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Composition, Structure, Function

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ISBN 978-3-642-54327-2 ISBN 978-3-642-54328-9 (eBook)
DOI 10.1007/978-3-642-54328-9
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014931509

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

For
Prof. Dr. Warwick Estevam Kerr

Preface

This new monograph, *Honeybee Nests: Composition, Structure, and Function*, was originally conceived as an extension or revision of an earlier monograph, *Honeybees and Wax: An Experimental Natural History*, published in 1986 by Springer Verlag, Berlin. That monograph was restricted to publications on *Apis mellifera* in the literature up until 1985. However, an extensive search of Google Scholar™ quickly revealed that the number of relevant scholarly references to *Honeybees and Wax* for all species of *Apis* published before 1986 is less than a third of that published since. While *Honeybees and Wax* did, and still does, enjoy fair coverage in contemporary literature, it is beyond doubt very out-of-date. Indeed, the need for a completely new work, and not merely a revision, became apparent when one considers that there has been more than a three-fold increase in the relevant literature.

It is fair to ask 'Is this a new or revised edition?' In the publishing trade it is obvious that there are subjective, qualitative differences between a 'revised edition' and a 'new edition' which hinges on whether the differences constitute something very different, or merely slightly different. In the present case, we can assure the reader that more than 60 % of the text, tables and figures in *Honeybee Nests: Composition, Structure, and Function* represents all new and relevant literature that has appeared in the scientific journals over the past quarter of a century.

We would like to introduce the chapters of this book by reference to the various forms of swarming. Given that there are well over 10,000 publications concerning swarming in honeybees that began to appear in the early eighteenth century, this matter has received intense scrutiny by observations and experiments. However, here we do not attempt a review of this subject, which would require its own volume, but rather restrict comments largely to the circumstances that precede swarming and in terms of 'wax-readiness' once a new nest site has been chosen and the colony has settled there.

The structure of the contents of this new volume is virtually opposite to that of its predecessor. In the 1986 book, the chapters began at the finest level on the nature and production of beeswax, including cytology of the wax gland system, composition and synthesis of beeswax and the energetic considerations that bear on these topics. This section was followed by chapters on the material properties of beeswax and the construction of cells and combs. The final section comprised

large-scale biological factors such as nectar flow, pollen and wax production, the brood nest, the queen and the microenvironment. On the suggestions of several colleagues, in this new work we proceed in precisely the opposite manner, from large and to small. So now it begins with a broad portrait on nesting, which is of fundamental interest to all honeybee biologists, and leaves the physics and chemistry of beeswax and molecular biology of silk to the end, which will have a considerably more finite appeal.

Of particular interest, we have seen, in the last quarter of a century, substantial incursions into the 'old honeybee preserve' of honeybees and wax by major scholarly publications in materials science, structural engineering, molecular biology, polymer chemistry, biomimetics and biomaterials, chemical chromatography, thermal chemistry, food chemistry, applied physics, mathematics, computer modelling and even archaeology. On the purely biological and apicultural side of the coin, there have many major areas of growth in nestmate recognition, social biology, toxicology, pheromones, biomechanics, biochemistry, endocrinology, behavioural ecology, olfactory physiology, evolutionary genetics and chemical ecology to cite a few.

Over the past 25 years studies have been conducted on wax synthesis and secretion in honeybees to identify specific cellular sites for the origin of hydrocarbons and fatty acids within the wax gland complex, and to establish the necessary ultrastructural correlates of this activity and of their transport. These include age-related changes in the composition of hydrocarbons and fatty acids in the epidermal cells, adipocytes and oenocytes. Similarly, with ever-more sophisticated instrumentation, there have been considerable refinements in the wax chemistry and characteristics of different honeybee species and races, their chemometric classification and analyses of the weighted frequency distributions in their carbon chain length variations. There have been major gains in our understanding of the material properties of beeswaxes, their tensile properties, crystal texture and crystallites and wax proteins. We know that honeybee silk is a ά-helical protein, how it behaves at different temperatures, the effects of solvents on silk, its relative crystallinity and molecular dynamics as well as the genetic basis of the honeybee ά-helical fibroin. Chemical differences between scale and comb wax have been determined and how these change in the conversion of newly secreted wax scales into comb.

Of perhaps equal importance is the previously catalogued literature on the Asian species of honeybees which has remained virtually unknown, otherwise untouched and never synthesised. It is apparent from a recent bibliography (Hepburn and Hepburn 2011) that, of some 4,000-odd publications on the Asian species of honeybees, there are several hundred publications dealing with the waxes and silk of these species. All of this hitherto unutilized material has now been analysed and incorporated, where appropriate, in this new text. It should be noted that references to Asian honeybees were sought from the Zoological Record (1864–2003), Apicultural Abstracts (1950–2004) and from Google Scholar™ (2005–2009). However, 95 % of this literature is post-WWII and much of it is now

available through Google Scholar™. Of interest here is a smallish group of papers on the analytical chemistry and physical constants of beeswax, nest site selection and beekeeping trade in beeswax.

The precursor of the present text, *Honeybees and Wax: An Experimental Natural History*, has not been entirely abandoned. Most of the chronological–historical chapters and paragraphs have been greatly edited, compressed, amended and retained. One will still find 'old references' (in fact very, very old) in this new book, which usually means references which only few computer-literate biologists can readily find or even bother to try. Nonetheless, one still encounters comments/queries from journals' referees and even editors as to why such an *old* reference has been cited. The answer here is very basic: we believe in the tradition of 'primacy of discovery'. This tradition has been falling by the wayside and there are particularly egregious examples of ignorance, or worse, incestuous self-aggrandisement in not citing the discoveries of those who came before us.

As an example, Jan Dzierżoń, who discovered parthenogenesis in honeybees and published his observations in 1845, is rarely cited, as a perusal of the literature on parthenogenesis in honeybees over the past 20 years readily confirm. A historical exception is that of François Huber (1814) whose treatise *Nouvelles Observations sur les Abeilles* remains a remarkably modern work. Similarly, the works of Dönhoff and Schirach remain primary source papers. By the same token, a list of Nobel laureates shows individual awards in physics, chemistry and physiology from 1901 until the end of WWII. Only then do we see 'multiple independent, discovery' awards to scientists who made discoveries working independently of each other, which in contemporary science, has become the norm (Merton 1973). The history of discoveries in apicultural science is yet to reach the 'multiple, independent discovery' phase. So we invoke the title of the famous 1897 painting of Paul Gaugin: *D'où Venons Nous/Que Sommes Nous/Où Allons Nous*, questions that are just as fundamental to human existence as to the course of science.

Another difference between the old *Honeybees and Wax* and the present volume is that the current text chapters include references and thus stand alone as journal review papers. This is to accommodate the distribution of electronic copies of individual chapters by the publisher. Readers of the complete text will note that several figures are used in more than one chapter. This occasional duplication arises because such figures are used in different arguments in each particular case.

The production of this volume would not have been possible without the quite considerable kindness of several people who have provided new information, references or reviewed various chapters in manuscript form:

Tara Sutherland (Canberra), Eileen Sheppard (Grahamstown), Anne Dufay (Sophia-Antipolis), Scott Camazine (Boalsburg), Mingxian Yang (Ya'an), Preecha Rod-im (Chom Bueng), Bhushan Karihaloo (Cardiff), Jürgen Tautz (Würzburg). On a personal note (C. W. W. Pirk), I like to thank my parents, Christel and Hartmut Pirk and my wife Catherine Sole for their support of my scientific endeavours.

We acknowledge permission of the publishers to use previously published figures from their journals. We owe a large debt of gratitude for years of financial support to the Council of Rhodes University (HRH), the Council of the University of Pretoria (CWWP), the National Research University Project of Thailand's Office of the Higher Education Commission and Thailand Research Fund (OD).

Finally, we owe an extraordinary debt of gratitude to Colleen Hepburn (Nelspruit) for reviewing the complete manuscript several times, and for her finely honed editorial skills and knowledge of the English language which certainly prevented this text from containing some abysmal errors.

Nelspruit, South Africa — H. R. Hepburn
Pretoria, South Africa — C. W. W. Pirk
Bangkok, Thailand — O. Duangphakdee

Contents

Chapter 1
General Introduction

Abstract This chapter consists of expanded abstracts of the contents of this book, beginning with the origin of beeswax, wax synthesis and secretion, and the ultrastructural correlates of its genesis. Honeybee nests are reviewed in terms of nest sites, space and density. Two hypotheses, a blueprint or a self-organization, have been proposed for the organization of the nest contents: the latter being highly probable. Honeybee variations in wax choice have also been assessed using waxes of several honeybee species as well as plant and mineral waxes. Waggle-dancers produce vibratory movements which are pulsed vibrations that increase during waggle runs. Honeybees secrete the same amount of wax whether queenless or queenright, and the actual secretion of wax is independent of queen status. The amount of wax produced is a linear function of the number of young bees in a colony, but the greatest amount of wax produced/bee, relative to colony size, occurs in small colonies. The nutrients that workers derive from pollen provide all the proteins, lipids, vitamins and minerals required for brood-rearing, and the primary consumers of pollen are nurse bees. Comb-building is conducted by many individuals, some in festoons, others not. Yet, the basic stimulus for comb-building is 'flowering', which provides the energy required for colony development. Comb-building pulses require a fullness of the comb threshold, with an excess or surplus brood and stored food. Cyclical changes of cellular organelles in the wax gland complex and the chemical composition of beeswax closely coincide with age-related rates of wax secretion. The mechanical properties and crystal structure of wax change with chemical additions by honeybees. Wax scales consist of aligned crystallites and their origin is probably due to a fusion of liquid products reaching the surface from the different cells in the wax gland complex. Analyses of derivatised comb waxes and their Euclidean distances are similar to those from morphometric, behavioural and DNA sequence analyses.

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_1,

1.1 The Origin of Beeswax

The first correct description of wax scales, their probable origin and uses, was made by Hornbostel (1744). In subsequent decades, microscopists observed the synchronised rise and fall of the elements of the wax gland complex of honeybees, epidermis, oenocytes and fat body, and thought that these were highly suggestive of a direct involvement of all three tissues in wax production. In an attempt to prove a necessary relationship between the simultaneous development of the wax gland epithelium, fat cells and oenocytes and wax secretion, Graber (1872) noted that the adipocytes are interspersed with 'oenocytes' (Wielowiejski 1886), and Holz (1878) offered the first alternative to the 'sweating' of beeswax hypothesis. Detailed studies were conducted that provided strong circumstantial evidence to support this proposition (Rösch 1927; Boehm 1965). Indeed, the wax mirror epidermis belongs to the Type 1 class of glandular cells (Noirot and Quennedey 1974), and consists of a system of microtubules that transport wax precursors from the fat body cells and oenocytes to the surface of the cuticle where they solidify and crystallise to become wax scales (Cassier and Lensky 1995). Studies of wax synthesis and secretion specifically identified sites for the origin of the hydrocarbon and fatty acid components within the wax gland complex, and established the necessary ultrastructual correlates of genesis and transport. The rates of wax secretion in honeybees of different ages have been measured, and the chemical composition of the tissues and ultrastructural changes corresponding with phases of wax production in relation to the division of labour, finally established (Hepburn et al. 1991).

1.2 Nests and Nesting

The nesting sites of open- and cavity-nesting honeybees are reviewed in terms of nest sites, space and honeybee density. Space comprises building space for new combs and living space for clustering bees. In a container of a fixed volume, a strong colony constructs more than a colony with a smaller population; but, the amount of comb constructed per bee decreases with increased density and increases in colony size (Freudenstein 1961). The quality aspects of space as a stimulus for comb-building include illumination and air movement. Volume, space and density will only operate on wax production when the colony has reached some critical, if yet indefinable, threshold. Wax bees move throughout the nest so there is a close synchrony between the 'needs' of specific comb-building areas and the presence of bees producing wax scales (Muller and Hepburn 1992; Pratt 2004). During comb-building there are concomitant changes in population size, population density, nectar and pollen influx, all of which affect honeybee/comb interactions. Nonetheless, 'space' is also a 'nearest neighbour' problem for colonies of cavity-nesting species, which translates to carrying capacity/km^2. On the other hand, it would appear that 'space' may well be a 'nearest neighbour' distance rule for the dwarf honeybees (Duangphakdee et al. 2013a).

1.3 Self-Organization of Nest Contents

The arrangement of the contents of both single vertical combs and horizontally arranged parallel combs are very similar among all species of honeybees; different areas of the comb are used repetitively for the same functions. They principally differ in the formation of their patterns which have been tacitly assumed for centuries to derive in some mysterious way as "in the nature of bees". Camazine (1991) erected a series of experiments to validate one of two mutually exclusive hypotheses for the comb patterns of *A. mellifera*: (1) a blueprint in which patterns develop in some pre-ordained and specified way intrinsic to bees; or, (2) a self-organization hypothesis ("a reaction–diffusion system" developed by Turing 1952), by which patterns emerge spontaneously from the dynamic interactions of the processes of placing, and then displacing, the different elements of the nests. The original self-organization hypothesis has been challenged, modified, and ultimately supported by rigorous mathematical analyses of this problem. The model of the self-organization hypothesis appears extremely robust and parsimonious and it remains the prevailing paradigm (Montovan et al. 2013). Explanations for pattern formation in the single comb dwarf and giant honeybee species are perhaps less difficult. Development of the vertical, single comb nest of *A. florea* is accomplished in four months after a swarm settles, and in only a few days the nest has already been partitioned into an area for honey (top of comb or 'crown'), an underlying pollen layer, below which both capped and uncapped larval cells occur. This basic pattern remains until the mature colony swarms some four months later. The major challenge is the construction of the crown comb (Duangphakdee et al. 2013b).

1.4 Interspecific Utilisation of Beeswax

A. florea was tested to determine whether they would salvage wax from their own deserted natal combs in preference to other conspecific combs and from heterospecific facsimiles of other species. Preferences for natal comb were significantly greater than for non-natal combs, with no wax being collected from heterospecific combs. Behavioural variations for wax choice were also assessed using the waxes of *A. capensis, A. florea, A. cerana, A. dorsata,* Japan wax, candelilla wax, bayberry wax and ozokerite which were tested in colonies of *A. m. capensis, A. florea* and *A. cerana. A. m. capensis* accepted only the beeswaxes. *A. cerana* and *A. florea* accepted the wax of *A. cerana, A. florea* and *A. dorsata* but rejected *A. m. capensis* and the other waxes (Hepburn et al. 2009). Comb-building in mixed-species colonies of *A. cerana* and *A. mellifera* was examined with foundation made from the waxes of these species and given to colonies with either an *A. cerana* or *A. mellifera* queen. The colonies did not discriminate between the waxes, and comb-building was a cooperative effort by both species (Yang et al. 2010).

The dwarf honeybees are unusual in that they regularly cannibalise wax from deserted nests (Pirk et al. 2011).

1.5 Communication of Vibrations and Scents

Communication across the combs of honeybees includes both distance and direction elements of waggle dances. Potential recruits attending a dancer emit vibrations which elicit a response to give the emitter a sample of nectar. Tooting and quacking by queens are both airborne sounds and substrate vibrations, which are carried mainly by the fundamental frequency component. The bees recognize these signals mainly by their temporal structure and comparisons of the threshold, emission level, and attenuation with distance, which suggests that they are used only within a restricted area of the comb (Michelsen 2012). When waggle-dancing honeybees move on comb they produce vibratory movements which indicates the location of the waggle dancer and the pulsed vibrations are increased during waggle runs, so amplifying the signals for remote dance-followers. Because sound intensity decreases with the density of the medium and with distance, beeswax is a suitable medium for sound transmission. Pheromones in combs serve as slow release systems with long time constants and include transmission of colony odour, queenrightness, cell capping, kin recognition, footprint pheromones, wax-salvaging behaviour etc. The specific dance sites that occur on combs are due to chemical tagging (Tautz and Lindauer 1997). Colony odour masking occurs when receiver bees are conditioned to the same comb source as introduced bees, which are accepted (Breed et al. 1988). A series of only a few methyl esters produced by queens, workers and drones are sufficient to induce the capping of mature brood (Le Conte et al. 1994); but it has also been proposed that workers cap cells depending on the depth of larvae in their cells and not on the ratios of ester emissions (Goetz and Koeniger 1992). Nonetheless, these results are not mutually exclusive in principle.

1.6 Wax Secretion, Comb Construction and the Queen

Discovery of queen substance led to the experimental dissection of the importance of these chemical signals in comb construction, especially because more combs are produced in the presence of mated queens than with virgin queens whose pheromonal bouquets differ substantially (Darchen 1956; Butler and Simpson 1958). In a series of experiments, Whiffler and Hepburn (1991) showed that bees secrete the same amount of wax whether queenless or queenright, with either mated or virgin queens, and living or dead. Moreover, removing mandibular glands or restricting workers access to the pheromones of queens has no effect on wax secretion.

Similarly, wax secretion does not differ significantly among colonies with caged or division board queens, with intact mandibular and abdominal tergite glands or not. The actual secretion of wax is independent of queen status. However, comb-building fundamentally differs from wax secretion because colonies headed by mated queens construct significantly more comb than queenless colonies, results consistent with other studies on *A. mellifera* and *A. cerana*. Collectively, these results indicate that the bouquet of the mandibular gland of queens cannot alone fully explain enhanced comb-building by queenright workers. Whatever the source of the comb-building stimulus, its effect requires direct contact with the queen because most comb is always built when workers have full access to a free-running, physical and chemical, mated queen; and, little comb is built when the colony has access only to the 'chemical' or 'physical' queen. The independence of wax secretion, as opposed to comb-building, from the pheromonal influence of the queen (Whiffler and Hepburn 1991) was subsequently confirmed in experiments by Ledoux et al. (2001).

1.7 The Significance of Brood

Differences in colony size among *Apis* species are not equated to the ratio of drones to workers or associated comb construction. Oviposition-related cell inspections reveal that a queen's decision to lay a fertilized egg or not, is determined by a specific stimulus generated on cell inspection. Uncapped or sealed queen cells cells are correlated to a reduction in the number of new cell constructions, possibly pheromonally mediated. Relative increases in the physiological activity of the wax glands in queenright bees are related to the age of the workers. Capped brood and broodlessness dampen the development of wax glands, while the presence of open brood stimulates their development, as under queenright conditions. Queenright bees produce much more comb than queenless bees; while queenless, broodright bees construct more comb than queenless, broodless bees. The amount of wax produced is a linear function of the number of young bees in a colony, but the greatest amount of wax produced/bee, relative to colony size, occurs in small colonies. Bees prevented from brood-rearing produce the same amount of wax as those engaged in both comb-building and brood-rearing. Colonies precluded from comb construction rear no more brood than those engaged both in brood-rearing and comb-building. The proportion of drone comb depends on the amount of drone comb and number of adult drones present in a colony, and is positively correlated to the number of workers. The combination of queenright and broodright colonies appears to be a more powerful stimulus than any other for comb-building.

1.8 The Role of Pollen in Comb Construction

The nutrients that workers derive from pollen provide all the proteins, lipids, vitamins, and minerals required for brood-rearing, and the primary consumers of pollen are nurse bees which feed the brood. The greatest net increase in the mass and nitrogen content of bees is obtained when bees are fed their normal diet based on pollen. The greatest rates of growth of young workers occur in their first week after eclosion, and pollen must be available for the normal development of the wax glands and for comb construction. Colonies provided with pollen begin brood-rearing earlier than the other colonies, and under temperate zone conditions, the relative abundance of pollen-rich plants flowering in spring drives brood-rearing. Likewise, increased access to pollen or protein resources is positively correlated with worker longevity. The need for pollen increases with the amount of brood which, in turn, can only increase in proportion to the availability of pollen. Pollen-fed bees produce considerably more comb than pollen-deprived bees. The control against an excess of stored pollen operate as a negative feedback loop.

Pollen foraging seems to be regulated by at least three mechanisms: young larvae, stored pollen, and empty space. The regulation of pollen foraging activity is based on the amount of brood which is a positive stimulus, while the quantity of stored pollen acts as an inhibitory stimulus. These two factors must eventually be integrated into single inhibitory signal on a sliding scale, which amounts to a mechanical analogue computer. Brood pheromone affects pollen foragers but not nectar-foraging behaviour. The dramatic increase observed for pollen foraging with supplemental brood pheromone suggests that the colony contains a pool of potential pollen foragers that are not actively foraging. These results support the stimulus response threshold hypothesis of division of labour. Camazine (1991) argued that the pattern of comb contents could be generated by a self-organizing algorithm of three simple rules: (1) the queen lays eggs in the centre of the comb; (2) workers deposit pollen and nectar at random; and (3) bees preferentially remove pollen and nectar from the brood nest relative to the honey storage area. Subsequent theoretical work supports this view.

1.9 Nectar Flows and Comb-Building

Comb-building is conducted in different areas of the nest by many individuals, some clustered in festoons, others not, while other wax-workings are often the efforts of individual bees (Lindauer 1952). Yet, the basic stimulus for comb-building is ‘flowering’, which produces the nectar and pollen essential to provide the energy required for colony development. These two factors allow colonies to grow and to complete annual cycles. If conditions are unfavourable colonies will abscond. This is borne out in the readily observed differences in bee behaviour between continents which have different climatic seasons. In temperate zones, the

onset of comb-building is associated with warm fronts, the more intense and closer together, the greater the colony response. European *A. mellifera,* are commonly dormant during winter, but Asian bees are active during the tropical dry season. Comb-building occurs during the dry season and the rainy season is their dormant period. Some plants flower during the rainy season and provide sufficient forage for the dwarf honeybees because they can still complete their comb within three weeks. Large *A. dorsata* colonies however cannot subsist on such meagre resources and seasonally migrate. Comb-building pulses require that comb fullness reach a threshold, with a balance of brood and stored food. Comb-building peaks are correlated with periods of high comb fullness, and with correlations between daily nectar intake and comb construction. Wax production is reduced in the absence of a nectar flow; likewise, the greater the supply of combs in the nest, the greater the increase in the number of nectar foragers. Nectar forage, empty combs and free building space within the nest are correlated with engorgement of the honey stomach and wax secretion. Once building has begun, the colony will track only nectar intake to control comb-building. They build when nectar can be collected in the field and, the combs are filled above their thresholds for comb fullness and nectar intake. The amount of wax is constant among age cohorts and across the seasons. About half of the wax in a colony is borne by festoon bees, the remainder from non-festoon bees, except in winter, when non-festoon wax production is higher than festoon wax production.

1.10 Construction of Combs

The construction of cells and the regulation of the space between combs are separate but related problems. The space between combs, affected by the bees themselves, is the very basis of contemporary practical beekeeping. Within a honeybee multiple comb nest there are several independent comb starts within the building cluster and at different attachment sites. Then Darchen's "rule of parallelism" comes into play, because the building bees modify their constructions so as to keep a reasonably equable and parallel space between combs. Parallelism overrides other considerations, such as the length of cells. Comb construction is the result of interplay of vertical and lateral forces acting on the combs which, over time, lead to many imperfections that are eventually hidden by retouching. A building cluster can independently exert torsional and tensile loading of a piece of comb. In the process of twisting comb, cell walls will inevitably be broken; however, the bees rapidly mend such tears and fractures. Honeybees achieve reasonably parallel sets of combs, but in the end, they have some means of both achieving this and of maintaining the distance between combs within limits that we can recognise as tolerances. This may be due to the detection of the vertical axis of gravity. Building bees might be able to exploit a sense of gravity that would allow them to build vertical combs. This was shown by disrupting the function of an organ and then observing the effects on comb construction. An unimpaired

sense organ of the honeybee neck is the instrument by which bees detect gravity and so orient themselves during comb construction, an interpretation supported by the discovery of magnetic material in a band across the abdomen. Indeed, different magnetic oxide nanoparticles, ranging from super-paramagnetic to multi-domain particles, were observed in all body parts of honeybees, but greater relative concentrations occurred in their abdomens and antennae. Mixed colonies of *A. mellifera* and *A. cerana* cooperate in normal building behaviour, only the number of irregular cells built was noticeable. In both pure controls, no worker brood was reared in the cells built on the foundation made of the wax of the opposite species. In pure *A. mellifera* colonies, cell size was modified, whereas those of *A. cerana* were constructed without modification but the cells based on *A. mellifera* wax were used only to rear drones or for storage.

1.11 Energetics of Honey/Beeswax Conversion

Consideration of the rates of wax production by *A. mellifera* (combs constructed), and the costs of construction (sugar required), developed during the period (1830–1840) of the application of the balance sheet. Moreover, it was known that the presence or absence of brood, pollen, combs and queens all had a bearing on wax production. A century later, sugar/wax conversion ratios were defined as the net amount of sugar consumed against wax production; but there was no insight as to how the energy assimilated by the bees was partitioned in the colony. Then, in Taranov's (1959) experiments, the total amount of wax produced was linearly related to the amount of sugar consumed. Others reported that comb construction was proportional to colony size and to nectar income, even though both groups lacked a measure of energy flow in their studies. The experiments and observations of this period suffered from a failure to separate the costs of colony maintenance vis-à-vis the production of wax.

An analytical refinement to compensate for the concentration of the sugar that the bees had stored in their combs provided a more accurate measure of sugar consumption. However, two major factors remained in the cost equation: (1) the relative importance of age structure in wax production; and (2) the problems of heat production as related to age, colony size and the synthesis of wax itself. Subsequently, Hepburn et al. (1984) calculated the rate of sugar consumption (corrected for attrition) and sugar stored in the nascent combs, as well as the rate of comb construction. The real metabolic rate, averaged over time for bees of different ages, showed that a plateau was reached in bees at about 12 days old, figures that included an adjusted metabolic rate as a function of bee age.

