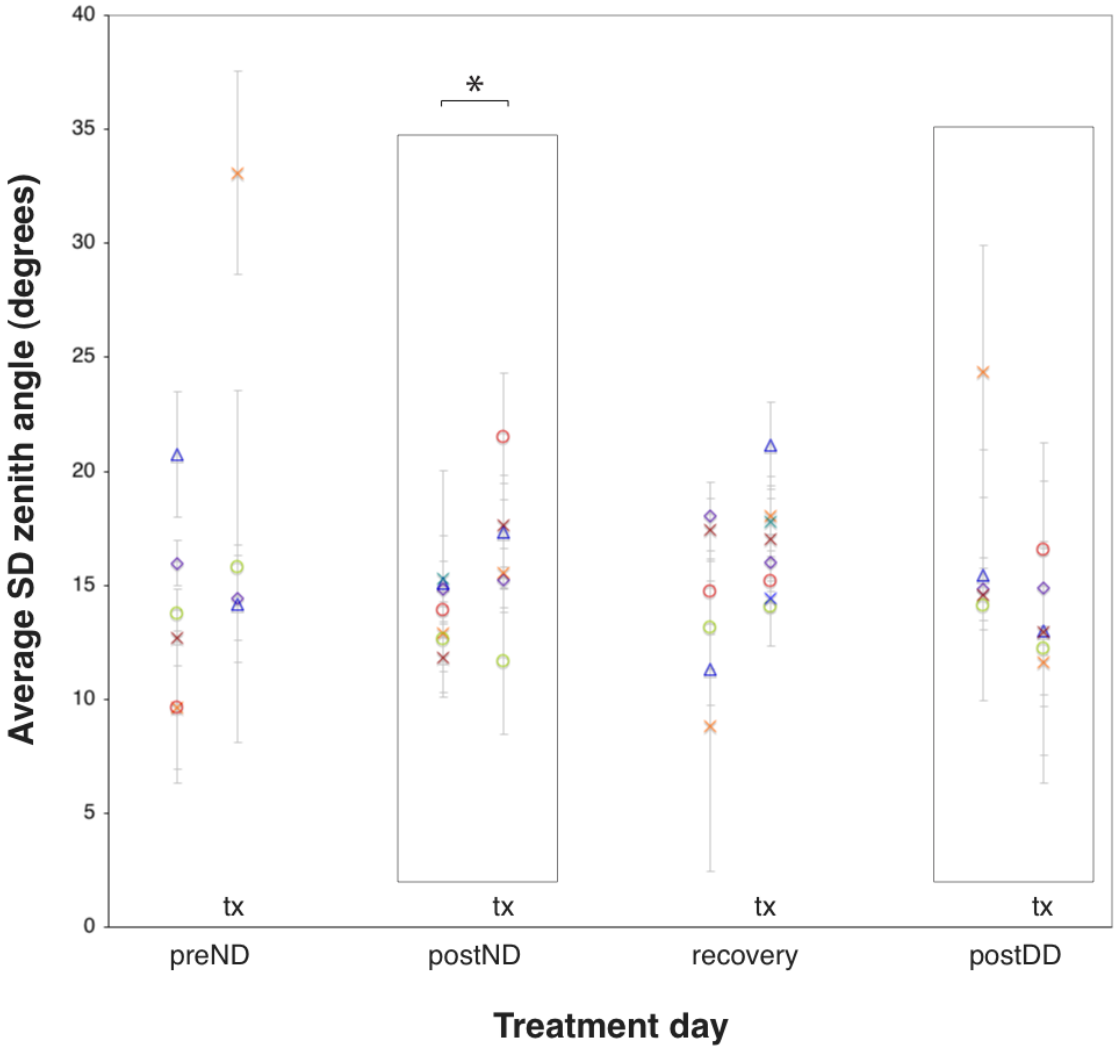

Fig. 32. Effect of sleep deprivation. Directional precision in treatment bees (tx) versus control bees on four different days of the experiment: the day prior to nighttime disturbance (preND), the day following nighttime disturbance (postND), two days after nighttime disturbance (recovery) and the day following daytime disturbance (postDD). The asterisk highlights a significant difference in directional precision between treatment (n = 6) and control bees (n = 7), based on a mixed-effects model test using treatment day as a fixed effect. Treatment bees exhibited greater SD averages than control bees during postND relative to postDD (in boxes). Greater SD of zenith angle = lower precision. Symbols represent averaged values for each bee for each treatment day (± s.e.m.).