Because oscillations in metabolic rate vary with age, this does not to imply that all the energy expenditure above a basal rate was diverted into wax production as such, because some expenditure would have been associated with the production of cluster heat. Within defined limits, it becomes cheaper for the bees to produce wax and to build comb as they become older. This trade-off, or cost calculation,

comes into play at both individual and colony levels. How wax synthesis and comb-building are constrained by thermal conditions is not well understood and there is only indirect evidence that bees cannot, or will not, sustain the costs of heat and comb production when both are very high. Both wax secretion and construction rapidly decline in autumn, and virtually cease during winter. It is not yet possible to adequately assess the relationship of wax synthesis and comb construction to the thermal conditions of a colony's nest.

1.12 Construction of Cells

In temperate zones, the onset of and sustained comb-building is always associated with warm fronts, the more intense and closer together, the greater the colony response (Koch 1961). European *A. mellifera,* are commonly dormant during the dry season (winter), but Asian bees are active during the tropical 'dry season'; nearer to the equator there are rainy and dry seasons, and sometimes, a cool or mild season. Comb-building occurs during the dry season while the rainy season is the dormant period. Some plants flower throughout the rainy season and the dwarf honey bees forage because they need smaller amounts of resources to establish new colonies, which they can complete within three weeks. Large *A. dorsata* colonies cannot subsist on such meagre resources, and seasonally migrate to find available forage (Duangphakdee et al. 2013a). Comb-building pulses require that for a colony currently collecting nectar, the fullness of the comb must reach a threshold, with a balance of brood and stored food. Comb-building peaks are correlated with periods of high comb fullness and weight gain, and with other correlations between daily nectar intake and comb construction on the following day and so on (Pratt 1998). Wax production is reduced in the absence of a nectar flow; likewise, the greater the supply of combs in the nest, the greater the increase in the number of nectar foragers, which is possibly stimulated by comb volatiles. Nectar forage and the availability of empty combs, as well as free building space within the nest are correlated with engorgement of the honey stomach and wax secretion. Bees build only when they collect nectar; but, comb-building is optimal when some threshold amount of honey is stored. The regulation of the timing to build is partly independent of the amount and duration of building. Once building has begun, the colony will track only nectar intake to control comb-building (Pratt 1999). They build when nectar can be collected in the field and the filling of the comb is above their threshold for comb fullness and nectar intake. The amount of wax is constant among age cohorts and across the seasons. About half of the wax in a colony is borne by festoon bees, the remainder by non-festoon bees, except in winter, when non-festoon wax production is higher than festoon wax production (Muller and Hepburn 1992).

1.13 Conversion of Scale Wax into Combs

The cyclical changes of cellular organelles and the chemical composition of beeswax precursors found in the haemolymph and gland tissues, closely coincide with age-related rates of wax secretion. It is one of the divisions of labour, and this coincidence of physiology and behaviour parallels other polyethisms (Hepburn et al. 1991). The mechanical properties and crystal structure of wax change with chemical additions by honeybees. Intact scales contain some non-lipoidal components and differ from comb wax in lipid composition. The mechanical properties of scale and comb wax vary with temperature. There is a linear relationship between load and elongation in the tensile stress–strain curves to the maximum sustainable load, so that the yield stress coincides with the ultimate strength of the material (Hepburn and Kurstjens 1988). New comb wax is an isotropic plastic whose mechanical properties depend on temperature. Larvae introduce silk into the comb in a random array so that the cells are structurally isotropic. The addition of silk improves the load-carrying capacity of the combs. With use, the combs become fibre-reinforced composite materials, with properties entirely different from the individual components (Hepburn and Kurstjens 1988; Zhang et al. 2010). Wax scales form as the liquid wax fractions transude from the pore canals onto the surface of the wax mirror, where these small droplets coalesce to form thin layers of wax, this process continues until a wax scale forms (Cassier and Lensky 1995). The relatively crystalline scale is reduced to an amorphous state during cell construction; but, given the warmth of the colony and the physical work done on the wax, an ordered texture is gradually introduced into the combs.

1.14 Material Properties of Scale and Comb Wax

Although the honeybee nest begins with the conversion of wax scales into combs, these two materials differ in their chemistry, crystal structure, tensile strength and stiffness which, in turn, are modified by the secretions of honeybees during comb-building (Hepburn and Kurstjens 1988). The strength of wax scales is about the same at temperatures between 25 and 35 °C, but above 35 °C, it declines. In contrast, comb wax is weaker and steadily decreases in strength with increasing temperature. The relative workability of scale wax is about the same between 25 and 45 °C, but is the converse with comb wax. Wax scales are stronger and more distensible, but less stiff than comb wax at 35 °C and require more energy to work than comb (Kurstjens et al. 1985). The reworking of constructed comb is significantly more cost-effective than starting a comb with wax scales. Salvaging old comb wax is also energetically advantageous (Pirk et al. 2011). Differences in the mechanical properties of scale and comb wax show that comb-building involves chemical modifications of the waxes. The relative amounts and kinds of lipids affect interspecific stiffness. Likewise, differing kinds and amounts of protein in

the waxes affect their mechanical properties. Highly-textured scales are converted from an anisotropic into an isotropic state. Lipases added during chewing modify the lipid composition of the scale in which stiffness is lost, but regained with the addition of proteins in comb-building. Beeswaxes are crystalline, the crystallites in wax scales are aligned, some perpendicular to the surface, others between 62° and 65° to the surface (Kurstjens et al. 1985). Their origin is probably due to a fusion of the liquid products reaching the surface from the different cells in the wax gland complex (Cassier and Lensky 1995).

1.15 The Wax Gland Complex

The first correct descriptions of wax scales, their probable origin and uses, were made by Hornbostel (1744). In subsequent years, microscopists observed the synchronised rise and fall of the epidermis, oenocytes and fat body of honeybees and thought that these were highly suggestive of a direct involvement of all three tissues in wax production. In an attempt to prove a necessary relationship between wax secretion and the simultaneous development of the wax gland epithelium, fat cells and oenocytes, Graber (1872) noted that the adipocytes are interspersed with 'oenocytes' (Wielowiejski 1886), and Holz (1878) offered the first alternative to the 'wax-sweating' hypothesis. Detailed studies were conducted that provided circumstantial evidence to support this proposition. Indeed, the wax mirror epidermis belongs to the Type 1 class of glandular cells, and indicates the reality of a system of microtubules to transport wax precursors from the fat body cells and oenocytes to the surface of the cuticle, where they solidify and crystallise to become wax scales. In earlier studies, Sanford and Dietz (1976) and Hepburn et al. (1991) both reported that the smooth endoplasmic reticulum (SER) is absent from wax secreting workers, and concluded that the epidermis mainly provides an elaborate system for wax precursor transport (Reimann 1952; Locke 1961; Hepburn 1986). Later studies of wax synthesis and secretion specifically identified sites for the origin of the hydrocarbon and fatty acid components within the wax gland complex, and established the necessary ultrastructual correlates of genesis and transport. Volume changes in the wax gland oenocytes, adipocytes and epidermis are described in terms of metabolic activity. However, in further electron microscopical studies of the wax gland complex, Cassier and Lensky (1995) reinvestigated the possible role of the epidermis and its transport modalities. They were able to show that there are indeed large cisternae of SER which are probably involved in the transport of wax precursors from the oenocytes to the pore canals, as well as carrying apolipophorins from the haemolymph to the wax mirrors. Although the entire discussion in this chapter is based on studies of *A. mellifera,* it can be noted that a brief paper on the ultrastructure of the wax gland of *A. cerana* confirms that this species is conformal with the details given here for *A. mellifera* (Du and Li 1991). The rates of wax secretion in honeybees of different ages have been measured, and the chemical composition of the tissues and ultrastructural

changes corresponding with phases of wax production in relation to the division of labour, finally established.

1.16 The Chemistry of Beeswax

Publications on the physical constants of the comb waxes of Asian and European beeswaxes first appeared a century ago. It was soon shown that carbon chain length was, on average, shorter in the Asian beeswaxes than in *A. mellifera,* which explains the lower melting points of the former. The Asian waxes are more alike than they are to *A. mellifera.* In Asian beeswaxes, the amounts of C_{31} and C_{33} in the pool of free fatty acids are reduced, but C_{25} hydrocarbons increased compared to that of *A. mellifera.* The major compound families in beeswax are alkanes, alkenes, free fatty acids, monoesters, diesters and hydroxymonoesters, while fatty alcohols and hydroxydiesters are minor constituents. There are notable species-specific differences among all honeybee species, but all share a complex mixture of homologous neutral lipids (Tulloch 1980; Frölich et al. 2000). The amounts of acylglycerols are the same in scale and comb wax, but diacylglycerols dominate the former and monoacylglycerols the latter. There are more double-bonded fatty acids in comb wax than in scale wax, and a greater saturation of the fatty acids in comb wax. Beeswaxes analysed with high temperature gas chromatography yielded a characteristic elution pattern for the waxes of each honeybee species (Aichholz and Lorbeer 1999). A parsimonious, unweighted, pair-group analysis based on the distributions of the chemical constituents for 82 elution peaks of the derivatized comb waxes of six species of honeybees, and the Euclidean distances for the beeswaxes, all present a very similar picture to that obtained from morphometric, behavioural and DNA sequence analyses (Phiancharoen et al. 2011). The wax glands and their products of secretion were highly conserved features during honeybee evolution.

1.17 Synthesis of Beeswax

The notion that honeybees secrete wax and not gather it from blossoms was first shown in the mid-18th century (Hornbostel 1744). Later, Huber (1814) observed that newly settled swarms do not gather pollen but construct combs and he concluded that beeswax was the secretory product of the glands of the wax mirrors and fuelled by honey. However, the actual amount of fatty material, present in bees before and after their incarceration in experimental cages and in combs constructed in the interim, had to be determined. This Dumas and Edwards (1843) did and they concluded that the amount of fatty material present at the onset of the experiment could not account for the wax produced by the end of the experiment; hence bees both synthesise and secrete wax. A century later, Piek (1961, 1964) fed

captive bees (1-^{14}C)-acetate, (UL-^{14}C)-glucose and deuterated water and recovered the labels both from bees and newly constructed combs. Then, Lambremont and Wykle (1979) incubated homogenates of the wax glands with (1-^{3}H)-tetracosanol and recovered the label only in the wax ester fraction, the ^{3}H wax ester fraction yielded a ^{3}H-fatty alcohol with the same R_f value as authentic tetracosanol. Blomquist and Ries (1979) showed that the incorporation of long-chain primary alcohols, fatty acids and the acyl group of acyl-CoA into wax monoesters and that (1-^{14}C)-palmitate entry into the monoester pool was enhanced by ATP, CoA and $MgCl_2$, while the addition of palmitoyl-CoA resulted in a fivefold yield increase. Subsequently, the specific cellular sites for the origin of hydrocarbons and fatty acids within the wax gland complex and the necessary ultrastructural correlates of this activity and of their transport were determined (Hepburn et al. 1991).

1.18 Material Properties of Honeybee Silk

Colourless honeybee silk, ~3 μm diameter, is produced through a spinneret at the tip of the labium-hypopharynx. Successive generations of brood apply silk to the cell walls, making the cells smaller as silk is deposited in the old brood combs. X-ray diffraction data show that honeybee silk contains ά-helical proteins ordered into coiled-coil structures with an axial periodicity of about 28 nm, and form a four-stranded array parallel to the fibre axis (Lucas and Rudall 1968). Honeybee fibroin is crystalline, but, when hydrated is only half as stiff as when dry, although they are equal in strength. The fibroin is hygroscopic and highly distensible when solvated because of its molecular conformation. The mechanical properties of silk are independent of temperature. Lithium thiocyanate and urea virtually eliminate the yield point of honey bee silk tested both dry and in distilled water, and values for stress in the slope of the solvent-related curves is reduced. The solvents act directly on hydrogen bonds and then the silks behave as unconnected bends during tensile deformation (Hepburn et al. 1979). The components, hierarchical structure and the conditions of their production all affect the mechanical properties of natural silks. The amino acid sequence in honeybee silk protein provides an explanation of why the coiled-coil packing is atypically tight; the most abundant core residue is the small amino acid, alanine. An atomistic simulation for the unfolding behaviour of ά-helical protein shows that two discrete transition states correspond to two fracture mechanisms. Six honey bee silk genes have now been identified, using a combination of genomic and proteomic techniques (Sutherland et al. 2010). Contemporaneously, Ackbarow et al. (2007, 2009) have begun to investigate multiple energy barriers and robustness in the fracture mechanics of ά-helical proteins and to elucidate why they are self-protective and flaw-tolerant.

References

Ackbarow T, Chen X, Keten S, Buehler MJ (2007) Hierarchies, multiple energy barriers and robustness govern the fracture mechanics of α-helical and β-sheet protein domains. Proc Nat Acad Sci 104:16410–16415

Ackbarow T, Sen D, Thaulow C, Buehler MJ (2009) Alpha-helical protein networks are self protective and flaw-tolerant. PLoS ONE 4(6):e6015. doi:10.1371/journal.pone.0006015

Aichholz R, Lorbeer E (1999) Investigation of comb wax of honeybees with high-temperature gas chromatography and high-temperature gas chromatography-chemical ionization mass spectrometry I. High-temperature gas chromatography. J Chromatogr A 855:601–615

Blomquist GJ, Ries M (1979) The enzymatic synthesis of wax esters by a microsomal preparation from the honeybee *Apis mellifera* L. Insect Biochem 9:183–188

Boehm B (1965) Beziehungen zwischen Fettkörper, Oenocyten und Wachsdrüsenentwicklung bei *Apis mellifica* L. Zeit Zellforsch Mikrosk Anat 65:74–115

Breed MD, Williams KR, Fewell JH (1988) Comb wax mediates the acquisition of nest-mate recognition cues in honey bees. Proc Natl Acad Sci 85:8766–8769

Butler CG, Simpson J (1958) The source of the queen substance of the honey-bee (*Apis mellifera* L.). Proc Roy Ent Soc Lond 33:120–122

Camazine S (1991) Self-organizing pattern formation on the combs of honey bee colonies. Behav Ecol Sociobiol 28:61–76

Cassier P, Lensky Y (1995) Ultrastructure of the wax gland complex and secretion of beeswax in the worker honey bee *Apis mellifera* L. Apidologie 26:17–26

Darchen R (1956) La reine d'*Apis mellifica* et les constructions cirières. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 243:439–441

Du ZL, Li N (1991) The ultrastructure of the wax gland cell of the worker honey bee, *Apis cerana*. Acta Ent Sinica 34:289–291 [in Chinese]

Duangphakdee O, Hepburn HR, Rod-im P (2013a) Seasonal movements of the red dwarf honeybee, *Apis florea*. (In ms.)

Duangphakdee O, Rod-im P, Hepburn HR (2013b) Nest development in the red dwarf honeybee *Apis florea*. (In ms.)

Dumas JB, Edwards HM (1843) Note sur la production de la cire des abeilles. Ann Sci Nat Paris 20:l–8

Freudenstein H (1961) Entwicklung und Leistung der Wachsdrüsen der Honigbiene. Biol Zentralbl 80:479–492

Frölich B, Tautz J, Riederer M (2000) Chemometric classification of comb and cuticular waxes of the honeybee *Apis mellifera carnica*. J Chem Ecol 26:123–137

Goetz B, Koeniger N (1992) Structural features trigger capping of brood cells in honey bees. Apidologie 23:211–216

Graber V (1872) Vorläufiger Bericht über den propulsatorischen Apparat der Insekten. Sber Akad Wiss Wien 65:189–204

Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin

Hepburn HR, Kurstjens SP (1988) The combs of honeybees as composite materials. Apidologie 19:25–36

Hepburn HR, Chandler HD, Davidoff MR (1979) Extensometric properties of insect fibroins: the green lacewing cross-β, honeybee α-helical and greater waxmoth parallel-β conformations. Insect Biochem 9:69–77

Hepburn HR, Hugo JJ, Mitchell D, Nijland MJM, Scrimgeour AG (1984) On the energetic costs of was production by the African honeybee, *Apis mellifera adansonii*. S Afr J Sci 80:363–368

Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax in honeybees. Apidologie 22:21–36

Hepburn H, Radloff SE, Duangphakdee O, Phiancharoen M (2009) Interspecific utilization of wax in comb-building by honeybees. Naturwissenschaften 96:719–723

Holz H (1878) Das organ der wachsbildung. Bienen-Ztg 34:183–184

Hornbostel HC (1744) (orig Melittophilus Theosebastus) Neue Entdeckung, wie das Wachs von den Bienen Komt. Hamburg Vermis Bibliothek 2:45–62
Huber F (1814) Nouvelles observations sur les abeilles [English translation, 1926]. Dadant, Hamilton
Koch HG (1961) Der Baubeginn der Bienenvölker als phänologische Ereignis. Z Angew Met 4:69–82
Kurstjens SP, Hepburn HR, Schoening FRL, Davidson BC (1985) The conversion of wax scales into comb wax by African honeybees. J Comp Physiol B156:95–102
Lambremont EN, Wykle RL (1979) Wax synthesis by an enzyme system from the honey bee. Comp Biochem Physiol 63B:131–135
Le Conte Y, Sreng L, Trouiller J (1994) The recognition of larvae by worker honeybees. Naturwissenschaften 81:462–465
Ledoux MN, Winston ML, Higo H, Keeling CI, Slessor KN, Le Conte Y (2001) Queen and pheromonal factors influencing comb construction by simulated honey bee (*Apis mellifera* L.) swarms. Insectes Soc 48:14–20
Lindauer M (1952) Ein Beitrag zur Frage der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 34:299–345
Locke M (1961) The cuticle and wax secretion in *Calpodes ethlius* (Lepidoptera: Hesperidae). Q J Microsc Sci 101:333–338
Lucas F, Rudall KM (1968) Extracellular fibrous proteins: the silks. In: Florkin M, Stotz EH (eds) Comprehensive biochemistry. Elsevier, Amsterdam, 26, pp 475–558
Michelsen A (2012) How do honey bees obtain information about direction by following dances? In: Galizia CG, Eisenhardt D, Giurfa M (eds) Honeybee neurobiology and behavior. Springer, Berlin
Montovan KJ, Karst N, Jones LE, Seeley TD (2013) Individual behavioral rules sustain the cell allocation pattern in the combs of honey bee colonies (*Apis mellifera*). J Theoret Biol 336:75–86. doi:10.1016/j.jtbi.2013.07.010;arXiv:1211.4298v2 [q-bio.PE]
Muller WJ, Hepburn HR (1992) Temporal and spatial patterns of wax secretion and related behaviour in the division of labour of the honeybee (*Apis mellifera capensis*). J Comp Physiol 171:111–115
Noirot C, Quennedey A (1974) Fine structure of insect epidermal glands. Ann Rev Entomol 19:61–80
Phiancharoen M, Duangphakdee O, Hepburn HR (2011) The biology of nesting. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 109–132
Piek T (1961) Synthesis of wax in the honey bee (*Apis mellifera* L.). Proc K Ned Akad Wet Ser C Biol Med Sci 64:648–654
Piek T (1964) Synthesis of wax in the honeybee (*Apis mellifera* L.). J lnsect Physiol 10:563–572
Pirk CWW, Crous KL, Duangphakdee O, Radloff SE, Hepburn HR (2011) Economics of comb wax salvage by the red dwarf honeybee, *Apis florea*. J Comp Physiol 181:353–359
Pratt SC (1998) Condition-dependent timing of comb construction by honeybee colonies: how do workers know when to start building? Anim Behav 56:603–610
Pratt SC (1999) Optimal timing of comb construction by honeybee (*Apis mellifera*) colonies: a dynamic programming model and experimental tests. Behav Ecol Sociobiol 46:20–42
Pratt SC (2004) Collective control of the timing and type of comb construction by honey bees (*Apis mellifera*). Apidologie 35:193–205
Reimann K (1952) Neue Untersuchungen über die Wachrdrüse der Honigbiene. Zoo1 Jahrb Abt Anat Ont 72:147–188
Rösch GA (1927) Über die Bautätigkeit im Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Zeit Vergl Physiol 6:265–298
Sanford MT, Dietz A (1976) The fine structure of the wax gland of the honeybee (*Apis mellifera* L). Apidologie 7:197–207
Sutherland TD, Young J, Weisman S, Hayashi CY, Merrit D (2010) Insect silk: one name, many materials. Ann Rev Entomol 55:171–188
Taranov GF (1959) The production of wax in the honeybee colony. Bee Wld 40:113–121

Tautz J, Lindauer M (1997) Honeybees establish specific sites on the comb for their waggle dances. J Comp Physiol A180:537–539
Tulloch AP (1980) Beeswax—composition and analysis. Bee Wld 61:47–62
Turing AM (1952) The chemical basis of morphogenesis. Phil Trans R Soc Lond 237(641):37–72
Whiffler LA, Hepburn HR (1991) The queen in relation to wax secretion and comb-building in honeybees. J Comp Physiol A169:209–214
Wielowiejski H (1886) Über das Blutgewebe der insekten. Z Wiss Zoo1 43:512–536
Yang MX, Hepburn HR, Duangphakdee O, Phiancharoen (2010) Comb construction in mixed-species colonies of honeybees, *Apis cerana* and *Apis mellifera*. J Exp Biol 213:1659–1664
Zhang K, Duan H, Karihaloo B, Wang J (2010) Hierarchical, multilayered cell walls reinforced by recycled silk cocoons enhance the structural integrity of honeybee combs. Proc Natl Acad Sci 107:9502–9506

Chapter 2
Nesting: Sites, Space and Density in Comb-Building

Abstract The nesting sites of open- and cavity-nesting honeybees are reviewed in terms of nest sites, space and honeybee density. Space comprises building space for new combs and living space for clustering bees. In a container of a fixed volume, a strong colony constructs more than a colony with a smaller population; but, the amount of comb constructed per bee decreases with increased density and increases in colony size. The quality aspects of space as a stimulus for comb-building include illumination and air movement. Volume, space and density will only operate on wax production when the colony has reached some critical, if yet indefinable, threshold. Wax bees move throughout the nest so there is a close synchrony between the 'needs' of specific comb-building areas and the presence of bees producing wax scales. During comb-building there are concomitant changes in population size, population density, nectar and pollen influx, all of which affect honeybee/comb interactions.

2.1 Introduction

Nesting is critical for the homeostasis, stability and ultimately the survival of honeybee colonies, and provides an arena in which colony growth unfolds in the usual annual colony cycle of swarming, reproduction and migration (Hepburn 2011). Once a swarm of bees has left its maternal nest it must find a new home, and descriptions of the process for *A. mellifera* abound as early as the early 18th century (Thorley 1744). The first studies of the ways in which colonies of the Asian *A. cerana, A. dorsata* and *A. florea* find new nest sites, have been described in a charming monograph by Lindauer (1961). Basically, the scout bees of a colony scour the countryside for potential nest sites and convey the information that they have reconnoitred to their nestmates. In the mother colony, this information is shared and "debated" until a consensus is reached as to the 'best' of the sites offered. Major studies on the nature of these debates, their duration and intensity, began with early studies on *A. mellifera* some 30 years ago, by Seeley and Morse (1978; Seeley et seq.), and have been recently summarised (Seeley 2010). These matters are discussed in detail below. 'Best' is the gist of the problem in a nutshell, and we suggest that 'best' can largely,

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_2,

but not entirely, be gleaned from a survey of natural honeybee nest sites (specifically excluding beekeeping hives) among the species of honeybees.

This is a review of the nesting sites of honeybees and the peculiarities and constraints of open-nesting and cavity-nesting. The honeybees comprise three groups with respect to nesting: the dwarf honeybees, *A. andreniformis* and *A. florea*, are single comb, open-nesting species; the medium-sized species, *A. cerana*, *A. koschevnikovi*, *A. mellifera, A. nigrocincta* and *A. nuluensis*, are multiple comb cavity-nesting bees; and the giant honeybees, *A. dorsata* and *A. laboriosa,* are also single comb open-nesting species (Phiancharoen et al. 2011). The ways in which the nests of these species are attached to a substrate further differentiate them: (1) there are no horizontal surfaces in the nests of the medium-sized and the giant honeybees, so communication using the dance language occurs in the vertical mode for both groups; whereas (2) in the dwarf species, dance language communication occurs in the horizontal mode. These factors clearly determine the suitability of potential nesting sites in the first instance for these species. Both open-air nesting and cavity-dwelling nesting sites further constrain the honeybees in terms of colony defense and micro-environmental homeostasis (Fuchs and Tautz 2011; Kastberger et al. 2011). Of equal importance is the nature of the nesting sites, mode of comb construction (Hepburn 1986) and the physico-chemical properties of the actual construction materials (Hepburn 1986; Phiancharoen et al. 2011).

2.2 Nesting Sites

2.2.1 The Dwarf Honeybees

Nests of *A. florea* occur in wooded areas, urban settings, areas with intensive agricultural activity as well as in the savanna (Franssen 1932; Free 1981; Matsuura 1983; Booncham et al. 1995; Nagaraja and Rajagopal 1999). *A. florea* nests are attached to a wide variety of plants (Basavarajappa 1998), and partially exposed to sunlight, wind and rain, and often have one surface of the comb exposed to direct sunlight for several hours a day (Fig. 2.1). *A. florea* are more likely to nest in diverse places, such as high up in tall trees in Southeast Asia, while in arid Western Asia they commonly nest in caves and buildings as well (Whitcombe 1984; Mossadegh 1990). *A. andreniformis* nests throughout Southeast Asia are most commonly observed in and near undisturbed, mixed deciduous and evergreen forests. Their nesting habitats are usually dark and shady places (20–35 % sun), well hidden and widely spaced. *A. florea* and *A. andreniformis* usually build single-comb nests in shrubs, bushes, and small trees, but double-comb nests have occasionally been reported for *A. florea* (Douglas 1886; Akratanakul 1977; Free 1981; Whitcombe 1984). *A. florea* nests are typically about 0.5–10 m above ground, but in towns and cities they are commonly found up to 15 m (Wongsiri et al. 1996; Wongsiri et al. 1997). *A. florea* are very adaptable and seem to find suitable nesting sites under extremely varied conditions (Mogga et al. 1989; Moritz et al. 2010).

Fig. 2.1 *Apis florea* nests with (*left*) and without (*right*) workers. On the *right*, one can see the differentially utilised parts of the comb. The crown with sealed honey above the twig, open brood or empty cells in the centre surrounded by sealed brood and newly constructed cells that are empty

Neither *A. florea* nor *A. andreniformis* form nest aggregations in the same tree or shrub (Wongsiri et al. 1996). However, they can achieve a relatively high concentration ranging between 7.1 and 14.3 colonies/km^2 (Duangphakdee et al. 2013a). Nesting density of *A. andreniformis* remains unreported; however, casual observations in Sabah State, Borneo (Duangphakdee, pers. obs.) and Sumatera, Indonesia (Hepburn, pers. obs.) indicate that they are diffusely distributed. Studies in north-western Thailand, have shown that *A. florea* have a wide range of nesting habitats and food sources, estimated from the number of tree species used for nesting (Akratanakul 1977; Oldroyd et al. 2008; Basavarajappa 1998). However, Rinderer et al. (2002) reported that *A. andreniformis* and *A. florea* colonies have a tendency to locate their nests near nests of their own species in south-eastern Thailand. *A. andreniformis* and *A. florea* colonies select similar nest sites, but the spatial correlations of these sites were significantly negative, indicating that colonies may avoid areas containing nests of the other species. It is rare to find nests of *A. florea* in the same tree as another honeybee species; but, curiously, an *A. florea* nest was once seen in the same tree as an *A. dorsata* nest, the former was about 6 m from the ground, the latter 13 m (Duangphakdee and Hepburn, unpubl. obs.). However, it has been stated that *A. florea* colonies build nests aggregated near one another forming spatial clumps (Rinderer et al. 2002; Wattanachaiyingcharoen et al. 2008).

2.2.2 The Cavity-Nesting Honeybees

The cavity-nesting bees show preferences for nesting sites which vary within races and among *Apis* species. Among the sympatric cavity-nesting species of Indonesia, different species nest in distinctly different habitats. *A. cerana* mainly nest in

agricultural or disturbed areas, while *A. nigrocincta* nest more deeply in the forests (Matsuura 1983; Kuntadi 1989; Hadisoesilo 1997). Similarly, *A. koschevnikovi* occur in primeval forests while *A. cerana* occur mostly in secondary forests, agricultural and urban areas in Peninsular Malaysia (Otis 1996). It is not evident whether these species specifically avoid aggregated nest sites, but some reports are suggestive to the contrary (Hadisoesilo 1997; Bakker 1999). There is no published information on nesting of *A. nigrocincta, A. koschevnikovi* and *A. nuluensis* as of yet.

Nest density is probably related to topographical variations and the availability of profitable forage. Nest density for *A. mellifera* ranges from 0.5 to 7.8 nests per km^2 whereas nest density in tropical bees is greater. For example, Inoue and Adri Salmah (1990) measured nest density of *A. cerana* in Padang, Sumatra and found 22 nests/km^2 with a mean distance of about 100 m between nests. Aggregations of nests are not well known in cavity-nesting bees; nevertheless, Rinderer et al. (2002) suggested that there is a tendency in *A. cerana* to form aggregated nests. The nest cavity volume of *A. cerana* is usually about 10–15 l, but ranges from 4.5 to 97 l (Inoue and Adri Salmah 1990; Oldroyd and Wongsiri 2006). Nest entrances may be about 1–2 m above ground, but they seem to have no real preference for height, because nests can also be many meters above ground or in cavities within the ground. Entrance sizes range between 2 and 100 cm^2 (Seeley et al. 1982; Inoue and Adri Salmah 1990; Oldroyd and Wongsiri 2006). Such studies are extremely few, but Bakker (1999) reported that *A. nigrocincta* may be less specific in its choice of nest sites. The first study of feral nests of European-derived *A. mellifera*, of which we are aware, is that of Seeley and Morse (1976), who analysed the structures of 21 such nests and found that nest cavities are vertically elongate, more or less cylindrical and 30–60 l in volume.

2.2.3 The Giant Honeybees

Unlike other *Apis* species, the giant bees, *A. dorsata* and *A. laboriosa,* build very exposed and easily visible nests (Starr et al. 1987; Reddy and Reddy 1989; Sattigi 2001; Woyke et al. 2001; Neupane et al. 2004; Reddy 1983). *A. dorsata* builds nests in inaccessible places, like vertical rock faces (hence the name ‘rock bee’ in India), in gorges along hill profiles, tall man-made structures such as water towers and buildings, and in the higher branches of remarkably, emergent tall trees which are highly visible in their surroundings (Fig. 2.2—Deodikar et al. 1977). Unusual nests, only 1 m above ground, have also been observed (Duangphakdee and Hepburn, unpubl. obs.). *A. dorsata* tend to build their combs in a north–south direction, minimising the exposure to strong wind and sunlight (Deodikar et al. 1977; Woyke et al. 2004). *A. laboriosa* apparently always build their nests beneath unweathered, light-coloured clear cliffs or rock overhangs, which have recently been analysed and described in great detail (Woyke et al. 2012). They have never been reported to nest on the branches of trees (Roubik et al. 1985;

Fig. 2.2 A tree with several *A. dorsata* nests and one unoccupied comb in the foreground

Underwood 1986); but, there is simply no information as to whether they are able to do so. Colonies of *A. dorsata* re-use preferred trees after an absence of several months (Neumann et al. 2000; Paar et al. 2000). Previous work has suggested that visual information is used by migratory colonies to relocate places where nesting has proven successful, although odour and tactile or chemical cues associated with the material of old combs seem more likely in determining the final choice (Neumann et al. 2000; Paar et al. 2000).

Giant honeybees vary quite considerably in their nesting habits and relative nest densities. *A. dorsata* and *A. laboriosa* are extremely gregarious species and 20–30 nests in a single tree are fairly common for the former, as are cliff overhangs for the latter (Roubik et al. 1985; Joshi et al. 2004; Woyke et al. 2004, 2012). Reports include a range of 67–256 colonies per tree for *A. dorsata* (Butani 1950; Lindauer 1956; Singh 1962; Deodikar et al. 1977). Oddly, Morse and Laigo (1969) found almost no aggregations in the Philippines. It could well be that the Philippine population is a distinct species (Lo et al. 2010), which would explain the difference in behaviour. In an extensive survey of *A. laboriosa* at 54 cliff sites in western Nepal, Joshi et al. (2004) reported an average aggregation of 6 nests per cliff, with a range of 1–37. Woyke et al. (2012) analysed some 23 nesting sites in Nepal, India and Bhutan, on which 587 colonies were established, with an average of about 25 colonies per site.

A. dorsata colonies nest gregariously; however, placing empty combs in previously occupied trees, or on nearby trees of the same species, did not attract more swarms; the same number of colonies that left trees returned to previously

occupied trees (Liu et al. 2007). Although it is believed that few individuals probably live long enough to make a return journey to their original nest site, some colonies nonetheless return to their exact former trees (Neumann et al. 2000; Paar et al. 2000; Liu et al. 2007). Because the longevity of workers has not been determined under field conditions, it may prove that there is nothing really 'magical' about a migrating swarm of *A. dorsata* returning to their original nests.

A. laboriosa is the largest species of *Apis* and is distributed along the Himalayas from Nepal to Vietnam (Hepburn and Radloff 2011). It builds exposed nests under rock ledges in deep, vertical river valleys, most commonly at 1,200–3,500 m (Roubik et al. 1985; Underwood 1986) and seems confined to areas higher than 2,500 m in the central and western areas of the Himalayas. The nests at 1,200–2,000 m could possibly be occupied throughout the year, but nest sites above 2,800 m are only occupied for a few months in summer (Underwood 1990). By late November, dropping temperatures make even the lower altitude cliff sites unsuitable for colony survival, and the colonies migrate to the forests and settle near the ground where they remain as combless winter clusters until late January (Underwood 1990). Those that nest below 1,200 m are reported not to migrate (Woyke et al. 2001).

2.3 Nest Cavities

The documentation for virtually every subspecies of African *A. mellifera* shows that the bees simply occupy cavities, natural or otherwise, including the hollows of trees and among their roots, in rock crevices, ridges of limestone, stony ground and even termite heaps (termitaria) excavated by aardvarks. The principal conclusion about nest site selection for this group of bees is that they will use any appropriate shelter that the natural terrain has to offer (Hepburn and Radloff 1998). Nest site preference is another matter, and is illuminated by interesting results from simple experiments using trap boxes to collect wild swarms of *A. m. scutellata* in Zambia, Kenya, Malawi (Nightingale 1983; Clauss 1992; Berg 1996) and *A. m. capensis* in South Africa (Hepburn and Radloff 1998), in which there was about a 10:1 greater catch in boxes 3–4 m above ground on building roofs, than at ground level. This fact is routinely exploited in traditional African beekeeping, the rule of thumb being "the higher the hive, the higher the occupation rate" (Mwangi 1985; Zulu 1970). Wherever tall trees occur in sub-Saharan Africa is where traditional beekeepers site their hives. Pressures for high sites include frequent fires, periodic flooding and predators.

When *A. mellifera* scout bees of European set out to find a new nest site, one of the criteria they use in selection is a measure of nest cavity volume (Seeley 1985; Seeley 1995). Here, the differences between European races and African races of *A. mellifera* are in stark contrast. Seeley and Morse (1976) found that natural cavity size preference for the former averaged about 45 l. The nest volumes of African *A. mellifera* ranged from about 5–150 l for *A. m. scutellata* in southern

Africa, but over 90 % of dozens of such measurements show that the average cavity volume hovers around 20 l, or only half that of European subspecies (Johannsmeier 1979; Berg 1996; McNally and Schneider 1996). As an aside, it is worth noting that various European experts, assessing the hives of traditional beekeeping in Africa, from Morocco and Ethiopia to Zimbabwe, state that they are too small. However, traditional man-made cylinders of straw, clay or log have a cavity size of about 25 l on average and are excellent facsimiles of nature.

Qualities, such as the compass direction of cavity opening with respect to the sun and possible distinctness of the apertures, have also been noted. Tests of trap boxes with distinct markings attracted no more colonies than unmarked boxes, nor did degree of a roof-overhang matter (Berg 1996). An analysis of compass orientation for the opening direction of about 140 wild nests in the Botswana swamps showed that they were randomly distributed (McNally and Schneider 1996). The actual nest size in nature is more problematical and it is doubtful as to whether many colonies stay at a fixed site for more than a season or two. In measurements of actual comb areas, based on about 80 established wild nests of *A. m. scutellata* in Botswana, McNally and Schneider (1996) found that the average comb area was about 6,000 cm^2, while Hassan and Bradbear (1994) recorded an average of about 5,000 cm^2 in Tanzania. Working with wax recovery figures from various parts of the continent, comprising decades of wax export trade, average recovery ranged between about 300 and 900 g of wax per colony (Estève 1932; Irvine 1957; Sheriff 1963; Silberrad 1976). These figures were calculated to be about 519 g/wax/colony/harvest/year (totally destructive harvest). Using a wax yield figure of 100 mg wax/cm^2 of comb (for *A. m. capensis, A. m. scutellata* and *A. m. adansonii*), Hepburn and Radloff (1998) estimated that nest comb area ranged from about 2,600–8,000 cm^2 for hundreds of thousands of colonies in Africa, and averaged about 4,500 cm^2, based on tonnage of beeswax exported (Hepburn and Radloff 1996).

Because there is a reasonable relationship between cavity volume and nest size, it appears that traditional beekeepers in Africa emulated nature well. But, there is also a southern hemisphere perspective, which is often lost on temperate zone biologists. Hepburn and Radloff (1996) performed time series and regression analyses of rainfall and beeswax exports from the woodland savanna of east central Africa, and determined that these two variables are most significantly and highly correlated when phase-lagged by one ‘bee year’ (running from July of year 1 to June of year 2). Rainfall and honey production are highly significantly correlated when lagged by one ‘bee year’. Honey and wax production are also highly correlated on a same ‘bee year’ basis. Thus, the beeswax harvest of any 1 year depends on the rainfall of the previous ‘bee year’. This is consistent with general effects of climate on vegetation, specifically to the fact that the bee trees of the African miombo flower in the dry season (Hepburn and Radloff 1996), as do the dipterocarp forests of Southeast Asia (Ridley 1901; Ashton et al. 1988; Sakai et al. 2002; Corlett 2011; Rattanawanee et al. 2012).

2.4 Colony Space and Density

2.4.1 Arrangement of Space

The importance of space for building combs in *A. mellifera* was observed by Huber (1814), who noted that when the nest cavity is packed with combs, building is curtailed; and, conversely, an absence of combs is an inducement to build. Gundelach (1842) asserted that when there is nectar afield, the bees are driven to build. Thus, nectar both arouses the drive to build and provides the fuel to do so. It was noted that bees only built when they hung under the combs (in a skep), and this only happened when there was not enough space to accommodate them among the combs. The drive to build is most notable in newly settled swarms on the branches of trees, rock overhangs or in empty skeps or hive boxes, where a whole nest of combs can be constructed within a week. Here, space can be considered in two ways: building space available for new comb construction and living space for clustering amongst the combs.

During summer, in the Caucasus, Muzalewskij (1933) experimentally extended the observations of Gundelach (1842). Using twenty 'average' *A. mellifera* colonies, he gave half of them a single building frame each, placed adjacent to the last frame of brood comb; each of the other ten colonies was given three such frames. Muzalewskij's basic thinking was that, if space is only a passive aspect of colony life, then one ought to obtain roughly the same amount of wax in the two experimental groups; however, if space acts in some way as an active stimulus for comb production, then clearly the hives with three empty frames should differ in the total amount of wax produced (Muzalewskij 1933). The results showed that those colonies given three empty frames produced around 808 g ± 24, which is some 32 % more than those given only one frame (550 g ± 77; Muzalewskij 1933). To eliminate any source of error that might have arisen from inequalities among the colonies, Muzalewskij simply performed the reciprocal experiment, and again, the colonies with three frames produced more wax (Muzalewskij 1933). One can also distinguish between the effects of space on comb-building and on the actual synthesis and secretion of wax scales (Hoffmann and Werner-Meyer 1960).

Given the perhaps unusual circumstances, in which there was simply no available space in which to construct new combs at a time when there was an autumn nectar flow, Dönhoff (1854) reported an extraordinary secretion of wax scales in *A. mellifera*. These scales were said to form large blocks of wax (possibly 2–3 mm in thickness) which greatly distended the abdomen. Similar examples, with the same interpretation as to cause were also noted in *A. mellifera* by von Buttel-Reepen (1900, 1915); Gwin (1931) and Minderhoud (1933), but this phenomenon has not been studied experimentally. These very large scales are probably genuinely distinct from other examples which appear to be either teratological or pathological in nature (Sendler 1938). Similarly, thick scales have also been observed on *A. cerana* workers in Zhejiang Province, China (cf. Fig. 2.3) and

Fig. 2.3 **a** A swarm of *A. cerana* settle in a stave barrel hive; **b** and **c** workers with wax pieces attached to them can be seen among other members of the swarm; **d**–**h** dead workers found at the entrance of the hive with wax pieces attached to their abdomens; **g** and **h** view of the same worker from opposite sides; **i** the swarm was able to construct comb with regular geometry (Zheng et al. 2011)

which could be a reason why some workers are sometimes trapped in wax during comb construction (Zheng et al. 2011).

The matter of spacing in the dwarf and giant honeybee species is peculiarly different from that of the cavity-nesting bees, because the former are not constrained by the sides of a cavity as are the latter. Nonetheless, 'space' as a 'nearest neighbour' problem still holds for cavity-nesting colonies, which translates to carrying capacity/km^2; but, there are no demographic studies of this kind for wild colonies of cavity-nesting bees. In the red dwarf honeybees, *A. florea*, it would appear that 'space' may well be a 'nearest neighbour' distance rule (Duangphakdee et al. 2013b). In a year long study of emigration and immigration of *A. florea* colonies in secondary, dry dipterocarp forests at Chombueng, Ratchaburi, Thailand, the standing population of *A. florea* colonies ranged from 20 to 41, with a mean of 34.25 colonies occupying a nesting area of 2.8 km^2. In terms of movement, this equates to a range of 7.1–14.3 colonies month/km^2 and an average carrying capacity of 12.2 colonies month/km^2 (Duangphakdee et al. 2013a).

Given significant immigration and emigration data, it is also of interest to consider the spacing of the colonies over the year. No two colonies occupied the same tree and a frequency calculation of the distances between 'nearest neighbouring' nests for 202 colonies over each month showed that over 90 % of the colonies were no more than 100 m apart, with an average distance between neighbouring colonies of 53.9 ± 114.74; the magnitude of the standard deviation being the result of the greater distance from 'nearest neighbours' by only 10 % of the colonies.

Unlike the dwarf bees, *A. dorsata* nest in aggregations and as many as 256 colonies have been observed in a single tree (Deodikar et al. 1977). Space in this context could include an average 'nearest neighbour' distance, so that nests do not overlap but enhance the defensiveness of densely packed clusters. A recent study by Kastberger et al. (2011) used stereoscopic motion analysis to obtain a three-dimensional analysis of the shimmering behaviour of clumped colonies of giant honey bees, which is an extremely accurate, non-invasive approach that holds much promise for spatial distribution studies.

2.4.2 Density Versus Space

In the experiments discussed above, the relative density (unmeasured) of bees would have changed with the construction of new combs, but without information on natural attrition or increase in the work force through brood production, the importance of density per se cannot yet be evaluated. The only study thus far that has attempted to assess the significance of the density of bees in a nest container is that of Freudenstein (1961). Using young bees of about the same age, he hived queenright colonies of *A. mellifera* in one-frame hives. These hives were either 0.5 l or 2 l in volume. Freudenstein first calculated how the amount of comb constructed per day varied with the size of the colony (Fig. 2.4).

Although the original data were presented in such a way as to preclude any rigorous statistical analysis, it is apparent that the small colonies of 500–1000 bees constructed about 7 cm of comb/day, while the larger colonies of 1000–4000 bees built three times that amount. Given a nest container of a fixed volume, the larger number of bees constructed relatively more comb; however, the density of bees per unit volume was increasing as was the population. If the data is viewed slightly differently (Fig. 2.5), one observes that the amount of comb constructed per bee decreased with increasing colony size and density in colonies exceeding 1000 bees. In either comparison, both density and the number of bees varied simultaneously. This of course is a conflation of variables thus precluding more precise interpretations.

To overcome these difficulties, Freudenstein (1961) then established colonies of *A. mellifera* of varying sizes, in either large (2 l) or small (0.5 l) nest boxes, to compare the performance of paired colonies of the same strength under a fourfold difference in density. In these experiments he measured only the mean height of

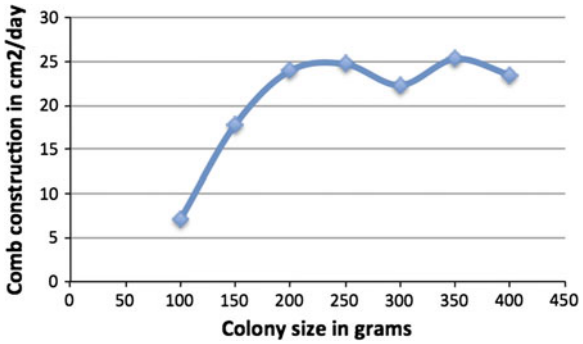


Fig. 2.4 Comb construction by *A. mellifera* colonies as a function of colony size (Freudenstein 1961)

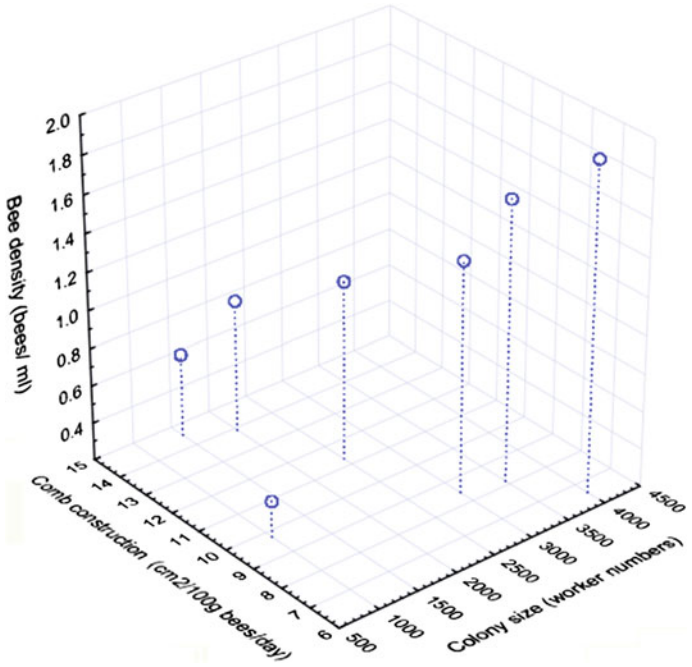


Fig. 2.5 Average comb construction by *A. mellifera* per 100 g/bees/day in relation to colony size and population density (Freudenstein 1961)

the wax gland epithelium as a function of bee density. He did not, unfortunately, provide any experimental data on the area or mass of wax comb—information rather crucial to the assessment of volume in relation to population density. Nonetheless, Fig. 2.6 shows that the height of the wax gland epithelium was

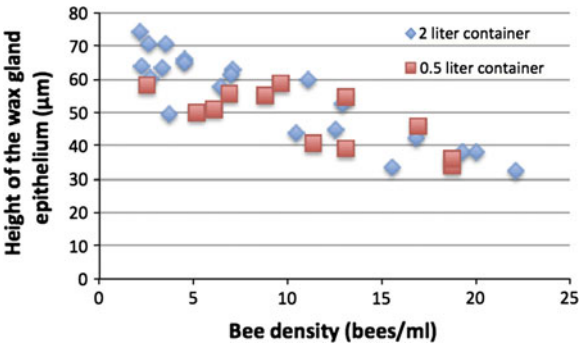


Fig. 2.6 Height of the wax gland epithelium of *A. mellifera* in relation to the bee density (Freudenstein 1961)

greater the lower the density of bees; the height of the epithelium decreased by half with an order of magnitude increase in bee density.

Szabo (1977) tried to establish the relationship between colony size and wax production. After the autumn flow had finished in Canada, he established 24 *A. mellifera* colonies ranging in size from 2 to 8 kg; to each of which he gave a single frame of brood and 19 frames of beeswax foundation. Each of the colonies was fed 14.5 kg of a 60 % sugar solution, and after 8 days the area of comb constructed was measured. Szabo (1977) found that wax construction was linearly related to the size of the colony and that there was an additional 50 g of wax produced with each kilogram increase in colony size. Unfortunately, the experiment was dominated by colonies of about 4.5 and 6.5 kg so that it was not possible to extrapolate the data any further, nor were possible individual contributions taken into account.

2.4.3 Reduction of Nest Size

Dealing with space in a slightly different way, Taranov (1959) suggested that the production of wax occurs only as a reaction of the colony to the absence of a nest (e.g. swarms newly arrived in an empty skep, reminiscent of Gundelach 1842), the unsuitability of an existing one, or serious disruption of the nest (e.g. colonies deprived of their combs as in Gontarski 1930). To test the effects of available space (perhaps better seen as nest shortage) Taranov (1959) established eight *A. mellifera* colonies, each of about 10,000 young bees of the same age. Four of these colonies contained a single frame full of honey (Group A), while in the other four, intact combs alternated with frames from which a portion of the comb had been cut away (Group B). Thus, all eight nests had been disrupted in some way. In Group A there was virtually no place for brood-rearing, while in B there was adequate space for food storage and brood-rearing. At the end of the experiment, duration unstated, the Group A colonies had produced an average of 728 g of wax, slightly more than double that of the Group B colonies which averaged 318 g of wax per colony (a highly significant difference). Interestingly enough, the two groups

differed by only 5 % or so in the average amount of brood reared: Group A produced an average of 23,546 young and group B some 22,197. The density of bees in a given space can obviously vary throughout the day and across the seasons.

An interesting observation from practical beekeeping with *A. m. scutellata* suggests, at first sight, that high density through heavy bee traffic may affect comb-building. Many producers of honeycomb know that bees tend to cap honeycombs in the back of a super in a Langstroth hive in preference to those closest to and just above the entrance. In consequence, beekeepers simply rotate the supers back to front once the back portion is almost complete, so giving the bees a new unworked back section. A natural experiment bearing on this problem came to light concerning a hive that was securely locked in a heavy-gauge steel cage but had been overlooked for 2 years. On its rediscovery, it was found to have three supers of completely capped honeycomb; however, the front quarters of those frames in the bottom super, nearest the entrance, were unworked and contained no honey (Hepburn 1986).