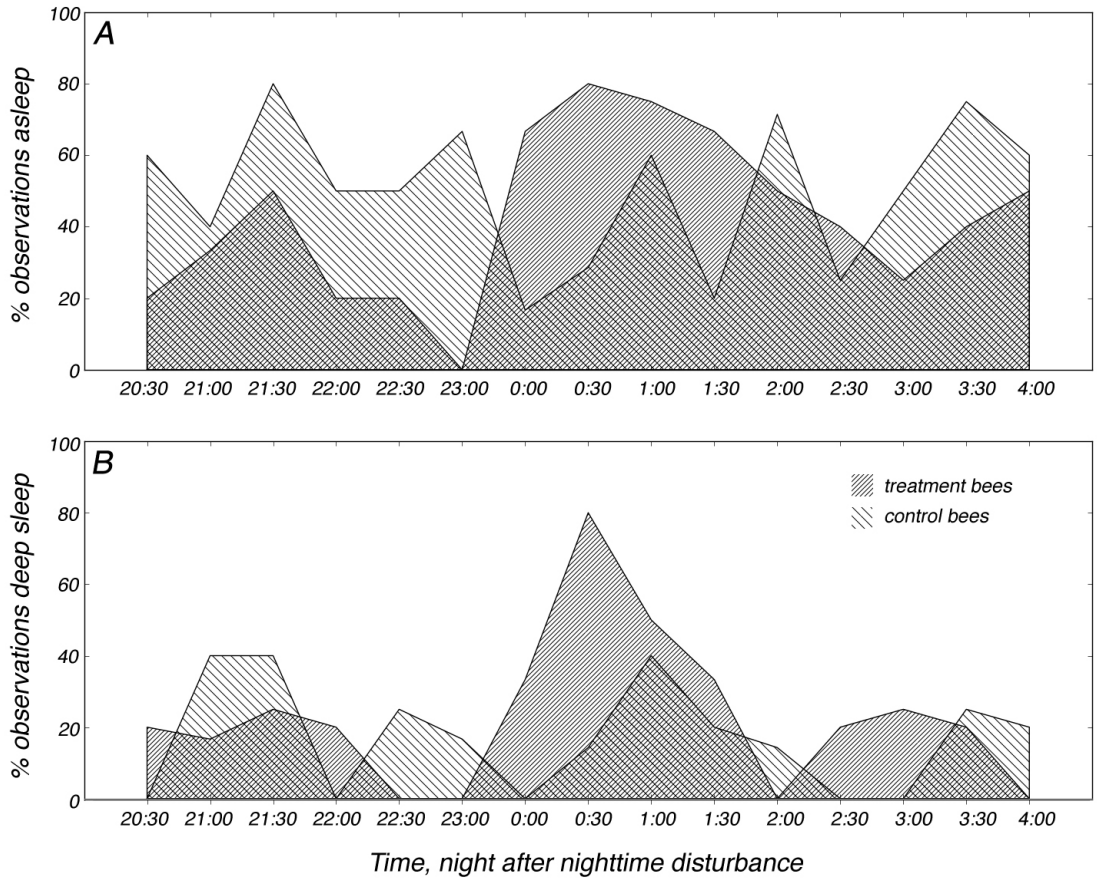

Fig. 33. Recovery sleep. Percent of observations during the night following nighttime disturbance that bees were (A) asleep and (B) exhibiting deep sleep. Treatment bees (n = 9) were more active at the beginning of the night, but experienced more sleep and more deep sleep than the control bees (n = 7) during the middle of the night.

Young (pre-foraging) adult bees are known to sleep extensively inside cells, but contrary to previous results (Klein et al. 2008), foragers slept inside cells a significant amount during this study (treatment: 8.4 ± 4.4%, control: 9.3 ± 4.6% of total observations, averaging measures per bee). Treatment bees slept less inside cells during the night following sleep deprivation than control bees (5.7 ± 3.0% vs. 21.7 ± 8.0% observations) relative to the night following daytime disturbance (z = -3.21, P = 0.0090) and relative to the night prior to sleep deprivation, although this latter comparison was not statistically significant (z = -2.22, P = 0.1400). I cannot presently identify how much deep sleep is obtained inside cells, or how sleep inside cells specifically contributes to recovery sleep.

DISCUSSION

We tested for a negative impact of sleep deprivation on precision of signal production in honey bees. Directional precision, but not distance precision, was impaired. Our measure of distance precision did not vary greatly and differences might have been obscured by a range of factors that affect waggle phase duration, including the optic flow an individual bee experiences (Tautz et al. 2004). Because only treatment bees were affected by disturbances and they were only affected when those disturbances occurred at night, our effects were specifically related to sleep and not to general disturbance. Treatment bees were effectively sleep-deprived, with recovery sleep obtained not only during the night, but likely during the day, with unknown partitioning of deep sleep within comb cells during either time.