These observations recalled Dadant's (1926) hypothesis, that returning nectar-laden foragers probably go up into the super just above the entrance, resulting in sufficiently dense traffic to prevent work in that area. This was tested on 12 hives as follows. A third of the hives were maintained as controls; in another third, a piece of fibre-board was placed so that incoming bees had to go one-third the length of the hive before reaching the super; in the remaining four hives, the bees were forced to go two-thirds the length of the hive to reach the super. The point of this little experiment was simply to shift the bees further into the hives during a spring flow, the anticipated effect of which would have been unworked comb at the experimentally induced new traffic jam sites. After several months the surprising result was that the front parts of the frames remained unworked, regardless of the point at which the bees could attain access the super (Hepburn 1986).

The quality aspects of space as a stimulus for comb-building can be partially derived from a related but slightly different experiment by Taranov (1959). Again using 10,000 queenright *A. mellifera* bees as a colony unit, he divided them into three groups of three colonies each: Group A had the bottom halves of their alternate combs cut away; Group B the top halves of alternate combs removed; and Group C was given a single comb filled with honey. At the end of the summer experiment, during which the bees were fed a 60 % sugar syrup, Taranov found that wax production varied enormously: Group A, which started without the bottom halves, averaged 155 g of wax; Group B, the one without the top halves 234 g, and Group C, with one full frame 385 g—all comparisons between groups being highly significantly different. The absence of a nest in Group C was a strong stimulus to construct comb. Finally it is probably fair to say that volume, space and density will only operate on wax production provided that the colony of bees has reached some critical threshold, even if we cannot yet specify such a limit.

2.4.4 Other Qualities of "Space"

It has been determined that a group of 50 bees and a queen are just sufficient for the production of comb by *A. mellifera* (Darchen 1957; Darchen 1957; Goetze and Bessling 1959). The factors that might affect this population level in the induction of wax working are open to discussion. The word 'space' has been used in several different ways in the preceding pages. Moreover, the qualities of space are extremely difficult to specify. Two additional aspects of this quality are wind or air currents and light, as well as the relative density of bees in different parts of the nest. These aspects are of obvious importance to *A. andreniformis* and *A. florea*, but have not as yet been investigated. However, open-air nesting by cavity-dwelling *A. cerana* is sufficiently infrequent that periodic notes on its occurrence have been reported (Sasaki and Okada 1988; Lazar 1995; Sugahara 1998; Akimoto 2000; Soman and Sawant 2001). The same applies to a description by Bouvier (1906) of *A. mellifera* colonies nesting in the open air in Paris, following which Darchen (1959a) investigated similar nests experimentally. His 'open air' nests were actually situated in very large clear boxes with open bottoms. He blew in a continuous current of air at a rate of 2–3 ms^{-1} in a direction parallel to existing combs (Fig. 2.7), and observed that the bees shifted away from the direct air current and confined their constructions downwind (Fig. 2.7b). Similar downwind building resulted when the direction of air was normal to the combs, as shown in Fig. 2.7.

The importance of illumination to comb construction is roughly indicated by the fact that we virtually never find nests of cavity-nesting bees like *A. mellifera* or *A. cerana* built in full sunlight, nor even the open-nesting species like *A. florea* or *A. andreniformis*. Colonies of *A. mellifera* found out-of-doors are invariably lodged below the limbs of trees or in bushes, where they receive dappled shade (Rau 1931; Avitabile 1975). In a brief note on swarms, Morse (1963) found that only one of 50 colonies kept in full sunlight built comb, even during a heavy nectar flow. Similarly, two colonies housed in transparent polyethylene cages did not build comb for an 8 week period, but they had synthesized wax, as evidenced by the many dropped wax scales that accumulated beneath their clusters. In another series of experiments on *A. mellifera*, Morse (1965) continued his studies on the effects of light and comb construction. Using about 10,000 bees per colony, he simulated the early April of New York in his flight room, with a daytime temperature of 22 °C. The bees were exposed to light and could forage for sugar syrup in the room. The bees constructed no combs but secreted wax. When the colony was covered with a wooden box lacking one side, it constructed about 50 cm of comb in the ensuing week. Morse then raised the temperature to 29 °C and in the following week the colony constructed 80 g of comb. He then exposed the bees to light (2250 lx) and they continued building combs.

Shifting to the field, Morse (1965) established six swarms, each with a caged queen, as follows: (1) each of two colonies was confined in its own box, from which one side had been removed, the consequent opening facing north; (2) two

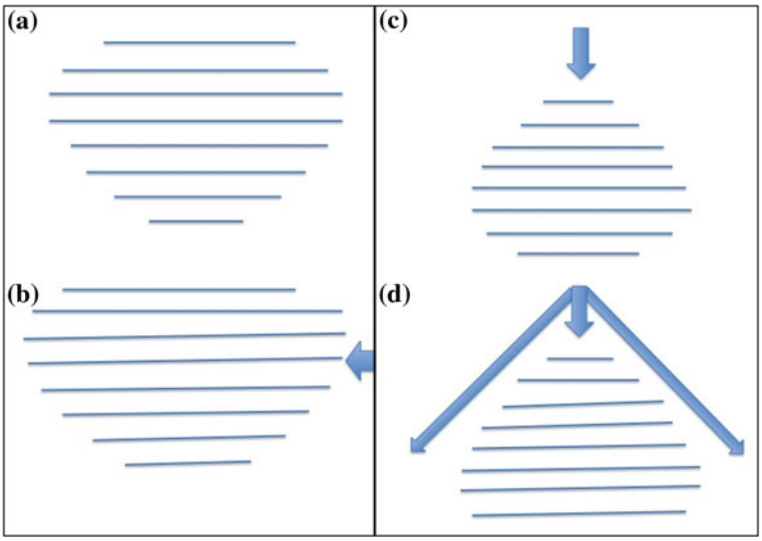


Fig. 2.7 The effect of a continuous current of wind on comb-building: **a** The existing comb structure at the onset of the experiment; **b** comb constructed after application of the air current. The effect of a continuous current of wind on comb-building by *Apis mellifera* where the direction of the air was normal to the combs; **c** combs before application of wind; and **d** combs constructed after the colony was subjected to wind (Darchen 1959a, b)

others were kept in gauze cages with one side open; and (3) two others were kept in wooden boxes. At the end of 3 weeks during a heavy nectar flow, the bees in Group (1): one colony had constructed only little comb, and the other none; for Group (2): there was no construction; finally, for Group (3): extensive combs were built with the exclusion of light. Thus Morse (1965) found that comb construction decreased with increasing (if unmeasured) light intensity. But in all three situations, wax scales had been produced, as evidenced by the scales beneath the colonies that had not built and the combs of those that had. Unlike wind, it appears that the direction of a light source has no effect on the pattern and arrangement of combs (Ifantidis 1978). Given the intensity of full sun, swarms of bees may well secrete wax but will not build combs. If, however, they have begun construction in darkness and are then exposed to light, construction continues; whether this will be at the same pace as that in darkness is unknown.

The fact that bees should first be kept in darkness, to stimulate comb construction, and then exposed to light for viewing has been known since Gundelach (1842), and is today a basic form of management for the use of observation hives (Showler 1978). It would appear that varying light intensity does not prevent the development of and secretion by the wax gland complex, but it certainly modifies building behaviour. The question of light naturally leads to a consideration of whether bees secrete wax and build combs during the daytime or during the night. To assess this, Darchen (1959a) set two colonies in huge glass boxes out of doors

and collected the debris that fell from the nests, assuming that the quantity of fallen wax scales was proportional to building activity. Dividing 4 days into nearly equal halves, he found that one colony dropped about twice as much wax during the day as at night, while the performance of a second colony was exactly the opposite. While light intensity exerts effects on comb construction, the day-night comparison also raises the question of circadian rhythms. How these factors operate together is simply unknown.

2.5 Seasonality, Space and Density

It has been well established experimentally that newly settled swarms of *A. mellifera*, *A. cerana* (Okada and Sakai 1960; Hadisoesilo 1990), *A. florea* (Duangphakdee et al. 2013b) and *A. mellifera* (Lee and Winston 1985; Hepburn 1986) are prodigious comb builders, but in a framework of space and time, comb-building only reaches parity with other wax working (capping and repairing) at the height of the colony growth cycle (Muller and Hepburn 1992). Comb-building is conducted in different areas of the nest by many individuals, some clustered in festoons, others not, while other wax works are often the efforts of individual bees (Lindauer 1952; Yang et al. 2010). Changing ratios of what work there is to be done and where it is carried out can be assessed by following the raw wax in a colony with the changing seasons.

Muller and Hepburn (1992) found that in the course of a year just as much wax is found on *A. mellifera* bees elsewhere among the combs as on festoon bees, but seasonal pictures are quite different (Fig. 2.8). It is our impression that the same, or a very similar scheme, would apply to *A. cerana* as well. It appears that wax-bearing bees can be found in the right places at the appropriate times (Pratt 2004). The wax bees shift from one area of the nest to another, for example, with heavy nectar flow for capping honey cells or to areas requiring brood capping. This ensures a close synchrony between comb area 'needs' and the presence of bees with wax scales. Although not all would agree (Fergusson and Winston 1988), the distribution of these wax bees is largely predicated on an underlying age-based cycle of glandular secretion (Hepburn et al. 1991).

The effects of storage space are elegantly illustrated in the statement "that strong nectar flows fuel comb-building", an explanation proposed for this relationship was formulated by Butler (1954), and, indeed is an old axiom of practical beekeeping (Langstroth 1853). Butler argued that the greater the influx of nectar into the colony, the longer the house bees must retain nectar in their honey stomachs. This, of course, requires the right combination of available storage space and ratio of foragers to house bees. Serving as distended reservoirs over time, these bees assimilate some of the nectar sugar and become stimulated to secrete wax. Enquiries were made at Rothamsted to find out whether these ideas had ever been tested, but had remained unpublished. In reply, we were informed in the negative. This sensible idea has proven far easier to appreciate than to test.

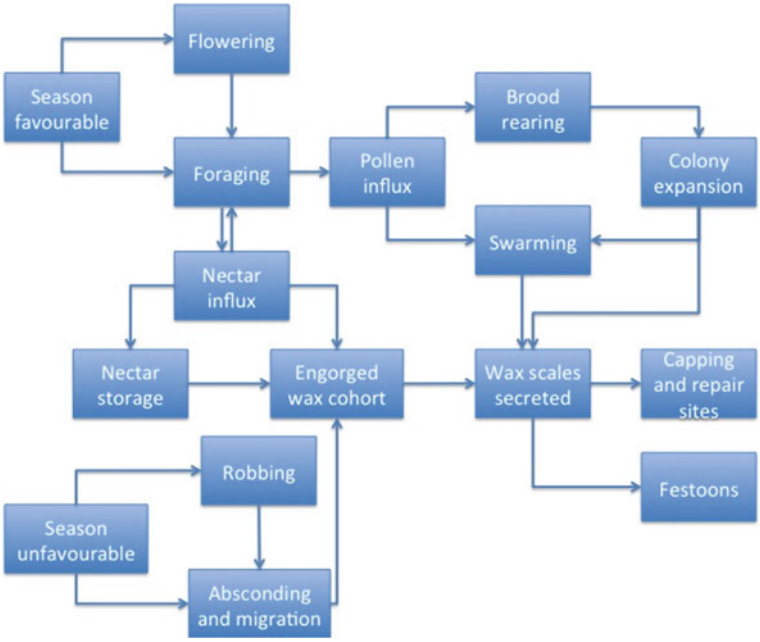


Fig. 2.8 Flow diagram for the stimulation of wax secretion in *A. mellifera.* The favourable season sequence should apply to all *A. mellifera*; but the unfavourable one only to the tropical races in Africa (Hepburn 1998)

Using queenright colonies in which comb available for nectar storage was experimentally reduced or entirely eliminated, a correlation between engorgement of the honey stomach and wax secretion was obtained (Hepburn and Magnuson 1988). This experiment did not distinguish between physical distension of the honey stomach and the time such a bee might spend in trying to disgorge and store the nectar. Nonetheless, the observation is indirectly supported by experiments in which either the deprivation of combs (Fergusson and Winston 1988), or lack of sufficient storage space (Seeley 1995), both led to increased foraging, accelerated wax secretion and, ultimately, comb-building. Collectively, the experimental data lead to a simple feedback system: forager dancing effectively recruits more nectar-foragers; when the incoming nectar is difficult to off-load, a special tremble or stop dance is performed, which inhibits further recruitment (Seeley 1992; Nieh 1993).

During comb-building there are concomitant changes in population size, population density, nectar and pollen influx, all of which affect honeybee-comb interactions. Of these, Harbo (1988) examined the relationship between colony size, brood production and combs for colonies that were equalized. He found that those *A. mellifera* colonies which had produced the largest amount of comb, also produced the largest number of brood and adult workers. To separate queens from comb effects, he performed a second experiment using large and small combs as the variables of interest. Comb effects were significant (queens not) and small

combs resulted in reduced brood production. But there is more to a colony in a cavity, and the variables richer than has thus far been assessed.

Harbo (1993) extended his findings to examine the effects of nest cavity (hive) volume on growth and productivity by adjusting the population density against volume. In winter, crowded bees consumed less honey per bee and reared less brood than less crowded colonies. During the flows of spring through autumn, the crowded colonies produced more honey but less brood than the less crowded ones. In another experiment, comb effects were tested against space effects. Both affected brood rearing and honey production. Colonies with combless, extra space produced less honey and more brood than those with the same amount of comb but less space (Harbo 1993). These results complement those of Taranov (1959) and Szabo (1977) who had shown that brood production and comb construction are not competitive activities: the exclusion of one activity does not accelerate the other.

If creativity in biology is partially the result of the discovery of variables, then we can take some solace from the status quo of our current knowledge on space and density. We know that space, volume, density and colony size all affect wax production. From first principles we also know that gas exchange and heat transfer weigh heavily in the equation. We also know that a scout can obtain information about an empty cavity that we translate into a measure of volume. Likewise, we have a few experimental observations to hand. It will be very rewarding indeed to see the development of experiments that might, 1 day, integrate them all.

References

Akimoto T (2000) Winter temperature in the exposed nest of the Japanese honeybee, *Apis cerana japonica*. Honeybee Sci 21:31–34 (in Japanese)

Akratanakul P (1977) The natural history of the dwarf honey bee, *Apis florea* F. in Thailand. Thesis, Cornell University, Ithaca

Ashton PS, Givnish TJ, Appanah S (1988) Staggered flowering in the Dipterocarpaceae: new insights into floral induction and the evolution of mast fruiting in the seasonal tropics. Am Nat 132:44–46

Avitabile A (1975) Exposed combs of honey bees. Am Bee J 115(436–437):456

Bakker DW (1999) Foraging and habitat selection by two species of honey bee near Lore Lindu National Park in Sulawesi, Indonesia. Thesis, University of Guelph, Guelph

Basavarajappa S (1998) Status of natural colonies of *Apis dorsata* in Maidan region of Karnataka, India. Indian Bee J 60:143–146

Berg S (1996) Investigations to increase the attractiveness of the Malawi standard hive for natural colonisation through bee swarms. Department of National Parks and Wildlife, Rumphi

Booncham U, Wongsiri S, Thirakupt K (1995) Niche differences between *A. florea* and *A. andreniformis* in dry-evergreen forest Thailand. In: Proceedings of international conference tropical bees environment, Pedu Lake, Kedah, p 33

Bouvier EL (1906) Nouvelles observations sur la nidification des abeilles l'air libre. Ann Soc Entomol Fr 75:429–444

Butani DK (1950) An *Apis dorsata* colony in New Delhi. Indian Bee J 12:115

Butler CG (1954) The world of the honeybee. Collins, London

Clauss B (1992) Bees and beekeeping in the north western Province of Zambia. Mission Press, Ndola
Corlett RT (2011) Honeybees in natural ecosystems. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 215–225
Dadant HC (1926) Behavior of bees in comb-building. No. 2. Am Bee J 26:278–280
Darchen R (1957) La reine d'*Apis mellifica*, les ouvrieres pondeuses et les constructions cirières. Insectes Soc 4:321–325
Darchen R (1959a) Observation et experimentation sur un essaim nidifiant artificiellement à l'air libre. Ann Abeilles 1:5–11
Darchen R (1959b) Un des roles des chaines d'abeilles: la torsion des rayons pour les rendre parallèles entre eux. Ann Abeilles 2:193–209
Deodikar GB, Ghatge AL, Phadke RP, Mahindre DB, Kshirsagar KK, Muvel DS, Thakar SS (1977) Nesting behaviour of Indian honeybees. III Nesting behaviour of *Apis dorsata* Fab. Indian Bee J 39:1–12
Dönhoff E (1854) Ueber den uebergang der wachsblättchen in wachstlümpchen. Schweiz Bienen-Ztg 15:279
Douglas JC (1886) The hive-bees indigenous to India and the introduction of the Italian bee. J Asia Soc Bengal Sci 55:83–96
Duangphakdee O, Hepburn HR, Rod-im P (2013a) Seasonal movements of the red dwarf honeybee, *Apis florea* (in ms.)
Duangphakdee O, Hepburn HR, Rod-im P (2013b) Development of the nest of *Apis florea*. (In ms.)
Estève G (1932) L'apiculture au Tchad. Agron Colon 21:161–165
Fergusson LA, Winston ML (1988) The influence of wax deprivation on temporal polyethism in honey bee (*Apis mellifera* L.) colonies. Can J Zool 66:1997–2001
Franssen CJH (1932) Notes on *Micrapis florea* F. Natuurk Tijdschr Ned-Indie 92:55–63
Free JB (1981) Biology and behaviour of the honeybee *Apis florea*, and possibilities for beekeeping. Bee Wld 62:46–59
Fuchs S, Tautz J (2011) Colony defence and natural enemies. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 369–395
Freudenstein H (1961) Entwicklung und leistung der wachsdrüsen der honigbiene. Biol Zentralbl 80:479–492
Goetze G, Bessling BK (1959) Die wirkung verschiedener fütterung der honigbiene auf wachserzeugung und bautätigkeit. Z Bienenforsch 4:202–209
Gontarski H (1930) Über das baualter und die wachserzeugung der bienen. Leipz Bienen-Ztg 45:284–286
Gundelach FW (1842) Die naturgeschichte der honigbienen. JJ Bohné, Cassel
Gwin CM (1931) Wax production by the honey bee colony. Wisc Beekeep 7:109–110
Hadisoesilo S (1990) A preliminary study on a swarm of *Apis cerana* at kuok, riau province, Indonesia. Bull Penelitian Kehutanan 5:357–363
Hadisoesilo S (1997) A comparative study of two species of cavity-nesting honey bees of Sulawesi, Indonesia. Thesis, University of Guelph, Guelph
Harbo JR (1988) Effect of comb size on population growth of honey bee (Hymenoptera: Apidae) colonies. J Econ Entomol 81:1606–1610
Harbo JR (1993) Worker-bee crowding affects brood production, honey production, and longevity of honey bees (Hymenoptera: Apidae). J Econ Entomol 86:1677–1678
Hassan LAM, Bradbear N (1994) Designs of low-cost and low-technology hives for beekeepers in the tropics.In: Proceedings of the 5th international conference Apic tropical climate, Trinidad and Tobago, pp 30–34
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR (1998) Reciprocal interactions between honeybees and combs in the integration of some colony functions in *Apis mellifera* L. Apidologie 29:47–66
Hepburn HR (2011) Absconding, migration and swarming. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 133–158

Hepburn HR, Magnuson P (1988) Nectar storage in relation to wax secretion by honeybees. J Apic Res 27:90–94
Hepburn HR, Radloff SE (1996) Beeswax exports and rainfall in the savanna woodlands of east central Africa. Apidologie 27:473–478
Hepburn HR, Radloff SE (1998) Honeybees of Africa. Springer, Berlin
Hepburn HR, Radloff SE (2011) Biogeography. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 55–67
Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax in honeybees. Apidologie 22:21–36
Hoffmann I, Werner-Meyer W (1960) Wachsdrusenentwicklung und Wachsbedarf bei der Honigbiene. Zool Beitr 5:337–343
Huber F (1814) Nouvelles observations sur les abeilles. (English translation (1926)) Dadant, Hamilton
Ifantidis MD (1978) Wabenorientierung im nest der honigbiene (*Apis mellifica* L.). Apidologie 9:57–73
Inoue T, Adri Salmah S (1990) Nest site selection and reproductive ecology of the Asian honey bee, *Apis cerana indica*, in central sumatra. In: Sakagami SF, Ohgushi R, Roubik DW (eds) Natural history of social wasps and bees in equatorial Sumatra. Hokkaido University Press, Sapporo, pp 219–232
Irvine FR (1957) Indigenous African methods of beekeeping. Bee Wld 38:113–128
Johannsmeier MF (1979) Termite mounds as nesting sites for colonies of the African honeybee. S Afr Bee J 51:9
Joshi SR, Ahmad F, Gurung MB (2004) Status of *Apis laboriosa* populations in kaski district, western Nepal. J Apic Res 43:176–180
Kastberger G, Maurer M, Weihmann F, Ruether M, Hoetzl T, Kranner I, Bischof H (2011) Stereoscopic motion analysis in densely packed clusters: 3D analysis of the shimmering behaviour in giant honey bees. Front Zool 8:833–856
Kuntadi TH (1989) Nesting habit of *Apis cerana* F in nature. Bull Penelitian Kehutanan 5:21–26 (in Indonesian)
Langstroth LL (1853) Langstroth and the hive and the honeybee. (Facsimile, 1977) Root, Medina
Lazar M (1995) *Apis cerana* nesting in the open. Indian Bee J 57:146
Lee PC, Winston ML (1985) The effect of swarm size and date of issue on comb construction in newly founded colonies of honeybees (*Apis mellifera* L.). Can J Zool 63:524–527
Lindauer M (1952) Ein beitrag zur frage der arbeitsteilung im bienenstaat. Z Vergl Physiol 34:299–345
Lindauer M (1956) Communication among the honeybees and stingless bees of India. Z Vergl Physiol 38:521–557
Lindauer M (1961) Communication among social bees. Harvard University Press, Cambridge
Liu F, Roubik DW, He D, Li J (2007) Old comb for nesting site recognition by *Apis dorsata?* Field experiments in China. Insectes Soc 54:424–426
Lo NH, Gloag RS, Anderson DL, Oldroyd BP (2010) A molecular phylogeny of the genus *Apis* suggests that the giant honey bee of the Philippines, *A. breviligula* maa, and the plains honey bee of southern India, *A. indica* Fabricius, are valid species. Syst Entomol 35:226–233
Matsuura M (1983) Notes on the nesting habits of the three honeybee species in Sumatra. Honeybee Sci 4:117–122 (in Japanese)
McNally LC, Schneider SS (1996) Spatial distribution and nesting biology of colonies of the African honey bee *Apis mellifera scutellata* (Hymenoptera: Apidae) in Botswana, Africa. Envir Ent 25:643–652
Minderhoud A (1933) Over de aanwezigheid van wasschubjes bij de bijen. Maandschr Bijent 36(3–7):29–30
Mogga JB, Abdin AMZE, Nagi SKA, Ali AM (1989) *Apis florea* in Sudan: some biological observations. In: Proceedings of the 4th international conference Apic tropical climate, Cairo, pp 422–424

Moritz RFA, Haddad N, Bataieneh A, Shalmon B, Hefetz A (2010) Invasion of the dwarf honeybee *Apis florea* into the near East. Biol Invasions 12:1093–1099
Morse RA (1963) Swarm orientation in honeybees. Science 141:357–358
Morse RA (1965) The effect of light on comb construction by honeybees. J Apic Res 4:23–29
Morse RA, Laigo FM (1969) *Apis dorsata* in the philippines. Philippine Association of Entomologists Inc, Laguna
Mossadegh MS (1990) Nesting behaviour of *Apis florea* F (Hymenoptera: Apidae) in Khuzestan, Iran. In: Proceedings of the 11th international Conference IUSSI, New Delhi, pp 669–670
Muller WJ, Hepburn HR (1992) Temporal and spatial patterns of wax secretion and related behaviour in the division of labour of the honeybee (*Apis mellifera capensis*). J Comp Physiol 171:111–115
Muzalewskij BM (1933) Wie ein leerer raum im nest die wachsausscheidung der bienen beeinflusst. Arch Bienenkd 14:1–145
Mwangi RW (1985) Reasons for the low occupancy of hives in Kenya. In: Proceedings 3rd International Conference Apic Tropical Climate, Nairobi, pp 61–63
Nagaraja N, Rajagopal D (1999) Colony establishment, nesting and foraging activity of the little honey bee, *Apis florea* F (Hymenoptera: Apidae). J Ent Res 23:331–338
Neumann P, Koeniger N, Koeniger G, Tingek S, Kryger P, Moritz RFA (2000) Home-site fidelity in migratory bees. Nature 406:474–475
Neupane KR, Hartfelder KH, de Jong D, de Pereira RA, Santos Cristino A, dos Morais MM, Tanaka ED, Silva AP, da Almeida GF, do Nascimento AM (2004) Nesting behavior of giant honey bees (*Apis dorsata*). VI Encontro sobre abelhas, Ribeirão Preto 351–357
Nieh JC (1993) The stop signal of honey bees: reconsidering its message. Behav Ecol Sociobiol 33:51–56
Nightingale J (1983) A lifetime's recollection of kenya tribal beekeeping. IBRA, London
Okada I, Sakai T (1960) A comparative study on natural comb of the Japanese and European honeybee, with special reference to cell number. Bull Fac Agric Tamagawa Univ 1:1–11 (in Japanese)
Oldroyd BP, Wongsiri S (2006) Asian honey bees. Harvard University Press, Cambridge
Oldroyd BP, Gloag RS, Even N, Wattanachaiyingcharoen W, Beekman M (2008) Nest-site selection in the open-nesting honey bee *Apis florea*. Behav Ecol Sociobiol 62:1643–1653
Otis GW (1996) Distributions of recently recognized species of honey bees (Hymenoptera: Apidae; *Apis*) in Asia. J Kans Ent Soc 69:311–333
Paar J, Oldroyd BP, Kastberger G (2000) Giant honeybees return to their nest sites. Nature 406:475
Phiancharoen M, Duangphakdee O, Hepburn HR (2011) Biology of nesting. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 109–131
Pratt SC (2004) Collective control of the timing and type of comb construction by honey bees (*Apis mellifera*). Apidologie 35:193–205
Rattanawannee A, Chanchao C, Lim J, Wongsiri S, Oldroyd B (2012) Genetic structure of a giant honey bee (*Apis dorsata*) population in northern Thailand: implications for conservation. Insect Conserv Diver 6:38–44
Rau P (1931) Exposed combs of the honeybee. Ecology 12:615–616
Reddy CC (1983) Studies on the nesting behaviour of *Apis dorsata* F. In: Proceedings of the 2nd international conference apic tropical climate, New Delhi, pp 391–397
Reddy MS, Reddy CC (1989) Height-dependent nest site selection in *Apis dorsata* Fabr. Indian Bee J 51:105–106
Ridley HN (1901) The timber of the Malay Peninsula. Agric Bull Straits and Federated Malay States 1. Government Printing Office, Singapore
Rinderer TE, Oldroyd BP, de Guzman LI, Wattanachaiyingcharon W, Wongsiri S (2002) Spatial distribution of the dwarf honey bees in an agroecosystem in southeastern Thailand. Apidologie 33:539–543
Roubik DW, Sakagami SF, Kudo I (1985) A note on distribution and nesting of the Himalayan honeybee *Apis laboriosa* smith (Hymenoptera: Apidae). J Kans Ent Soc 58:746–749

Sakai S (2002) General flowering in lowland mixed dipterocarp forests of South-east Asia. Biol J Linn Soc 75:233–247
Sasaki M, Okada I (1988) Feral nest of *Apis cerana japonica* constructed in an open space. Honeybee Sci 9:77–78 (in Japanese)
Sattigi HN (2001) Nesting behaviour of rock bee selection of nesting height. Karnataka J Agric Sci 14(3):664–667
Seeley TD (1985) Honeybee ecology. Princeton University Press, Princeton
Seeley TD (1992) The tremble dance of the honey bee: message and meaning. Behav Ecol Sociobiol 31:375–383
Seeley TD (1995) The wisdom of the hive. Harvard University Press, Cambridge
Seeley TD (2010) Honeybee democracy. Princeton University Press, Princeton
Seeley TD, Morse RA (1976) The nest of the honey bee (*Apis mellifera* L.). Insectes Soc 23:495–512
Seeley TD, Morse RA (1978) Nest site selection by the honey bee, *Apis mellifera*. Insectes Soc 25:323–337
Seeley TD, Seeley RH, Akratanakul P (1982) Colony defense strategies of the honeybees in Thailand. Ecol Monogr 52:43–63
Sendler O (1938) Über ungewöhnliche wachs-ausscheidung der honigbiene. Nat Volk Frankf 68:87–92
Sheriff JS (1963) Beeswax and honey production—the Nyasaland potential. Ministry of National Resources and Surveys, Zambia
Showler K (1978) The observation hive. Bee Books New and Old, Steventon
Silberrad REM (1976) Bee-keeping in Zambia. Apimondia, Bucharest
Singh S (1962) Beekeeping in India. Indian Council of Agricultural Research, New Delhi
Soman AG, Sawant SB (2001) Nesting of Indian hive bees in open. Indian Bee J 59:240
Starr CK, Schmidt PJ, Schmidt JO (1987) Nest-site preferences of the giant honeybee, *Apis dorsata* (Hymenoptera: Apidae), in Borneo. Pan-Pac Entomol 63:37–42
Sugahara M (1998) Feral colonies of Japanese honey bees in the northeastern part of Osaka Prefecture. Honeybee Sci 19:37–41 (in Japanese)
Szabo TI (1977) Effect of colony size and ambient temperature on comb-building and sugar consumption. J Apic Res 16:174–183
Taranov GF (1959) The production of wax in the honeybee colony. Bee Wld 40:113–121
Thorley J (1744) Melisselogia or, the female monarchy: being an enquiry into the nature, order, and government of bees. Thorley, London
Underwood BA (1986) The natural history of *Apis laboriosa* Smith in Nepal. Thesis, Cornell University, Ithaca
Underwood BA (1990) The behaviour and energetics of high-altitude survival by the Himalayan honeybee, *Apis laboriosa.* Thesis, Cornell University, Ithaca
von Buttel-Reepen H (1900) Sind die bienen reflexmaschinen? Biol Zentralbl 20:1–82
von Buttel-Reepen H (1915) Leben und wesen der bienen. Vieweg, Braunschweig
Wattanachaiyingcharoen W, Wongsiri S, Oldroyd BP (2008) Aggregations of unrelated *Apis florea* colonies. Apidologie 39:531–536
Whitcombe RP (1984) The biology of *Apis* spp in Oman with special reference to *Apis florea* Fab. Thesis, University of Durham, Durham
Wongsiri S, Thapa R, Oldroyd BP, Burgett MD (1996) A magic bee tree: home of *Apis dorsata*. Am Bee J 136:196–199
Wongsiri S, Lekprayoon C, Thapa R, Thirakupt K, Rinderer TE, Sylvester HA, Oldroyd BP, Booncham U (1997) Comparative biology of *Apis andreniformis* and *Apis florea* in Thailand. Bee Wld 78:23–35
Woyke J, Wilde J, Wilde M (2001) Swarming, migration and absconding of *Apis dorsata* colonies. In: Proceedings of the 7th international conference apic tropical climate, Chiang Mai, pp 183–188

Woyke J, Wilde J, Reddy CC (2004) Open-air-nesting honey bees *Apis dorsata* and *Apis laboriosa* differ from the cavity-nesting *Apis mellifera* and *Apis cerana* in brood hygiene behaviour. J Invert Path 86:1–6

Woyke J, Wilde J, Wilde M (2012) Swarming and migration of *Apis dorsata* and *Apis laboriosa* honey bees in India, Nepal and Bhutan. J Apic Res 56:81–91

Yang MX, Tan K, Radloff SE, Phiancharoen M, Hepburn HR (2010) Comb construction in mixed-species colonies of honeybees, *Apis cerana* and *Apis mellifera.* J Exp Biol 213:1659–1664

Zheng HQ, Hu FL, Pirk CWW (2011) Errors in comb-building behaviour in *Apis cerana cerana* that result in entrapped workers. Insectes Soc 58:413–415. DOI: 10.1007/s00040-011-0158-x

Zulu GM (1970) Bark-hive beekeeping in Zambia. Bull 2A. Forest Department, Lusaka

Chapter 3
Self-Organization of Nest Contents

Abstract The arrangements of the contents of both single, vertical and horizontally arranged parallel combs are very similar among all species of honeybees, and different areas of the combs are repetitively used for the same functions. They principally differ in the formation of their patterns, which have been tacitly assumed for centuries, to derive in some mysterious way as "in the nature of bees". Camazine (1991) conducted a series of experiments to validate one of two mutually exclusive hypotheses for the comb patterns of *A. mellifera*; (1) a blueprint hypothesis in which patterns develop in some pre-ordained and specified way intrinsic to bees; or, (2) a self-organization hypothesis (a reaction–diffusion system), by which patterns emerge spontaneously from the dynamic interactions among the processes of placing, and then displacing, the different elements of the nests. Camazine's original self-organization hypothesis has been challenged, modified, and ultimately, supported by rigorous mathematical analyses of this problem. The model and the self-organization hypothesis appear extremely robust and parsimonious and remains the prevailing paradigm (Montovan et al. 2013). Explanations for pattern formation in the single-comb dwarf and giant honeybee species are perhaps less difficult. Development of an *A. florea* vertical, single comb nest is accomplished in 4 months after a swarm settles. In only a few days the nest has already been partitioned into areas for honey (top of comb), an underlying pollen layer below, and a central area which both capped and uncapped larval cells occur. This basic pattern remains until the mature colony swarms some 4 months later. The major challenge is the construction of the crown comb.

3.1 Introduction

Honeybees are a group of largely wild insects of which only the two medium-sized, cavity-nesting species, *A. cerana* and *A. mellifera,* have successfully been semi-domesticated to the extent that they can be maintained in artificial cavities such as woven skeps, clay or log hives or man-made boxes, such as the ubiquitous

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_3,

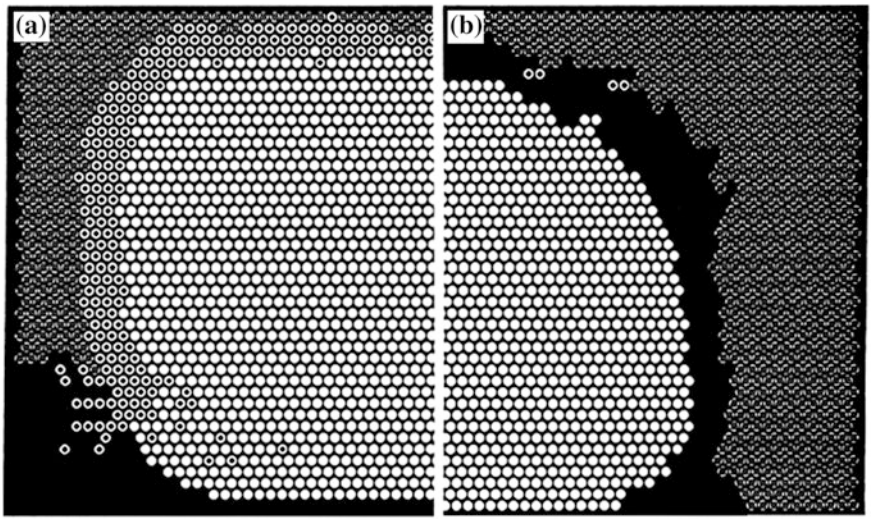


Fig. 3.1 **a** Characteristic nest pattern of *A. mellifera*: centrally located brood, a band of pollen above and to the side of the brood area and a honey storage region at the periphery (*left*). **b** Comb pattern when little pollen was available with an empty area where pollen was previously stored (*right*). *White circles*—brood; *white circles with black dot*—pollen; *grey areas*—honey; and *black*—empty cells (Camazine 1991)

Langstroth hive (Free 1982; Crane 1999), while the other species are entirely wild. Nonetheless, the arrangement of comb contents are very similar among all species of honeybees, whether the nest be a single vertical comb (*A. andreniformis, A. dorsata, A. florea* and *A. laboriosa*), or collections of horizontally arranged parallel combs (*A. cerana, A. koschevnikovi, A. nigrocincta* and *A. mellifera*). For the latter, brood, honey and pollen are stored in a series of parallel wax combs so that a characteristic, well-organized pattern develops on the combs, consisting of three distinct concentric regions: a central brood area, a surrounding rim of pollen, and an outer large, peripheral region of honey (Fig. 3.1a and b). For the cavity-nesting species this pattern is most pronounced on the central combs, which intersect a large portion of the roughly spherical volume of brood (Camazine 1991).

The arrangement and distribution of the contents of European bees' nests, *A. mellifera*, seems to have been basically understood by the middle of the eighteenth century (Dublin Society 1733; Thorley 1744). Indeed, there was a proliferation of texts on honeybees in the early nineteenth century and some of the more important, subsequently influential, and still pertinent ones are those of Huber (1814), Dzierzon (1852) and Langstroth (1857). Among the more recent works describing the natural nests of honeybees and the distribution of their contents are: for *A. andreniformis* (Wongsiri et al. 1997); *A. cerana* (Tokuda 1924, 1935; Sakagami 1959); *A. dorsata* (Koeniger et al. 2010); *A. florea* (Sakagami and Yoshikawa 1973; Rinderer et al. 1996; Duangphakdee et al. 2013); *A. laboriosa* (Underwood 1986) and *A. mellifera* (Seeley and Morse 1976). Oddly enough,

natural (non-beehive) nests of *A. mellifera* have seldom been investigated, but Seeley and Morse (1976) did so and also provided a summary of the characteristics of the nests of *A. florea* based on data from Benton (1896), Rahman and Singh (1946), Lindauer (1956), Ruttner (1968), Sakagami and Yoshikawa (1973); as well as *A. dorsata* based on data from Benton (1896), Grassé (1942), Rahman and Singh (1946), Kallapur (1950), Lindauer (1956), Singh (1962), Ruttner (1968), Morse and Laigo (1969) and *A. mellifera* (Seeley and Morse 1976).

3.2 Pattern Formation in Combs

3.2.1 Reaction–Diffusion Systems Pattern Formation

It is evident that different areas of the comb are used repeatedly for the same functions in all honeybee species. It has been tacitly assumed for centuries that the patterns observable in the arrangement of nest contents in *A. mellifera* are in some mysterious way "in the nature of bees"; or as Pappus suggested, "bees have a certain geometrical forethought by which the most economical container to be made of wax was, in fact, the hexagonal configuration". However, the observations that pollen and honey are regularly deposited in empty cells within the brood area during the day, only to be removed to their 'proper' places during the night, led to an especially seminal paper on pattern formation of comb use in honeybees by Camazine (1991). While Camazine's ideas are certainly original, they stem from two sources; his childhood wonderment as to why sand dune ripples looked so much like patterns of clouds in the sky (so-called cloud streets—Camazine, pers comm.), and the application of reaction–diffusion equations formulated by Turing (1952) to explore pattern formation. Turing's model demonstrates self-organization, and remains a classical paradigm in studies of morphogenesis.

Camazine (1991) conducted a series of experiments to validate one of two mutually exclusive hypotheses: (1) a blueprint or template hypothesis, in which patterns develop in some pre-ordained and specified way intrinsic to bees; or (2) a self-organization hypothesis in which patterns emerge spontaneously from the dynamic interactions among the processes of placing and then displacing the relevant nest elements. In a series of classically simple and illuminating observations and experiments, Camazine (1991) noted that the brood pattern is initiated by the laying habits of the queen, who must take into account the presence of nearby brood and, perhaps, the comb boundaries. This given, the queen lays eggs and the bees deposit both nectar and pollen haphazardly among the combs in the first instance. Possibly informed by the presence of young nurse bees, the queen does not lay eggs outside the nascent brood area, but continually searches for empty cells near other eggs or brood.

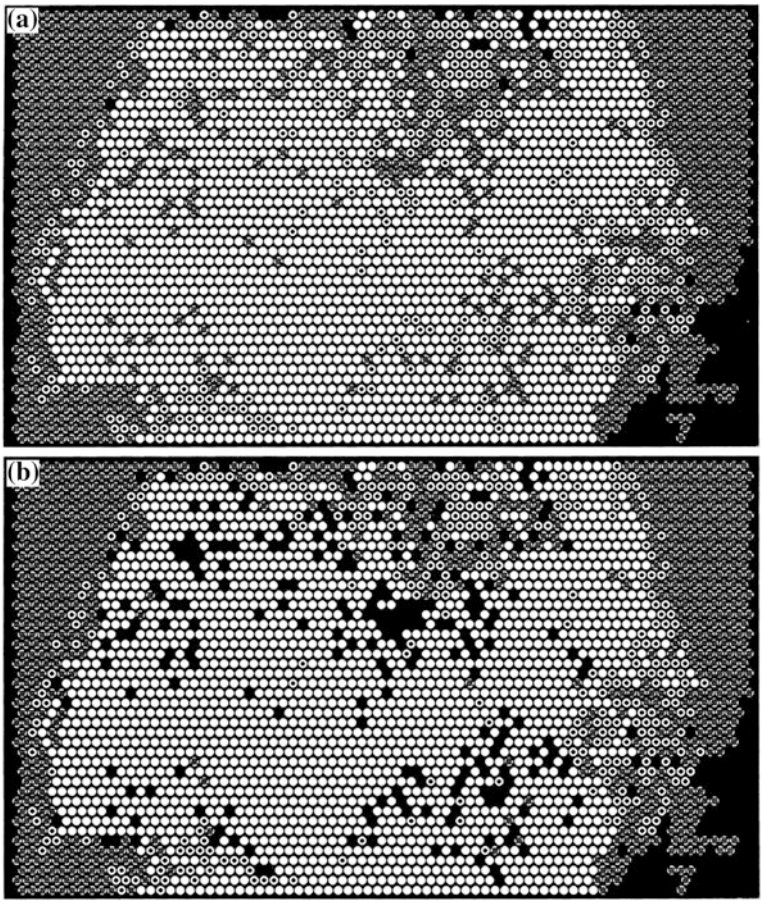


Fig. 3.2 Comb of an *A. mellifera* nest showing preferential removal of honey and pollen away from brood: **a** *upper trace* made at 19:00 at the end of foraging; **b** *lower trace* of the same comb the following morning at 08:00. Cell symbols as in Fig. 3.1 (Camazine 1991)

Cells in the brood area filled with honey or pollen are preferentially emptied of their contents. This was experimentally shown by the distribution of cell emptying from the brood area, which is a function of distance from the nearest brood cell (Fig. 3.2a and b).

Brood cells emptied of nectar and pollen, are then found by the queen who lays in them, and so the pattern develops. Camazine and colleagues (Jenkins et al. 1992) then proceeded to develop a computer simulation model to establish pattern-forming rules, as estimated from the actual experiments. Using the empirical events from observation hives as the parameter values, they were able to reveal interacting processes that contribute to pattern formation. The simulation also produced the final pattern observed in observation hives and confirmed the interpretation of pattern formation (Fig. 3.3a–c). The model and the self-organization

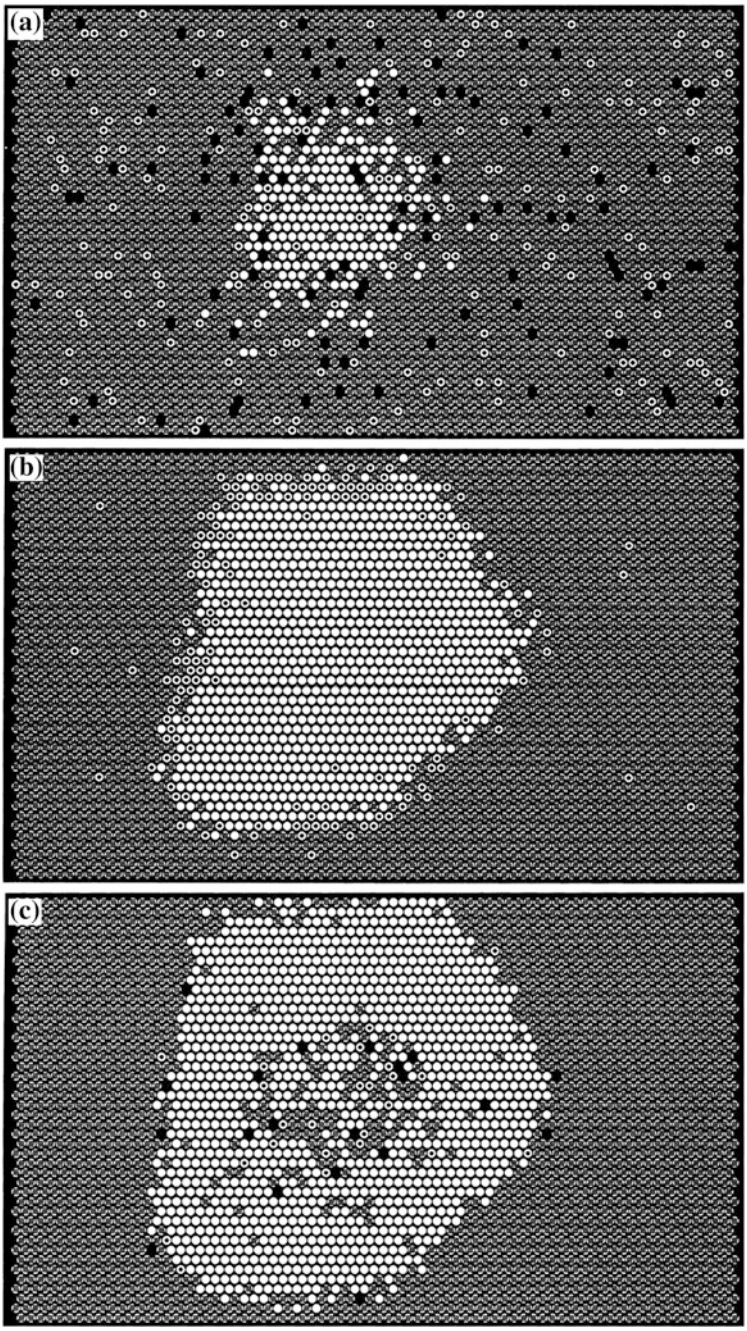


Fig. 3.3 Computer simulation of pattern formation of *A. mellifera* comb: **a** day 1; **b** day 7; **c** day 22. Cell symbols as in Fig. 3.1 (Camazine 1991)

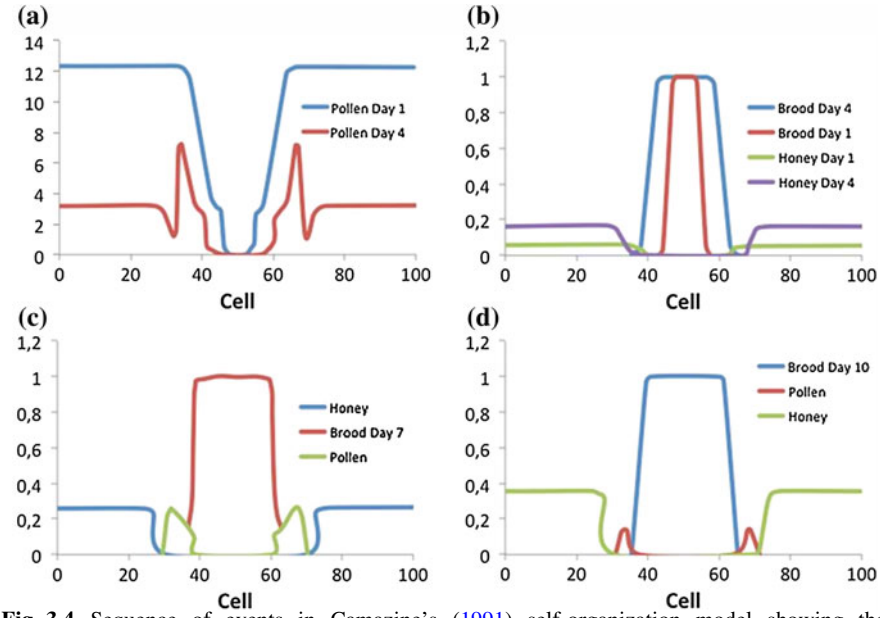


Fig. 3.4 Sequence of events in Camazine's (1991) self-organization model showing the formation patterns of brood, pollen, and honey, observed in a drawn frame in an observation hive of *A. mellifera*. **a** Pollen distribution when input is low; the concentration of pollen initially increases at the periphery, but then decreases and is low everywhere; **b** the brood area has expanded over the first few days with an increase in honey at the periphery of the comb. (In this particular model there was a 3-day pollen burst over days 5–7). The interface between honey and pollen consists of a zone of empty cells due to a low level of pollen; **c** by day 7, the pollen band has developed rapidly and a typical pattern of brood, pollen and honey has formed. Honey concentration is increasing at the periphery of the comb, and the brood area is expanding; **d** by the end of day 10, the pattern is intact, but the pollen band is slowly decreasing. Equilibrium is reached when the brood is surrounded by honey and pollen and the queen can find no empty cells in which to lay eggs (after Camazine 1991; Jenkins et al. 1992)

hypothesis appear extremely robust and parsimonious. This idea has been further analyzed mathematically by Jenkins et al. (1992), who derived rate constants for the removal and re-deposition of honey and pollen in order to achieve their characteristic bands and positions above the brood area (Fig. 3.4). Camazine's approach and interpretations have subsequently been endorsed by Bonabeau et al. (1997) and Theraulz et al. (2003).

3.2.2 Template Effects?

In the intervening years since the works of Camazine (1991), Bonabeau et al. (1997) and Theraulz et al. (2003) were published more detailed knowledge of worker behaviour has been reported. For example, Johnson and Baker (2007) observed that nectar-receiving bees tend to deposit their nectar loads near the top

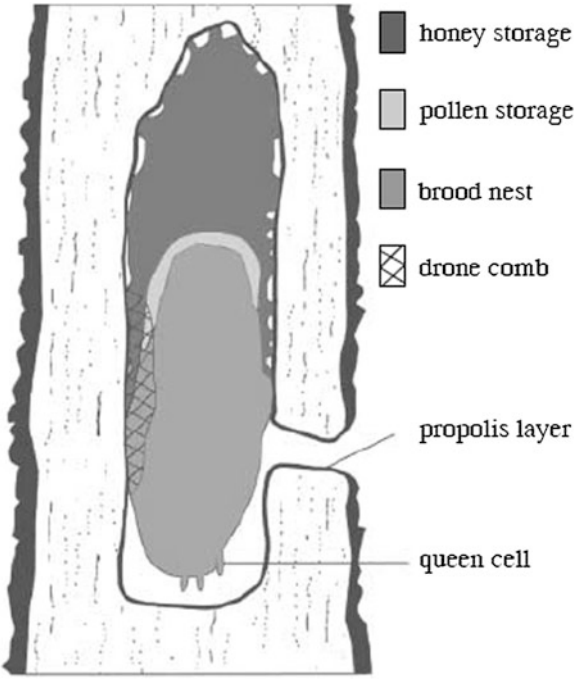


Fig. 3.5 Characteristic pattern of comb organization in a tree cavity occupied by an *A. mellifera* nest (Johnson 2009, after Seeley and Morse 1976)

of the comb where the nectar band occurs. Likewise, Dreller and Tarpy (2000) showed that foragers must have direct contact with the brood and pollen areas to regulate their foraging for pollen and preferentially deposit pollen in cells near the brood area. Inevitably, these observations and subsequent analyses required some refinements. Johnson (2009) re-examined pattern formation on combs in relation to four groups of bees: the queen, nectar-receiving bees, pollen foragers and nurse bees. He concluded that the vertical pattern of honey at the top of the comb and brood at the bottom is owing to a gravity-based template effect, while the band of pollen depends on both a self-organization effect as well as a queen-based template. Johnson's model is based on the distribution of comb contents in a tree-dwelling colony, described by Seeley and Morse (1976) and illustrated in Fig. 3.5.