Operation of the insominator jostled only the treatment bees. Not all bees could be monitored for response to the insominator at all times (e.g., bees inside cells or on the opposite side of the hive), but most treatment bees exhibiting immobility responded by moving their bodies and antennae, grooming, or walking after the array of magnets swept by. Response to jostling induced by the insominator or the handheld magnet varied among treatment bees, especially in the latter part of the night.

We initially examined divergence angles (absolute value of the difference between dance angles performed subsequent to a left and right turn) as a possible measure of directional precision (Towne and Gould 1988, Weidenmüller and Seeley 1999, Gardner et al. 2007), but eliminated this from our report due to the recently questioned interpretation or relevance of this measure (Tanner and Visscher 2006, Tanner and Visscher 2010).

Our study has revealed that sleep deprivation degrades the ability of bees to precisely encode directional information in their waggle dances. Deciphering precise direction information may suffer when either dancers or potential recruits lack sleep, and we expect this would result in less efficient foraging. Testing this hypothesis will be the next step to understanding sleep deprivation's potential consequences on the fitness of a colony. In studying the effects of sleep deprivation on honey bee communication, we begin to see the functional significance of sleep to societies.

APPENDIX

Computer code used for transcribing waggle dance information.

The following computer program is the JavaScript component of the web page (http://www.pupating.org/bees/map_points.php) used to record bee positions and orientations presented below a transparent browser. This program calls the JavaScript libraries jQuery (1.2.1) (http://jquery.com/) and Vector Graphics Library (3.0.3) (http://www.walterzorn.com/jsgraphics/jsgraphics_e.htm), and the PHP program store_points.php to store the coordinates (X1,Y1) and (X2,Y2) of mouse clicks in a MySQL database.

```
<script type="text/javascript" src="scripts/jquery.js"></script>
<script type="text/javascript" src="scripts/wz_jsgraphics.js"></script>
<script type="text/javascript">
 $(document).ready(function(){
   var offsetX = 0;
   var offsetY = 0;
   var line_offsetX = offsetX
   var line_offsetY = offsetY
   jQuery(document).ready(function(){
     var X = [];
     var Y = [];
     $("#window").mousemove(function(e){
       $('#live').html((e.pageX-offsetX) +', '+ (e.pageY-offsetY));
     });
     $("#window").click(function(e){
       X[X.length] = e.pageX-offsetX;
       Y[Y.length] = e.pageY-offsetY;
       if (X.length==1) {
         $('#click1').html(X[0] +', '+ Y[0]);
       } else if (X.length==2) {
         jg.setColor("#ff0000");
         jg.setStroke(Stroke.DOTTED);
         jg.drawLine(X[1]+line_offsetX,Y[1]+line_offsetY,X[0]+line_offsetX,Y[0]+line_offsetY);
         jg.paint();
         $('#click2').html(X[1] +', '+ Y[1]);
         $.get("store_points.php", { X1:X[0],Y1:Y[0],X2:X[1],Y2:Y[1] });
         $("div.main_insert").find("p.warning:visible").slideUp("slow");
       }
     });
   })
 });
</script>
```

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VITA

Born after humble beginnings as a hemi-zygote to artists and art gallery owners Arnold and Karen Anne Klein, Barrett Anthony Klein embraced his love of insects by majoring in entomology at Cornell University. Advised by George Eickwort and mentored by Thomas Seeley, Barrett graduated in 1993 and assisted James Nieh to study stingless bee communication in Panama. Barrett then translated his entomophilic energies into freelance scientific illustration and filmmaking, followed by fabrication of natural history exhibits at Chase Studio Inc. in southwest Missouri, then spent several years working in the Exhibition Department at the American Museum of Natural history, roaming its half-lit halls by night and creating insects, giant viruses, and working in both education and exhibition by day. After working on the Hall of Biodiversity, various temporary and traveling exhibits, and displays for the Bronx Zoo's Congo rainforest, Barrett returned to entomology research as a Master's student at the University of Arizona in Daniel Papaj's and Diana Wheeler's labs. Barrett studied subsocial spiders, aphid dietary responses to symbionts, insects and arachnids as art media, and produced a thesis defining sleep in the paper wasp *Polistes flavus*. Since graduating in 2003, Barrett has been co-advised by Ulrich Mueller and Lawrence Gilbert. Aside from working on social aspects of honey bee sleep, he has collaborated with Margaret Wray on honey bee communication, studied hitchhiking in leaf-cutter ants, illustrated various fungus growing ant projects and a damselfly field guide authored by John Abbott, designed and fabricated faux frogs for collaborative research with Ryan Taylor, Joey Stein and Michael Ryan on multimodal signaling in Panama, and produced works celebrating cultural entomology.

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This manuscript was typed by the author.