In models, colonies using the more complex scheme (proposed by Johnson 2009), during a period of high nectar inflow is shown in Fig. 3.6. It is followed by pattern formation obtained by a self-organization model and two template effects during a period of high nectar inflow shown in Fig. 3.7.

3.2.3 Recent Models

The modifications that Johnson's (2009) scheme suggests remain open to argument and debate. In a rigorous mathematical analysis of this problem by Montovan et al. (2013), these authors support Camazine's original proposition, that the combined

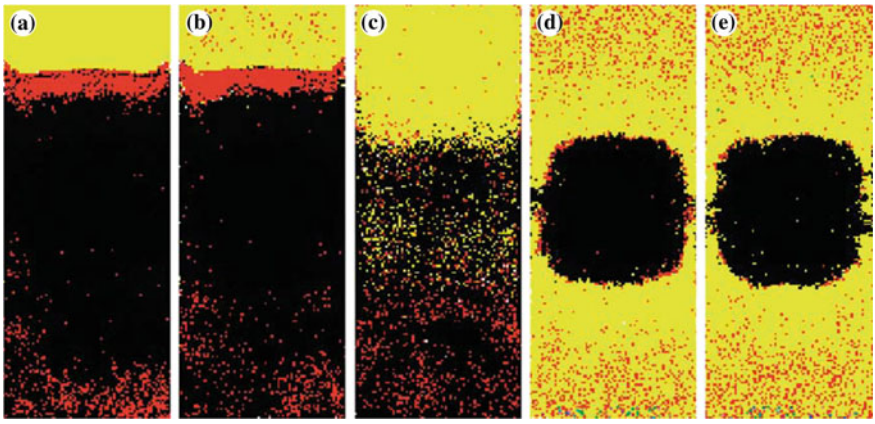


Fig. 3.6 Mechanisms underlying comb pattern formation in an *A. mellifera* nest: SO—self-organization, T1—gravity-based template, T2 queen-based template. Each of the frames (**a**–**e**) shows the pattern at 14 days. The full model (with rain) is shown in (**a**) so (SO + T1 + T2); **b** without the queen-based template, pollen is scattered throughout the honey zone (SO + T1); **c** without the self-organizing mechanism, a pollen band does not form, and the brood and honey areas are indistinct (T1 + T2); **d** without the gravity-based template, the pattern remains concentric as opposed to vertical so that (SO + T2); **e** original self-organization model of Camazine does not lead to pattern formation under realistic parameter settings (SO) (Johnson 2009)

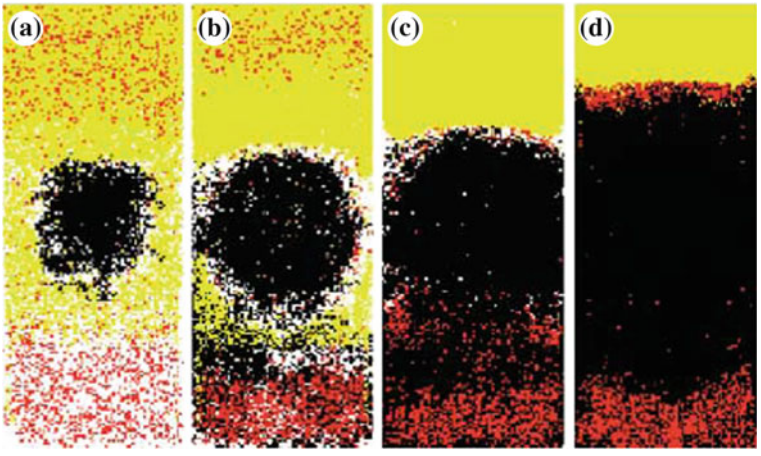


Fig. 3.7 Johnson's (2009) pattern formation in an *A. mellifera* nest obtained by self-organization and two template effects during a period of high nectar inflow: **a** day 1, **b** day 4, **c** day 7, and **d** day 14. Cells of honey are *yellow*, those of pollen *red* and brood cells *black*. On day 1, pollen was scattered about the comb but once brood was present, pollen was shifted to the bottom of the comb. Nectar was preferentially unloaded near the top of the comb by the nectar-receiving bees. By day 7 the comb pattern was almost formed except for the band of pollen cells, which had formed by day 14 (Johnson 2009)

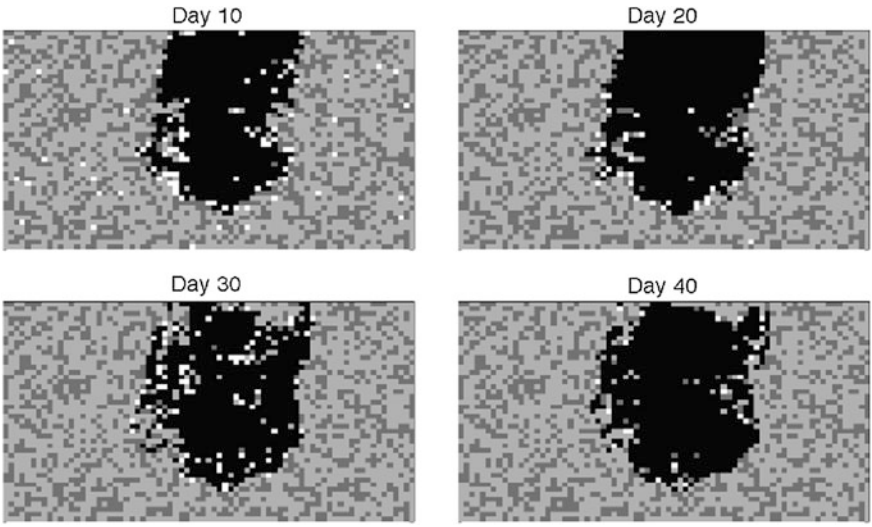


Fig. 3.8 Simulation of Model 4 beginning with an empty comb but after 20 days the pattern is maintained when the emerging adults leave their cells (Montovan et al. 2013)

actions of many individual bees could produce the comb pattern with which we are familiar, using rather simple but biologically meaningful rules. However, as they pointed out, the Camazine model does not explain how the comb pattern is maintained with subsequent generations of brood. In their analyses, their Model 1 is the original Camazine model; in Model 2, an alternate queen movement method is employed while leaving the remaining rules identical to those of Model 1. Model 3 uses alternate honey/pollen consumption rules while all other rules are identical to those of Model 1. Model 4 employs the alternate methods of both queen movement and honey/pollen consumption (Fig. 3.8). Model parameter values were varied over a wider range than were used, so that the sensitivity of the model to choices of parameter values could be assessed. For queen movements, Montovan et al. (2013) used a Gaussian distribution of directions with a mean toward the centre of the comb. For honey and pollen they defined the probability of selecting a particular cell that is linearly proportional to the number of brood cells within a chosen distance, which includes the idea that nurse bees take more honey/pollen from cells nearer to brood, without assuming that nurse bees make multiple trips from the same cell.

The model of Montovan et al. (2013) contains the basic processes that Camazine described, but to check that their models would in fact create the initial pattern of a compact brood region surrounded by a ring of pollen, they simulated the first 20 days for all four models. For the Camazine model, their simulations reproduced Camazine's results in Model 1, but the desired pattern formed in the first 20 days gradually dissolved as brood cells are vacated. They found that all the models were able to form the initial pattern. In a simulation of Model 4, the initial

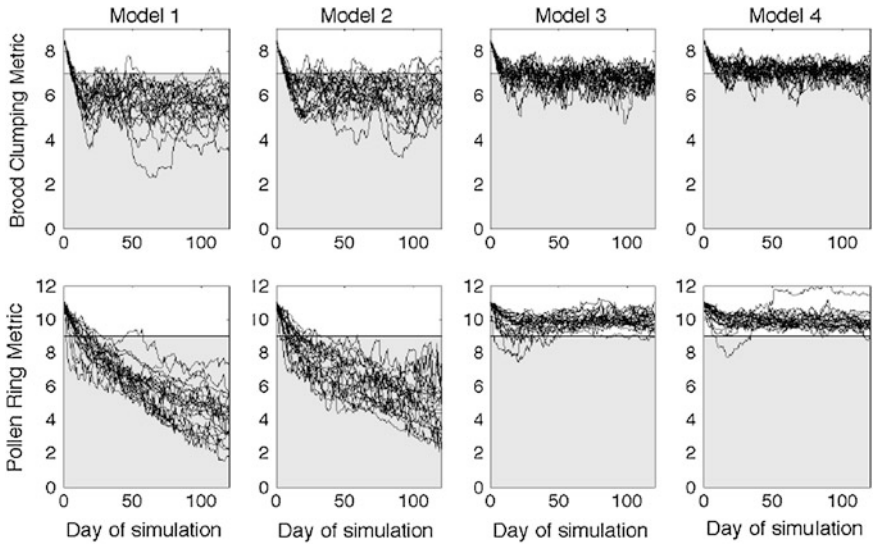


Fig. 3.9 Trajectories for all four models which illustrates that the combined modifications for queen movement and honey/pollen removal used in Model 4 provides the best result for *A. mellifera* nests (Montovan et al. 2013)

pattern is not perfect, but a compact brood region forms, as does a ring of pollen. Models 1 and 4 form similar patterns initially, but Model 1 cannot maintain the pattern, while Model 4 is able to both create and maintain the pattern. The overall differences between the four models lie in the ability to maintain a compact brood region and a pollen ring over time and are apparent in the trajectories of the brood and pollen metrics through 120 days of simulation for each model (Fig. 3.9). To conclude this section, Camazine's work showed a highly developed prescience when he was able to demonstrate that the centuries-old belief "in the nature of bees" (which equates to "a certain geometrical forethought" as postulated by Pappus) as an explanation for patterns in combs, could be bettered.

3.3 Developmental Cycles of *Apis florea* Nests

When we consider that Camazine's observations were made on *A. mellifera* combs in observation hives, there is a more natural comparison which can be made to the nests of the dwarf honeybees, *A. florea*, and we present this material for its heuristic value. *A. florea* nests are single, exposed combs, vertically attached to one or two thin branches in trees or bushes throughout Southeast Asia, and have been described quite thoroughly many times (cf. Hepburn and Hepburn 2011). Analyses of the structure of these nests have been comprehensively reported in an especially relevant publication by Sakagami and Yoshikawa (1973), who described and

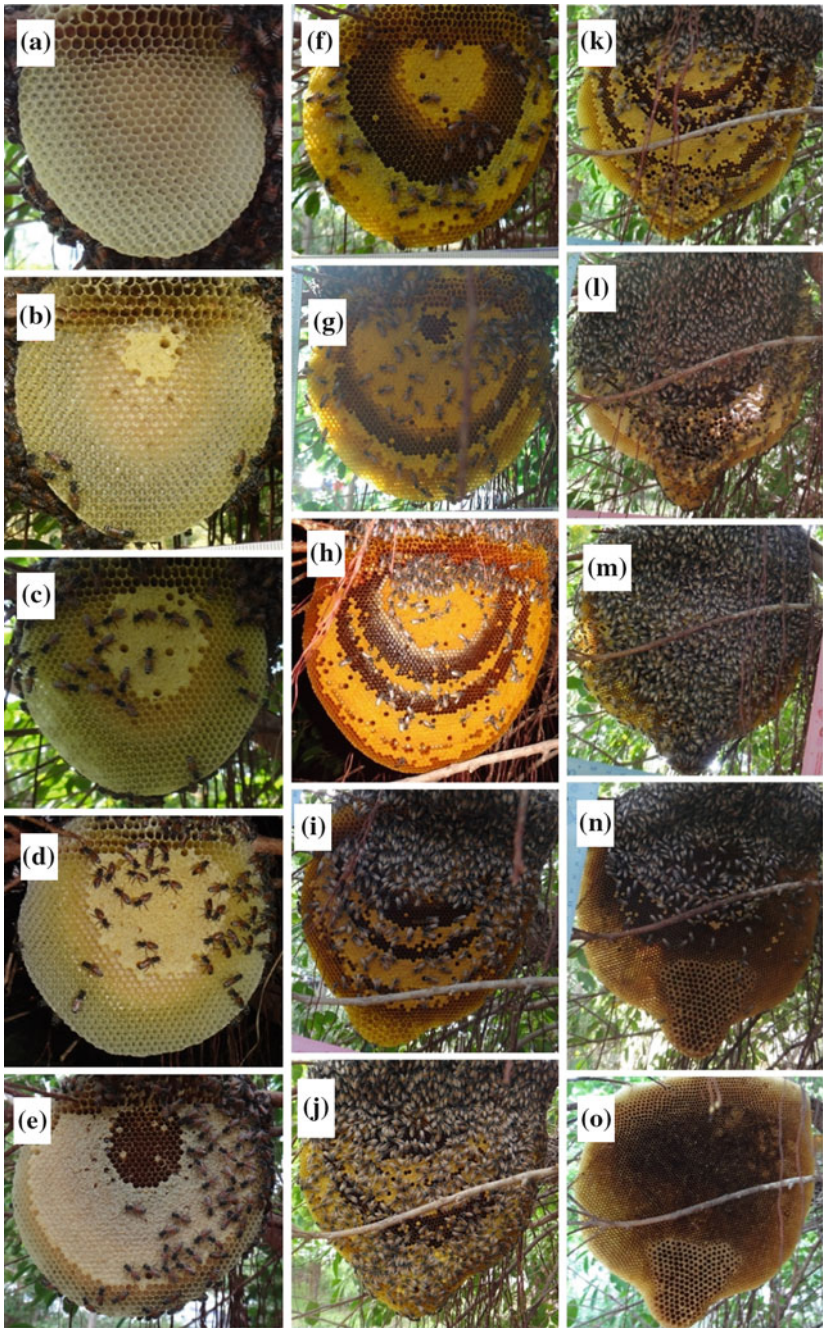
illustrated the arrangement of honey, pollen, worker and drone brood cells as well as reproductive queen cells. Further details on the nests of *A. florea*, the arrangement and dimensions of cells and their physical relationships to one another were tabulated by Rinderer et al. (1996).

Although we now have accurate descriptions of the nest structure of *A. florea* as reported by Sakagami and Yoshikawa (1973) and Rinderer (1996), virtually all the nest specimens of *A. florea* that they and others examined were purchased at Chatuchak Market in Bangkok. Because of the presence of drone cells at the bottom of the combs purchased, they were rightly adjudged to be mature nest specimens. So, these works provide what are literally 'snapshots' in time of mature nests on their day of harvest for market. More recently, Duangphakdee et al. (2013) photographically documented the chronological growth and development of *A. florea* nests at Chom Bueng, Thailand, by newly settled swarms, from their inception until their final days before reproductive swarming or absconding.

The areas of brood comb in examples of the dwarf, medium-sized and giant honeybees, all consist of concentric regions in the plane of the comb. However, in the medium-sized, cavity-nesting honeybees, the use of multiple parallel combs means that, in a three-dimensional perspective, the concentric rings of sequential brood combs approximate a sphere, while those of honey and pollen are ovals or inverted saucers. Because the dwarf honeybees usually construct a single comb, we use this species to illustrate the chronological changes that occur from the onset of building to maturity of the comb and its final abandonment as documented by Duangphakdee et al. (2013). However, the vertical arrangement of specialized areas is the same as in *A. mellifera,* as described by Seeley and Morse (1976).

A. florea nests were collected and moved at dusk. They were hung on small trees, maintaining the vertical position of the combs. Colonies were allowed to adapt to their new environment and resumed normal activities and foraging. After few days the brood comb, extending below the crown, was cut away at dusk, and removed to induce absconding (Woyke 1976; Duangphakdee et al. 2012). The following day, a new nest site selection process was conducted when the whole colony took off to a new nesting site. The authors then followed each swarm until the colony settled in a new nesting tree. Their results are shown in a chronological series of photographs (Fig. 3.10).

The dimensional growth curves of the nests of two colonies of *A. florea* show daily changes in comb length, width and area from inception of the nest to its maturity and completion (Figs. 3.11, 3.12 and 3.13). Initially, both the lengths and widths of the nests double in parallel, following a logarithmic form over the first 10 days (Figs. 3.11 and 3.12). Then the rates of change gradually begin to decrease in subsequent weeks, but nonetheless do so in tandem. In consequence, the rate of change in the area of the comb yields a linear constant (Fig. 3.13).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◀ **Fig. 3.10** Development of an *A. florea* vertical, single comb nest of over 16 weeks once a swarm settled. By day 4 (**b**) the nest has already been partitioned into an area for honey (top of comb), an underlying pollen layer, below which both capped and uncapped brood cells occur. This basic pattern remains until the mature colony swarms some 4 months later. In the sequence of photographs shown: **a** on day 2, the darker wax honey crown is being developed above the brood area which contains eggs and larvae in a concentric pattern; **b** by day 4, some of the brood cells have been capped and more eggs and larvae are in the cells below, maintaining the concentric pattern; **c** on day 6 the progression of cell cappings continues as does the expansion of the uncapped brood area; **d** by day 8 the concentric rings of capped and uncapped brood increased and workers began storing nectar in the crown; **e** on day 16 the oldest patch of brood emerged as adults, and extensive capping of brood cells continued (note that the brood area does not extend to the periphery of the comb); **f** on day 23, the previously empty cells of (**e**) now contained capped brood of what will be the second generation of adults, the cells in the area surrounding this contains newly laid eggs, while the outer band contains capped brood; **g–k** sequential occurrences between days 30 and 93, showing the staggered distribution of concentric brood of various ages and generations with drone cells finally constructed by day 93; **l** by day 100 drones emerged from their cells at the bottom of the comb; **m** on day 107 the drones have left the nest; **n** by day 114 there are no new eggs, no uncapped brood and only very few capped cells; **o** on day 121 the colony absconded (Duangphakdee et al. 2013)

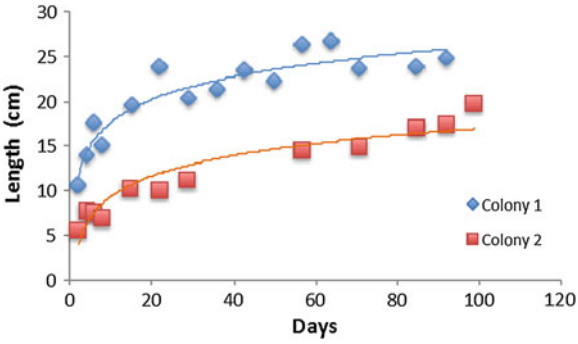


Fig. 3.11 Change in length of *A. florea* comb

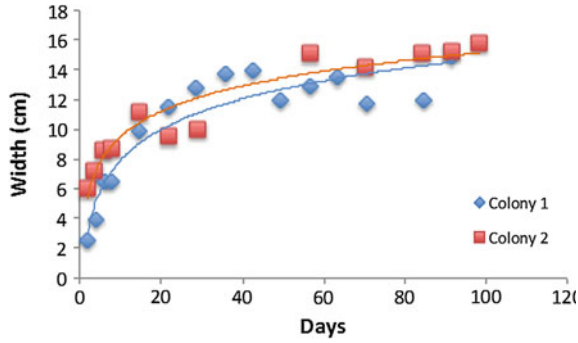


Fig. 3.12 Change in width of *A. florea* comb

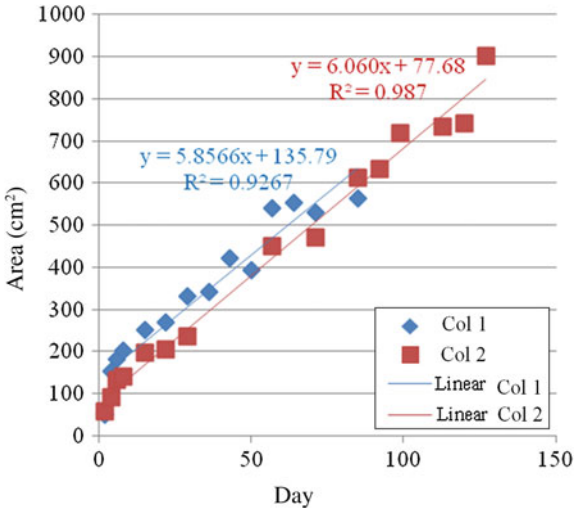


Fig. 3.13 Change in area of *A. florea* comb

References

Benton F (1896) The honey bee. US Govt Printing Office, Washington

Bonabeau E, Theraulaz G, Deneubourg JL, Aron S, Camazine S (1997) Self-organization in social insects. TREE 12:188–193

Camazine S (1991) Self-organizing pattern formation on the combs of honey bee colonies. Behav Ecol Sociobiol 28:61–76

Camazine S (2001) Self-organizing systems. In: Anderson PW, Epstein JM, Foley DK, Levin SA, Nowak MA (eds) Self-organization in biological systems. Princeton University Press, Princeton

Crane E (1999) The world history of beekeeping and honey hunting. Taylor and Francis, London

Dreller C, Tarpy DR (2000) Perception of the pollen need by foragers in a honeybee colony. Anim Behav 59:91–96

Duangphakdee O, Hepburn HR, Radloff SE, Pirk CWW, Rodim P, Wongsiri S (2012) Waggle dances in absconding colonies of the red dwarf honeybee, *Apis florea*. Insectes Soc 59:571–577

Duangphakdee O, Hepburn HR, Rodim P (2013) Developmental stages in the nests of *A. florea*. (In ms.)

Dublin Society (1733) Instructions for managing bees. A Rhames, Dublin

Dzierzon J (1852) Nachtrag zur Theorie und Praxis des neuen Bienenfreundes. CH Beck'schen Buchhandlung, Brieg

Free JB (1982) Bees and mankind. Allen and Unwin, Australia

Grassé PP (1942) Les abeilles sociales et leur evolution. In: Caullery M (ed) Biologie des abeilles. Presses Universitaires de France, Paris

Hepburn HR, Hepburn C (2011) Bibliography of the Asian species of honeybees. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 473–657

Huber F (1814) Nouvelles observations sur les abeilles. Barde, Genève

Jenkins MJ, Sneyd J, Camazine S, Murray JD (1992) On a simplified model for pattern formation in honey bee colonies. J Math Biol 30:281–306

Johnson BR (2009) Organizing model for task allocation via frequent task quitting and random walks in the honeybee. Am Nat 174:537–547

Johnson BR, Baker N (2007) Adaptive spatial biases in nectar deposition in the nests of honey bees. Insectes Soc 54:351–355
Kallapur SK (1950) An experiment in the collection of wild honey. Indian Bee J 12:122–124
Koeniger N, Koeniger G, Tingek S (2010) Honey bees of Borneo: exploring the centre of *Apis* diversity. Natural History Publication (Borneo), Kota Kinabalu
Langstroth LL (1857) A practical treatise on the hive and the honey-bee. CM Saxton & Co, New York
Lindauer M (1956) Ueber Verstandigung bei indischen Bienen. Z Vergl Physiol 38:521–557
Montovan KJ, Karst N, Jones LE, Seeley TD (2013) Individual behavioral rules sustain the cell allocation pattern in the combs of honey bee colonies (Apis mellifera). J Theoret Biol 336:75–86. doi:10.1016/j.jtbi.2013.07.010; arXiv:1211.4298v2 [q-bio.PE]
Morse RA, Laigo FM (1969) Apis dorsata in the Philippines. Monogr Philippine Assoc Ent 1:1–96
Rahman KA, Singh S (1946) Size of the cell of the brood comb of the Indian honey bees. Indian Bee J 8:154–159
Rinderer TE, Wongsiri S, Kuang BY, Liu JS, Oldroyd BP, Sylvester HA, de Guzman LI (1996) Comparative nest architecture of the dwarf honey bees. J Apic Res 35:19–26
Ruttner F (1968) Systématique du genre *Apis*. In: Chauvin R (ed) Traité de biologie d'abeille, vol 1. Masson, Paris
Sakagami SF (1959) Regularities in the pattern of comb utilization in the honeybees, illustrated by the life history of a Japanese honeybee colony forced to live in a Langstroth hive (Studies on the Japanese honeybee, *Apis cerana cerana* Fabr. VII). Insectes Ecol 7:117–128 [in Japanese]
Sakagami SF, Yoshikawa K (1973) Additional observations on the nest of the dwarf honeybee, *Apis florea* (Hymenoptera, Apidae). Kontyû 41:217–219
Seeley TD, Morse RA (1976) The nest of the honey bee (*Apis mellifera* L.). Insect Soc 23:495–512
Singh S (1962) Beekeeping in India. Indian Council of Agricultural Research, New Delhi
Theraulaz G, Gautraisi J, Camazine S, Deneubourg JL (2003) The formation of spatial patterns in social insects: from simple behaviours to complex structures. Trans R Soc Lond 361:1263–1282
Thorley J (1744) Melisselogia, or, the female monarchy: being an enquiry into the nature, order, and government of bees. Thorley, London
Tokuda Y (1924) Studies on the honeybee, with special reference to the Japanese honeybee. Trans Sapporo Nat Hist Soc 9:1–26
Tokuda Y (1935) Study on the honeybee, with special reference to the Japanese honeybee. Bull Imp Zootech Exp Sta 15:1–27
Turing AM (1952) The chemical basis of morphogenesis. Phil Trans R Soc Lond 237(641):37–72
Underwood BA (1986) The natural history of *Apis laboriosa* Smith in Nepal. Thesis, Cornell University, Ithaca
Wongsiri S, Lekprayoon C, Thapa R, Thirakupt K, Rinderer TE, Sylvester HA, Oldroyd BP, Booncham U (1997) Comparative biology of *Apis andreniformis* and *Apis florea* in Thailand. Bee Wld 78:23–35
Woyke J (1976) Brood-rearing efficiency and absconding in Indian honeybees. J Apic Res 15:133–143

Chapter 4
Intraspecific and Interspecific Comb-Building

Abstract *A. florea* was tested to determine whether they would salvage wax from their own deserted natal combs in preference to other conspecific combs and from heterospecific facsimiles of other species. Preferences for natal comb were significantly greater than for non-natal combs, no wax being collected from heterospecific combs. Behavioural variations for wax choice were also assessed using *A. capensis, A. florea, A. cerana* and *A. dorsata* waxes, Japan wax, candelilla wax, bayberry wax and ozokerite, which were tested in *A. m. capensis, A. florea* and *A. cerana* colonies. *A. m. capensis* accepted only the beeswaxes. *A. cerana* and *A. florea* accepted the *A. cerana, A. florea* and *A. dorsata* waxes, but rejected *A. m. capensis* and the other waxes. Comb-building in *A. cerana* and *A. mellifera* mixed-species colonies was examined with foundation made from the waxes of these species and then given to colonies having either an *A. cerana* or an *A. mellifera* queen. The colonies did not discriminate between the waxes and comb-building was the combined efforts of both species.

4.1 Introduction

Aside from competition during foraging, nest site selection, and robbing, few other interspecific interactions among honeybee species have been investigated in any depth. But, among these, the activities of the Cape honeybee, *A. m. capensis*, have become notorious as exemplars of intraspecific parasitism (Moritz et al. 2011); and, likewise, interspecific social parasitism is widespread in *A. cerana* (Nanork et al. 2006), and *A. florea* (Chapman et al. 2009). There have also been studies of reciprocal transfers of *A. cerana* workers with *A. koschevnikovi* (Koeniger et al. 1996), *A. cerana* with *A. nuluensis* (de Guzman et al. 1996), heterospecific queen rearing with *A. cerana* and *A. mellifera* (Oschmann 1965; Ruttner and Maul 1983; Potichot et al. 1993), interspecific ovarial activation (Hepburn 1994; Tan et al. 2009), communication (Su et al. 2008; Tan et al. 2008), thermoregulation in *A. cerana* and *A. mellifera* mixed-species colonies (Yang et al. 2010a, b, c), and

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_4,

defense behaviour (Tan et al. 2010). Such heterospecific studies are at a nascent stage; but the complexity of the behaviours observed produce data that reveal activities which have survived speciation in honeybees. In this chapter we discuss a new dimension to such studies relating to the intraspecific and interspecific utilization of different waxes and of comb-building in mixed-species colonies of honeybees.

The nature of speciation has been analysed and reconstructed in quite considerable detail, particularly with emphasis on reproductive isolation (Wilson 1971; Bush 1975; Via 2001). However, many aspects of physiology and behaviour remain shared among species after speciation, but have been little touched upon, and their study illuminates the extent to which some important features have been conserved. For example, it was shown recently that *A. cerana* and *A. mellifera* mixed-species colonies are quite viable (Tan et al. 2006) and, indeed, each can interpret the waggle dances of the other (Su et al. 2008; Tan et al. 2008). Turning to beeswax, intraspecific comparisons of the beeswaxes among the races of *A. mellifera* show that they can only be distinguished after careful calculation of the peak-elution patterns of selected compounds (Brand-Garnys and Sprenger 1988; Fröhlich et al. 2000a), which obviously indicates that speciation within *A. mellifera* is an on-going process. However, there are notable species-specific differences in beeswaxes among species (Aichholz and Lorbeer 1999). Although the waxes vary, they all share a complex mixture of homologous neutral lipids in common (cf. Chap. 16).

4.2 Intraspecific Comb Wax Salvage

The secretion of wax and construction of combs represents a large metabolic investment by honeybees, so that desertion of the nest, for whatever reason, constitutes an energetically hefty expenditure (Hepburn et al. 1984; Pirk et al. 2011). Nonetheless, nest desertion by absconding or migrating colonies is a common feature of tropical honeybees in Africa (Hepburn and Radloff 1998) and Asia (Oldroyd and Wongsiri 2006; Hepburn 2011). Despite the possible cost effectiveness of cannibalising wax from a deserted nest and reusing it in the construction of a new one (Pirk et al. 2011), this behaviour is thus far only known for three species of honeybees, *A. andreniformis* (Duangphakdee, pers. obs.; Wongvilas, pers. obs.), *A. florea* (Akratanakul 1977; Dutton and Free 1979; Wongsiri et al. 1997; Hepburn et al. 2009, 2010), and *A. m. capensis* (Hepburn and Radloff 1998).

Inasmuch as *A. florea* will accept heterospecific beeswaxes inserted into their nests (Hepburn et al. 2009), Hepburn et al. (2010) conducted experiments on absconding *A. florea* colonies to determine whether these bees would preferentially salvage wax from their own, original natal comb over that of other conspecific combs; and, whether they would salvage wax from crown comb facsimiles of *A. florea* combs fashioned from the combs of *A. cerana, A. dorsata,* and

A. mellifera. Because *A. florea* colonies also tend to nest near one another (Rinderer et al. 2002; Wattanachaiyingcharoen et al. 2008), this demographic characteristic invites competition for accessible, free-standing, empty combs, the wax of which is a valuable and metabolically expensive resource (Hepburn et al. 1984; Pirk et al. 2011).

Hepburn et al. (2011) studied *A. florea* colonies that occur naturally in a small wood of about 3.65 ha, at King Mongkut's University of Technology, Chom Bueng, Thailand. The occurrence of these non-experimental colonies constitutes possible intercolonial competition for wax salvage among *A. florea* colonies. Over two seasons, *A. florea* colonies were collected, three or four at a time, moved at dusk, and each nest was suspended under its own open-sided bamboo shelter at the edge of a copse. The following day, about a thousand workers from each colony on the crowns of the combs were marked with a dot of craft paint, one unique colour per colony. At dusk the same day, the brood comb extending below the crown was cut away and removed to induce absconding. The comb crown is strictly a honey store, which prior to absconding, is virtually emptied of honey to provide fuel for the ensuing absconding flight (Hepburn et al. 2011). The next day, the colonies were continuously observed until they absconded and settled in a new tree, after which compass directions and the distances flown were measured for each colony.

In the first experiment, as soon as a colony absconded, two additional empty *A. florea* comb crowns were placed adjacent to the original crown (about 10 cm apart) of the recently absconded nest. The relative positions of the three crowns were assigned using a different set of random numbers for each colony and set of comb crowns. Within an hour of absconding and settling elsewhere, colonies issued foragers which returned to their original nest sites to scavenge wax. One hour after absconding, the three experimental crowns at each shelter were checked and the numbers of colony-specific colour-marked bees on the combs were counted; this was repeated three times at 30 min intervals. Then the positions of the combs relative to one another were changed again on the basis of random numbers, and the numbers of marked bees arriving at each comb were again counted.

Foragers from six colonies which had absconded returned to their natal nests to salvage wax. When these wax-salvage foragers reached the shelter and encountered three adjacent but different *A. florea* comb crowns, including their own original natal one, their preferences for the combs from which they salvaged wax differed significantly. Some of the colour-marked foragers reconnoitred all three combs but only landed on and recovered wax from their own original natal combs. Foragers from one-third of the colonies collected wax from all three combs. In two wax-scavenging episodes, foragers retrieved more wax from the non-natal combs than their own natal ones (Table 4.1). It is worth noting that some unmarked *A. florea* foragers also salvaged wax from these combs, but because these individuals could not be linked to a specific colony source, such bees were not counted. Some of these bees could have been unmarked bees from the natal colony that absconded; however, among them were bees whose departing flight paths were different from the compass directions in which the test colonies had flown

Table 4.1 Preferences of six, wax-salvaging *A. florea* colonies from natal and non-natal combs. Numerical values represent the sum of wax-scavenging events (Hepburn et al. 2010)

Combs					
Colony	Natal	Non-natal	G-value	df	*P*-value
1	106	0	232.9	1	<0.0001
2	112	0	246.1	1	<0.0001
3	95	0	208.7	1	<0.0001
4	6	19	1.0	1	0.3102
5	2	0	4.4	1	0.0361
6	13	62	9.7	1	0.0019
Total	334	81	702.8	6	<0.0001
Heterogeneity G			312.9	5	<0.0001
Mean ± SD	56.7 ± 53.7	13.5 ± 24.9			

after absconding. These bees were designated as 'free-lance' wax-scavengers from other colonies in the vicinity.

The results from this experiment demonstrate that *A. florea* wax-salvaging foragers from different colonies differed significantly as to whether they would cannibalize wax from non-natal *A. florea* combs; some did, others not. Given that the hydrocarbons of comb waxes vary among colonies of the same species and that *A. cerana* and *A. mellifera* worker bees can discriminate intraspecifically between combs of different colonies (Breed et al. 1988, 1995; Sasaki et al. 2000; Wilde et al. 2001), Hepburn et al. (2010) interpreted this data to indicate that (1) *A. florea* has just as a refined level of discriminatory ability as do the other two species; and, (2) that the observed differences in wax-salvage behaviour probably reflect genetic differences for this trait in *A. florea*. When two *A. florea* non-natal comb crowns were placed with the natal one, many returning marked foragers indiscriminately cannibalized wax from all three combs. At the same time, there were other foragers, the so-called 'free-lance' wax scavengers derived from other nests in the vicinity that retrieved comb crown wax. It appears that a deserted *A. florea* nest is a resource worth securing by any colony of this species in the surrounds.

4.3 Interspecific Wax Salvage

On completion of the above tests with three *A. florea* comb crowns, a second experiment was conducted; but this time *A. florea* comb crowns were tested against facsimiles made from *A. cerana, A. dorsata,* and *A. mellifera* combs. Test specimens of *A. cerana* and *A. mellifera* combs were prepared from frames of drawn combs by cutting away about 3 cm of drawn comb and adhering this to the top bars of whole frames. *A. dorsata* combs were cut into 3 cm strips which were wax-melted onto bare frame top bars. Thus, all combs were about 3 cm high and 12 cm wide, very similar to an *A. florea* crown when the top bars of the test combs

Table 4.2 Preferences of five *A. florea* wax-salvaging colonies for conspecific *A. florea* as well as *A. dorsata, A. cerana* and *A. mellifera* crowns. Numerical values represent the sum of wax-scavenging events (Hepburn et al. 2010)

Crown combs				
Colony	*A. florea*	*A. dorsata*	*A. cerana*	*A. mellifera*
1	7	0	0	0
2	62	0	0	0
3	22	0	0	0
4	89	0	0	0
5	56	0	0	0
Total	236	0	0	0
Mean ± SD	47.2 ± 32.8	0	0	0

were inverted to simulate the *A. florea* crown combs. The positions of the four wax crowns were again assigned randomly. Procedurally, this experiment was exactly like the first experiment. In both experiments none of the comb specimens had been used for brood rearing, were about of the same light colour, and therefore, probably of the same age, and were collected in the same area.

In this experiment, wax-salvaging by *A. florea* foragers from the experimental crowns of *A. florea, A. cerana, A. dorsata* and *A. mellifera* was observed. In separate trials of five different colonies, the number of paint-marked *A. florea* bees that salvaged wax from *A. florea* crowns was 47.2 ± 32.8, while paint-marked *A. florea* foragers did not retrieve waxes from the crowns of *A. cerana, A. dorsata* or *A. mellifera* (Table 4.2). The five colonies differed significantly in the numbers of wax-salvaging bees on *A. florea* crowns compared to the other species combs. Some unmarked *A. florea* foragers salvaged wax from the *A. cerana* crown, and approached but did not salvage wax from the *A. dorsata* and *A. mellifera* crowns. Because these bees were unmarked, they were excluded because of the possibility that such individual bees were not from the test colonies.

In this experiment the results were significant in that paint-marked *A. florea* foragers did not salvage wax from *A. cerana, A. dorsata,* or *A. mellifera* crowns. This indicates an unequivocal sensory capacity of *A. florea* foragers to distinguish between *A. florea* and non-*A. florea* waxes. However, the fact that unmarked *A. florea* foragers also salvaged wax from *A. cerana* comb suggests a greater behavioural plasticity than indicated just by the experimental colonies.

When the data of these experiments are juxtaposed to similar ones of heterospecific wax utilization within combs (Hepburn et al. 2009), some context-specific anomalies appear. In an experiment with heterospecific waxes, small squares of beeswax foundation fashioned from comb waxes of *A. florea, A. cerana, A. dorsata* and *A. mellifera* colonies were inserted in 'windows' cut in the middle of *A. florea* combs. All the *A. florea* colonies unequivocally accepted the wax inserts of *A. cerana, A. dorsata* and *A. florea* and built on them, but rejected the *A. mellifera* wax inserts. However, in this experiment, paint-marked *A. florea* foragers did not salvage wax from the combs of *A. cerana, A. dorsata* or *A. mellifera*. This contrast obviously

indicates that sensory discrimination of waxes by *A. florea* is exercised in the field but not in the nest; a context in which it should be unnecessary in the absence of heterospecific nest parasitism by other honeybee species.

4.4 Interspecific Wax Discrimination

Because *A. mellifera* can distinguish olfactory differences between combs of different colonies (Fröhlich et al. 2000b) and different ages of the same species (Breed et al. 1998), questions arise as to what extent is there flexibility for wax choice among honeybee species? Do they discriminate among waxes that they might naturally encounter (as in the Southeast Asian species), compared with waxes foreign to them? Finally, what are the Euclidean distances based on chemical composition of beeswaxes of different sister-groups, and are these similarities and differences related to wax choice in different species of honeybees?

Although *A. andreniformis* (Duangpakdee, pers. obs.; Wongvilas, pers. obs.), *A. florea* (Akratanakul 1977; Hepburn et al. 2009, 2010) and *A. m. capensis* (Hepburn and Radloff 1998), are known to scavenge wax conspecifically from abandoned combs, there have not yet been any reports of heterospecific salvage. To assess behavioural flexibility for wax choice using several beeswaxes, plant and mineral waxes as the test materials, Hepburn et al. (2009) used the Cape honeybee, *A. m. capensis,* colonies in South Africa, and *A. cerana* and *A. florea* colonies in Thailand. *A. m. capensis, A. florea, A. cerana* and *A. dorsata* beeswaxes, three plant waxes (Japan wax—ex: *Toxicodendron*, candelilla wax—ex: *Euphorbia*, and bayberry wax—ex: *Myrica*) and ozokerite, a mineral wax, were moulded into small sheets of wax foundation (inserted in the grooves of normal frame top bars), or, were cut into small squares and inserted in 'windows' cut from the host combs.

Photographs were taken to document the results once the combs were drawn. All four kinds of beeswax were accepted by colonies of *A. m. capensis* and cells were constructed on them (Fig. 4.1a). (As an aside, the honeyguides that damaged the combs shown in Fig. 4.1 are specialist feeders on beeswax and have the enzymic capacity to digest it (Downs et al. 2002; Diamond and Place 2008). The Asian orange-rumped honeyguide also consumes beeswax (Cronin and Sherman 1976; Underwood 1992), but no physiological studies have been reported on its digestive capacity). Both the Japan and bayberry waxes were gnawed away and removed by the bees, while candelilla and ozokerite waxes remained untouched (Fig. 4.1b; Table 4.3). The *A. cerana* colonies accepted the strips of *A. cerana, A. florea* and *A. dorsata* wax and extended their combs on these; however, they either gnawed or avoided the wax of *A. m. capensis* as well as all plant and mineral waxes (Table 4.3).

After 1 week the *A. florea* colonies had repaired the wax inserts in their combs. However, they did not refashion the larger cell base to *florea*-size, but constructed new cell walls much thicker than normal so that cell diameter was a close approximation to normal size. Over subsequent weeks, all the *A. florea* colonies had accepted

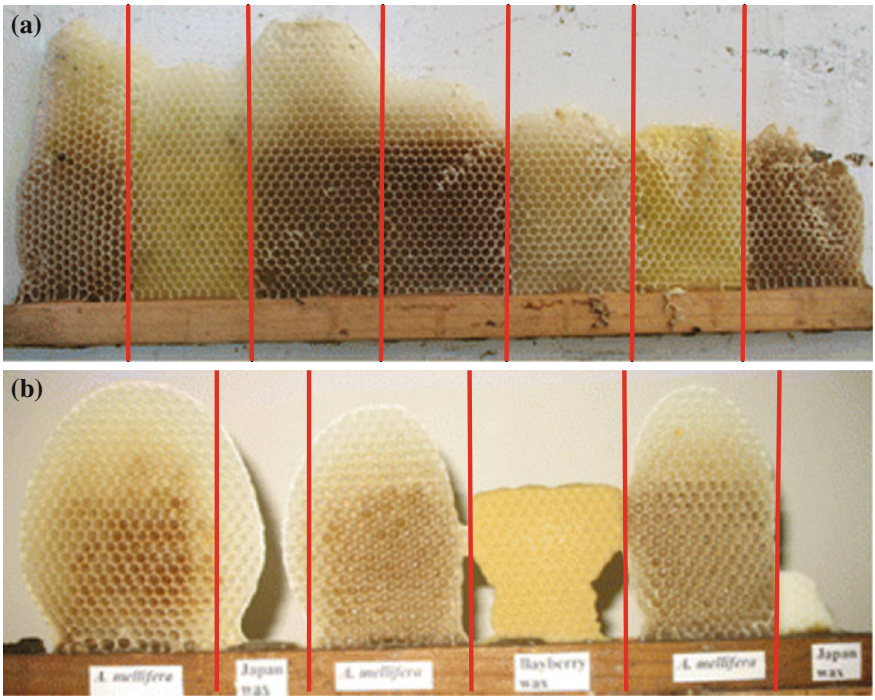


Fig. 4.1 **a** Comb construction by *A. m. capensis* on foundation sheets made from (*left* to *right*) the waxes of *A. m. capensis, A. cerana, A. m. capensis, A. florea, A. dorsata, A. cerana,* and *A. m. capensis*; **b** Comb construction by *A. m. capensis* on foundation sheets made from (*left* to *right*) the waxes of *A. m. capensis*, Japan wax, *A. m. capensis,* bayberry, *A. m. capensis* and Japan wax. The obvious damage to the combs in a) was inflicted by the Lesser Honeyguide, *Indicator minor,* feeding on them (Hepburn et al. 2009)

Table 4.3 Reactions of *A. m. capensis, A. cerana* and *A. florea* honeybees to thin sheets of different beeswaxes, plant and mineral waxes (Hepburn et al. 2009)

Waxes		Host colonies		
		A. m. capensis	*A. cerana*	*A. florea*
Beeswax	*A. cerana*	Accepted builds	Accepted builds	Accepted builds
	A. florea	Accepted builds	Accepted builds	Accepted builds
	A. dorsata	Accepted builds	Accepted builds	Accepted builds
	A. m. capensis	Accepted builds	Gnawed/untouched	Gnawed
Plant wax	Bayberry	Gnawed	Untouched	Not tested
	Japan wax	Gnawed	Untouched	Not tested
	Candelilla	Untouched	Untouched	Not tested
Mineral wax	Oxokerite	Untouched	Untouched	Not tested

the *A. cerana* and *A. dorsata* foundation wax inserts (Fig. 4.2a). One colony accepted the *A. mellifera* wax, but the other two simply gnawed away at the wax. *A. cerana* colonies readily and equally accepted the waxes of only three species (*A. cerana, A. dorsata* and *A. florea*), and partially or completely rejected that of *A. m. capensis* (Table 4.3, Fig. 4.2b). Based on a parsimonious cluster analysis (cf. Chap. 16, Fig. 16.3), the giant honeybee group (*A. dorsata* and *A. laboriosa*) is clearly segregated from the other species, as are the dwarf species (*A. andreniformis* and *A. florea*), while *A. mellifera* is placed close to its sister-group, *A. cerana*.

Given the ubiquitous nature and abundance of surface waxes throughout the plant kingdom (Kolattukudy 1976), it is perhaps surprising that elaborate glands for wax synthesis and secretion evolved in honeybees in the first place (Hepburn et al. 1991). This is particularly curious as many other apoid bees utilize various plant exudates for building their nests (Roubik 1992). Nevertheless, all *Apis* species have such glands, but their product of secretion, beeswax, has also changed with speciation, there being only 13 out of 82 chromatographic elution peaks shared in common across all species (Aichholz and Lorbeer 1999, cf. Chap. 16). The plant and mineral waxes were uniformly rejected, possibly because they lack some or all of the 13 shared compounds present in all beeswaxes and/or are actually repellent for other reasons (Sackin 1998). The plant waxes often contain terpenoid compounds, which are known honeybee repellents (Hamilton 1995). Given that the alkanes, monoesters and diesters, hydroxymonoesters, hydroxydiesters are shared in common within all beeswaxes, these compounds could be interpreted as the 'essence' of beeswax, which may be necessary and sufficient to induce bees to build comb.

The rejection of the *A. m. capensis* wax by *A. cerana* and *A. florea* is difficult to account for, but could possibly be due to the presence of a series of saturated fatty acids, C_{22}–C_{36}, all of which are absent from *A. cerana, A. florea* and largely from *A. dorsata* wax. Indeed, using the proboscis extension reflex technique, Fröhlich et al. (2000b) showed that *A. mellifera* workers can recall and distinguish the fatty acids and hydrocarbons of wax. It is also pertinent to mention cell size in relation to the foundation strips of wax given to the bees in this experiment. The *A. m. capensis* cell size used in making the moulds was about 4.8 mm in width; however, the cells of *A. florea* are 2.9 mm and *A. cerana* 4.3 mm. Inasmuch as both *A. florea* and *A. cerana* readily accepted the *A. cerana, A. dorsata* and *A. florea* wax foundation made to the *A. m. capensis* cell size (4.8 mm), then rejected the *A. m. capensis* wax, cannot be attributed to differences in cell size.

Considering the Euclidean distances of the beeswaxes, the dwarf and giant honeybees are distinct groups, but *A. mellifera* is only slightly skewed away from *A. cerana*. It is somewhat curious that both *A. cerana* and *A. florea* accepted the *A. dorsata* wax, which, based on chemical cladistics for wax, is the most distant from both. Perhaps this would appear to be a small discrepancy in light of the currently prevailing phylogenies for *Apis* based on nesting sites (Lindauer 1956), morphometrics (Alexander 1991), DNA sequences (Arias and Sheppard 2005) and behaviour (Raffiudin and Crozier 2007). In any event, the close proximity of the

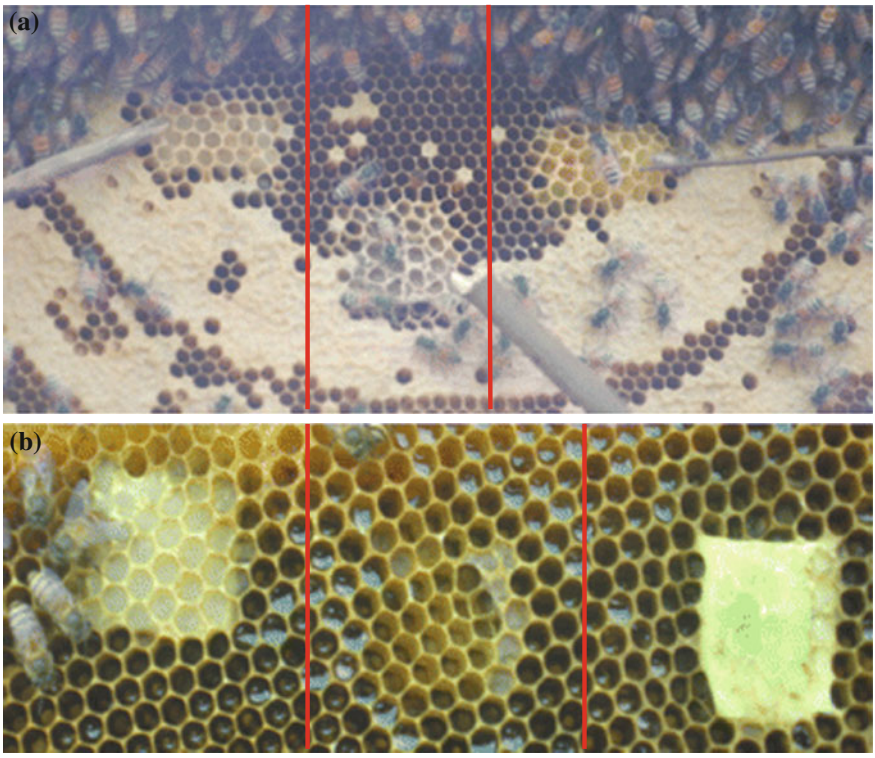


Fig. 4.2 **a** Comb construction by *A. florea* on foundation 'windows' made from (*left* to *right*) the waxes of *A. dorsata, A. m. capensis* and *A. cerana*; **b** Comb construction by *A. cerana* on foundation 'windows' made from (*left* to *right*) the waxes of *A. florea, A. m. capensis* and *A. dorsata* (Hepburn et al. 2009)

beeswax cluster groups to those based on DNA and morphometrics suggests that the wax glands were a highly conserved feature during honeybee evolution.

Previous studies on comb-building in *A. mellifera* have shown that some very simple building rules (Darchen 1954 et seq.; Hepburn and Whiffler 1991) which, coupled to the physico-chemical properties of beeswax as a building material (Pirk et al. 2004; Buchwald et al. 2006), can parsimoniously explain several aspects of comb-building behaviour. Indeed, regulation of behaviour through self-organisation (Bonabeau et al. 1997; Boomsma and Franks 2006; Detrain and Deneubourg 2006), specifically in honeybee societies, can be used to interpret behaviours including comb construction (Belic et al. 1986; Hepburn 1998), the arrangement of food-storing and brood-rearing in the combs (Camazine et al. 1990; Camazine 1991), and the regulation of food collection behaviour (Jenkins et al. 1992).

4.5 Comb-Building in Mixed-Species Colonies

Mixed-species colonies of honeybees offer us a valuable opportunity to investigate the relationships within and between the two species and provide us with a new perspective to examine the theories of self-organisation in honeybees and investigate the evolution of behaviour. Division of labour in mixed-species colonies remained an intriguing issue, which was not previously considered until quite recently with experiments by Yang et al. (2010c). They examined the comb-construction behaviour of mixed-species colonies of *A. cerana* and *A. mellifera* to answer several questions: (1) Will mixed-species colonies accept each other's waxes? (2) Will colonies of pure *A. cerana* accept *A. mellifera* wax and vice versa? (3) Given that the bees are presented with beeswax foundation of different cell base sizes, are these accepted as such, or are they modified during comb-building? (4) Do *A. cerana* and *A. mellifera* workers co-operate heterospecifically in comb-building or do they form separate, conspecific festoons? (5) Under the various conditions above, what cell sizes would emerge in the newly constructed combs? And (6) once constructed, how are these cells used in the economy of the nest?

4.5.1 Organisation of Mixed-Species Colonies and Wax Foundation

Yang et al. (2010c) established mixed-species colonies of both *A. cerana* and *A. mellifera* workers: three colonies were headed by *A. cerana* queens, and reciprocally, three colonies were headed by *A. mellifera* queens. Frames of sealed brood about to emerge as young adults of each species were placed in the colonies of the other species (Tan et al. 2006). Observations were made on the wax-building behaviour when the newly emerged workers of the two species were about 10–18 days old, the peak age of wax secretion (Rösch 1927; Hepburn et al. 1984; Seeley 1995). Pure *A. cerana* and *A. mellifera* colonies with the same age cohort of workers were also selected as control colonies, which were equalised for size, number of combs, adult bees, nectar and pollen stores and brood. In these experiments, beeswax was extracted from both *A. cerana* and *A. mellifera* combs and used to make small sheets of beeswax foundation of the two worker cell sizes: *A. cerana*, about 4.75 mm in diameter (Ruttner 1988), and *A. mellifera*, 5.35 mm in diameter (Winston 1987), using silicon rubber moulds (Hepburn et al. 2009). Both *A. cerana* and *A. mellifera* cell-size foundation was introduced into pure *A. cerana* and pure *A. mellifera* colonies. The experiments on cell size and wax discrimination, and comb-building cooperation were conducted with colonies of *A. cerana* and *A. mellifera* at an apiary at the King Mongkut's University of Technology, Chom Bueng, Thailand. The four types of beeswax foundation sheets (two wax types and two cell sizes) were fixed on the top bars of frames, and their

relative positions in the hives determined by random number assignment; they were then inserted into the centre of the hives.

Video-recordings were made of comb-building activity for the test and control colonies at 10 s intervals three times a day and every day for the replicates (Table 4.4). On replaying the video clips, detailed information was obtained on: (1) how many workers of each species were engaged in which type of comb-building; (2) how many starting sites were used to extend the building of new combs; (3) whether the festoon bees formed a mixed-species building chain and cooperated with each other in comb-building. (4) how many workers of each species were in each festoon; and (5) when comb building was complete. When the foundation sheets had been extended beyond their original lengths by the addition of several cm of new wax, the combs were removed from the hives and replaced with new top bars with the same four kinds of foundation (Yang et al. 2010c).

4.5.2 Cell-Size and Wax Discrimination

Pure *A. cerana* colonies ignored all beeswax foundation and began building new combs either from the top bar, or from the lower edges of the foundation sheets (Fig. 4.3a). By contrast, the pure *A. mellifera* colonies accepted both the *A. cerana* and *A. mellifera* foundation sheets and built cells on both cell sizes (Fig. 4.3b). In the two types of mixed-species colonies, all four types of foundation were accepted (Fig. 4.3c, d); workers of both species were seen building cells on the foundation (Fig. 4.4, Table 4.5). None of these mixed-species colonies showed any preference to a particular type of foundation with respect to wax type or cell size.

4.5.3 Cell-Size Modification of Foundation Sheets

All the *A. mellifera* cell-size sheets of foundation were built to their original size without any modification (Table 4.5); but the *A. cerana* cell-size foundation sheets were modified in all colonies, except for the pure *A. cerana* colonies. Some of these cells were squeezed to make space for enlarging neighbouring cells. The percentages of combs that had modified cells in the test and control groups are shown in Table 4.5. In *A. mellifera* queen-headed mixed-species colonies, all the *A. cerana* foundation sheets were modified, and in the pure *A. mellifera* colonies, nearly all were modified which was significantly different to the *A. cerana* queen-headed mixed-species colonies and pure *A. cerana* colonies (Yang et al. 2010c).

Table 4.4 Numbers (mean ± S.D.) of worker bees engaged in comb-building on four types of foundation (Yang et al. 2010c)

Foundations		Host colonies					
Waxes	Cell sizes	*A. cerana* queen-headed mixed colonies (N = 3, n = 14 replicates)		*A. mellifera* queen-headed mixed colonies (N = 3, n = 10 replicates)		Pure *A. cerana* colonies (N = 3, n = 12 replicates)	Pure *A. mellifera* colonies (N = 3, n = 12 replicates)
		A. cerana workers	*A. mellifera* workers	*A. cerana* workers	*A. mellifera* workers	*A. cerana* workers	*A. mellifera* workers
Apis cerana	*A. cerana*	3.5 ± 2.2	18.0 ± 5.7	3.3 ± 2.1	18.2 ± 9.0	–	16.8 ± 9.8
	A. mellifera	5.1 ± 2.4	16.6 ± 6.1	2.5 ± 2.3	17.0 ± 7.5	–	21.2 ± 9.7
Apis mellifera	*A. cerana*	4.1 ± 2.4	17.0 ± 3.3	1.4 ± 1.2	18.1 ± 8.2	–	19.3 ± 10.4
	A. mellifera	3.4 ± 3.3	16.5 ± 4.9	1.9 ± 2.0	19.2 ± 4.5	–	15.8 ± 10.6
	P-value	0.221	0.743	0.110	0.863		0.216

N is the number of pure colonies and *n* is the number of repetitions

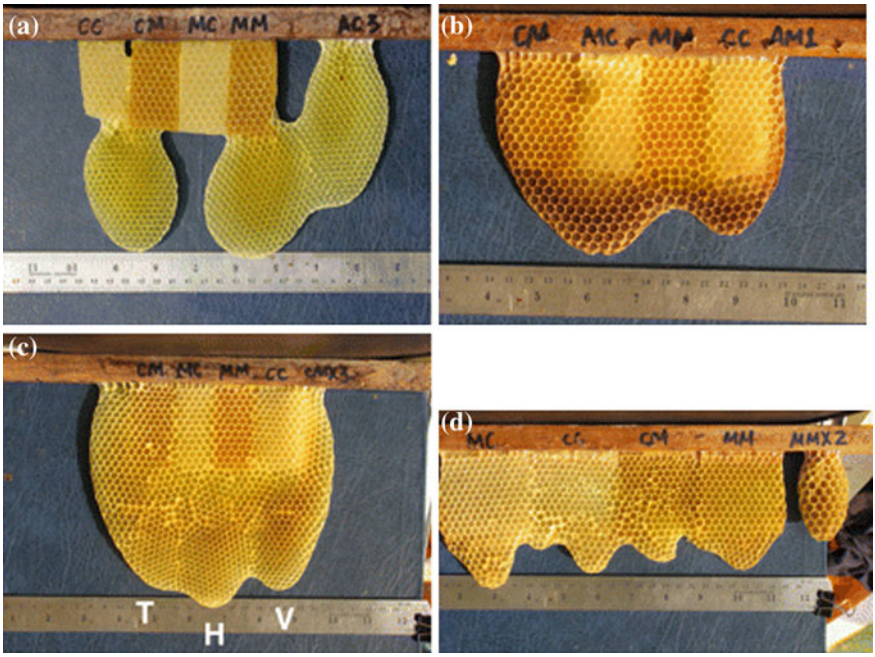


Fig. 4.3 Comb built by an *A. mellifera* queen-headed, mixed-species colony. Combs built in the four types of colonies: **a** Pure *A. cerana*; **b** Pure *A. mellifera*; **c** *A. cerana* queen-headed; and **d** *A. mellifera* queen-headed colony. Abbreviations on the *top bars* are: *CC*, *A. cerana* cell-size foundation made from *A. cerana* wax; *CM*, *A. cerana* cell-size foundation made from *A. mellifera* wax; *MM*, *A. mellifera* cell-size foundation made from *A. mellifera* wax; *MC*, *A. mellifera* cell-size foundation made from *A. cerana* wax. Cell direction patterns of newly built combs: *V* vertical; *H* horizontal; *T* tilted. AC3, an example comb built by a pure *A. cerana* colony (Colony 3); AM1; an example comb built by a pure *A. mellifera* colony (Colony 1); CMX3, an example comb built by an *A. cerana* queen-headed, mixed-species colony (Colony 3); MMX2, an example comb built by an *A. mellifera* queen-headed, mixed species colony (Colony 2) (Yang et al. 2010c)

4.5.4 Freely-Built Combs

On completion of the comb-building trials using different species, waxes and cell sizes on the artificial foundation sheets, the workers from the four types of colonies were observed starting to build new combs at several sites (Table 4.6). Pure *A. mellifera* colonies and *A. mellifera* queen-headed mixed-species colonies had significantly more festoons at new comb-building sites than pure *A. cerana* and *A. cerana* queen-headed colonies (Table 4.6). In *A. cerana* queen-headed mixed-species colonies, workers of both species were seen working together in festoons, although significantly more *A. mellifera* workers (57.9 ± 6.2 %) were involved than *A. cerana* workers (42.1 ± 6.2 %). Similarly, in the *A. mellifera* queen-headed mixed-species colonies, significantly more *A. mellifera* workers (67.5 ± 4.8 %)

Fig. 4.4 Comb-building by a mixed-species, wax-building chain of *A. cerana* and *A. mellifera* workers (Yang et al. 2010c)

Table 4.5 Percentages of *A. cerana* cell size foundation with modifications (Yang et al. 2010c)

Colony type	*A. cerana* cell-size foundations		*A. mellifera* cell-size foundations	
	Number	Percentage with modified signs (%)	Number	Percentage with modified signs (%)
Pure *Apis cerana* ($N = 3$, $n = 12$ replicates)	24	0	24	0
Pure *Apis mellifera* ($N = 3$, $n = 12$ replicates)	24	83.3	24	0
A. cerana queen-headed mixed colony ($N = 3$, $n = 14$ replicates)	28	10.7	28	0
A. mellifera queen-headed mixed colony ($N = 3$, $n = 10$ replicates)	20	100	20	0

N is the number of pure colonies and n is the number of repetitions

than *A. cerana* workers (32.5 ± 4.8 %) were engaged in comb-building in the festoons (Table 4.6). In total, significantly more workers were engaged in comb-building in the mixed-species colonies than in the pure *A. cerana* and pure *A. mellifera* colonies (Table 4.6).

As for irregular cells on the new combs, pure *A. cerana* and *A. mellifera* colonies built significantly fewer irregular cells (0.8 % and 2.7 %, respectively), than did the mixed-species colonies (9.1 % and 10.8 %, respectively); most of which were located at the seams of combs which had been started at different sites

Table 4.6 Characteristics of freely-built combs, mean ± SD (Yang et al. 2010c)

Parameter	*A. cerana* queen-headed mixed colonies (N = 3, n = 14 replicates)	*A. mellifera* queen-headed mixed colonies (N = 3, n = 10 replicates)	Pure *A. cerana* colonies (N = 3, n = 12 replicates)	Pure *A. mellifera* colonies (N = 3, n = 12 replicates)
Number of festoons	$2.3^{b} \pm 0.5$	$4.2^{a} \pm 1.4$	$1.9^{b} \pm 0.9$	$3.9^{a} \pm 1.1$
Number of *A. cerana* workers on the festoons	61.4 ± 13.4	36.8 ± 10.7	108.0 ± 29.1	–
Number of *A. mellifera* workers on the festoons	84.6 v 16.1	75.6 ± 16.3	–	90.3 ± 25
Total number of two species of workers on the festoons	$146.1^{a} \pm 22.0$	$112.4^{b} \pm 24.5$	$1\text{-}8.0^{b} \pm 29.1$	$90.3^{b} \pm 25.0$
Percentage of irregular cells (%)	$9.1^{a} \pm 3.6$	$10.8^{a} \pm 4.7$	$0.8^{b} \pm 0.5$	$2.7^{b} \pm 1.7$
Patterns of newly built combs: V = vertical H = horizontal T = tilted R = rosette	V + H: 29 % V + H + T: 22 % V + T: 21 V: 14 %; T: 7 % V + H + R: 7 %	V + H: 60 % V: 40 %	V: 75 % V + H: 17 % T: 8 %	V: 83 % V + H: 17 %
Cell size of newly built combs (mm)	$5.41^{b} \pm 0.27$	$5.93^{a} \pm 0.61$	$4.38^{c} \pm 0.06$	$5.74^{a,\ b} \pm 0.61$

Means within one row followed by the same letter are not significantly different (Tukey multiple comparisons: $p > 0.05$). N is the number of pure colonies and n is the number of repetitions (Yang et al. 2010c)

(Table 4.6). The *A. cerana* queen-headed mixed-species colonies showed significantly greater variation in the patterns of cell orientation on the newly built combs than *A. mellifera* queen-headed mixed-species colonies, pure *A. cerana* and *A. mellifera* colonies; different festoons on one comb built patterns different to those built on other combs (Table 4.6). *A. mellifera* queen-headed, mixed-species colonies built new combs mainly in vertical and horizontal patterns (Fig. 4.3d); in pure *A. cerana* and *A. mellifera* colonies, the patterns of cell orientation were more homogeneous and mainly vertical (Fig. 4.3a, b; Table 4.6).

The different mixed-species colonies built significantly different sized cells (Table 4.6). The largest cells were built by *A. mellifera* queen-headed mixed-species colonies. The cells built in the pure *A. mellifera* colonies and *A. mellifera*

Fig. 4.5 Utilisation of combs built on two types of cell size foundation in pure *A. cerana* colonies; *A. mellifera* size cells (*left*) were used for storing food, while the *A. cerana* size cells (*right*) were used for brood rearing (Yang et al. 2010c)

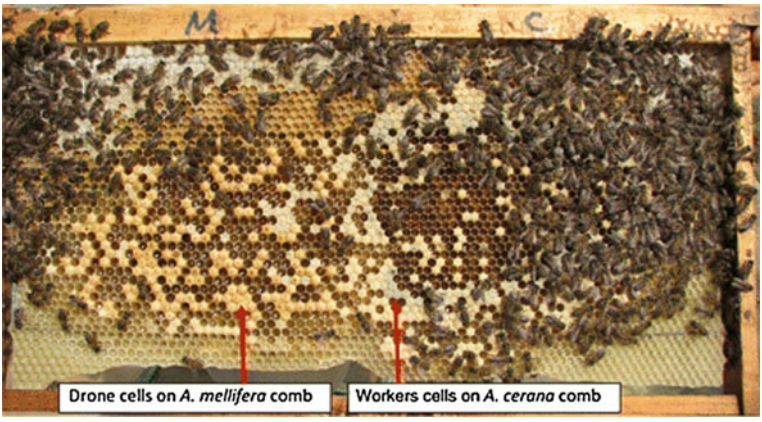


Fig. 4.6 Utilisation of combs built on two types of cell size foundation in pure *A. cerana* colonies; *A. mellifera* size cells (*left*) were used for drone brood rearing (with typical capping apertures), while the *A. cerana* size cells (*right*) were used for rearing worker brood (Yang et al. 2010c)

queen-headed mixed-species colonies were similar to *A. mellifera* drone cells (European type, 6.0–6.3 mm), whereas in the *A. cerana* queen-headed mixed-species colonies, the cells had a diameter of 5.41 ± 0.27 mm, which is like the normal *A. mellifera* worker size cells. The pure *A. cerana* colonies built cells 4.38 ± 0.06 mm in size, which is the normal *A. cerana* worker size cell.

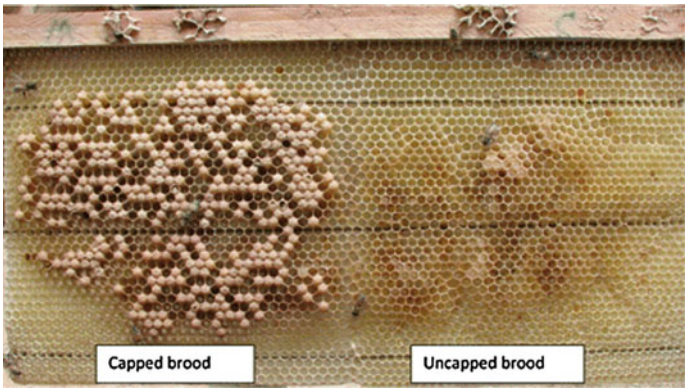


Fig. 4.7 Utilisation of combs built on two types of cell size foundation in pure *A. mellifera* colonies, the brood cells on the *A. mellifera* cells (*left*) are already capped but the larvae on the *A. cerana* cell size foundation (*right*) still need about three more days until capping, suggesting that the queens first laid eggs on the *left side* and only laid eggs in the *A. cerana* size cells somewhat later (Yang et al. 2010c)

4.5.5 Utilisation of the Newly Built Combs

In their experiments, Yang et al. (2010c) inserted both *A. cerana* cell size (4.75 mm in diameter) and *A. mellifera* cell-size (5.35 mm diameter) foundation strips into pure *A. cerana* and pure *A. mellifera* colonies, with the following results. Pure *A. cerana* colonies accepted both foundation types and built cells without altering the original cell base; while pure *A. mellifera* colonies accepted both foundation wax types but changed the *A. cerana* cell size to their normally larger cells, with the inclusion of many irregular cells.

Once the control combs had been constructed, *A. cerana* colonies differed from the *A. mellifera* colonies in the subsequent use of these cells. The pure *A. cerana* colonies used the *A. mellifera* size cells either for food storage (Fig. 4.5) or drone brood rearing, while the *A. cerana* size cells were normally used for rearing worker brood (Fig. 4.6). In pure *A. mellifera* colonies, queens mainly laid eggs in both *A. mellifera* and *A. cerana* size cells, but they all showed a preference for *A. mellifera* size cells and laid eggs in these cells first and more regularly (Fig. 4.7).

4.5.6 General Comb-Building

It is common knowledge that cavity-dwelling honeybees build multiple, parallel combs and that this parallelism is recognised as a building rule (Darchen 1954; Hepburn 1986; Hepburn and Muller 1988). Comb-building bees work in a dark

cavity or hive where there is no central source of information. When construction begins, the workers cling together in elongated chains or festoons, forming a dense cluster that facilitates an equable temperature for wax secretion and manipulation (Hepburn 1986). Numerous comb-building workers with active wax glands engage in the task of comb construction. But, instead starting to build at a single site, several festoons begin at independent sites, constructing starting strips of cells several cells (hence combs) simultaneously, and only later do they connect these using irregular transitional cells (Hepburn 1986; Hepburn and Whiffler 1991). In this case, the parallelism rule can only be achieved indirectly, at the finishing stage of comb-building, with many irregular cells and seam connections between several new combs started at separate sites (Hepburn and Whiffler 1991).

4.5.7 Comb-Building in Mixed-Species Colonies

A. cerana and *A. mellifera* workers cooperate heterospecifically in the same festoons in comb-building (Yang et al. 2010c); but it is somewhat strange that in the pure *A. cerana* colonies, none of the four types of foundation (foundation made from *A. mellifera* wax in both *A. mellifera* and *A. cerana* worker sell sizes and foundation made from *A. cerana* wax both in *A. cerana* and *A. mellifera* worker size cells) were accepted, although two of the four foundations were embossed with normal *A. cerana* size cell. In sharp contrast to this, in the pure *A. mellifera* colonies, workers were seen building cells on both types of wax foundation and of both cell sizes. These results indicate that *A. mellifera* workers are more tolerant of cell size factors in wax foundation. This contrast is revisited in both types of mixed-species colonies where more *A. mellifera* workers than *A. cerana* workers were seen building comb, irrespective of the species of host queen.

However, interestingly, *A. cerana* workers did engage in comb-building on foundations of both waxes and the two cell sizes in the both types of mixed-species colonies (Table 4.6). This certainly suggests that comb-building workers *A. mellifera* can somehow stimulate *A. cerana* workers to start building comb. A comb-building stimulus appears reciprocal because in pure *A. mellifera* colonies, while 83.3 % of the *A. cerana* cell size foundation sheets were modified and expanded to *A. mellifera* cell size, only 10.7 % were modified in mixed-species colonies headed by *A. cerana* queens. In the *A. cerana* queen-headed mixed-species colonies, more *A. mellifera* workers were engaged in comb-building festoons, so it is not surprising that the cell sizes were similar to normal *A. mellifera* worker-sized cells.

It is interesting to note that in an *A. cerana* queen-headed mixed-species colony, the festoons were formed predominately by *A. mellifera* workers with fewer *A. cerana* workers joining them. However, the combs built in the mixed-species colonies did have more irregular cells than were observed in any of the pure *A. cerana* or *A. mellifera* colonies. This seems to indicate that the *A. cerana* workers also play a role in determining final cell-size. Although they did cooperate in festoons, the two species cannot really perform the comb-building tasks

harmoniously. The fact that the combs in the pure *A. mellifera* colonies and *A. mellifera* queen-headed colonies mixed-species were built to normal *A. mellifera* drone-sized cells may be related to the season in which in the experiment was conducted.

In conclusion, *A. cerana* workers as colonies did not accept any type of beeswax foundation, but as individuals were stimulated by *A. mellifera* workers to engage in comb-building. So, the results are consistent with the idea that honeybee comb-building behaviour is an example of self-organisation. It was also confirmed that in the mixed-species colonies, these two closely related honeybee species did in fact cooperate in comb-building, even though irregular cells arose through their joint efforts. It can also be inferred that, although the comb-building workers are poorly informed and lack a central controller (Pratt 2004), comb-building is really a task that can only be finished by smaller groups, in which individuals cooperate closely to achieve progress. This might explain, in part, why *A. mellifera* workers do not dominate the comb-building effort.

The results presented here, based on mixed-species colonies, reinforce the conclusion that this experimental method is extremely useful for testing underlying mechanisms that evoke or suppress certain behaviours. Such an experimental context has been successfully used to elucidate disruption of social networks as in ovarial activation (Tan et al. 2009), stimulation of social networks, dance language (Tan et al. 2008) and retinue behaviour towards queens (Yang et al. 2010a). The results from the comb-building experiments provide additional evidence for the value of mixed-species colonies as experimental probes for investigating pre- and post-speciation behaviour in honeybees.

References

Aichholz R, Lorbeer E (1999) Investigation of combwax of honeybees with high-temperature gas chromatography and high-temperature gas chromatography-chemical ionization mass spectrometry. I: high-temperature gas chromatography. J Chromatogr A 855:601–615

Akratanakul P (1977) The natural history of the dwarf honey bee, *Apis florea* F. in Thailand. Thesis, Cornell University, Ithaca

Alexander BA (1991) A cladistic analysis of the genus *Apis*. In: Smith DR (ed) Diversity in the genus *Apis*. Westview, Boulder, pp 1–28

Arias MC, Sheppard WS (2005) Phylogenetic relationships of honey bees (Hymenoptera: Apinae: Apini) inferred from nuclear and mitochondrial DNA sequence data. Mol Phylogenet Evol 37:25–35

Belic MR, Skarka V, Deneubourg JL, Lax M (1986) Mathematical model of honeycomb construction. J Math Biol 24:437–449

Bonabeau E, Theraulaz G, Deneubourg JL, Aron S, Camazine S (1997) Self-organization in social insects. TREE 12:188–193

Boomsma JJ, Franks NR (2006) Social insects: from selfish genes to self organisation and beyond. TREE 21:303–308

Brand-Garnys EE, Sprenger J (1988) Beeswax—new aspects of a classic raw material for cosmetics. Seifen Öele Fette Wachse 114:547–552 [in German]

Breed MD, Leger EA, Pearce AN, Wang YJ (1998) Comb wax effects on the ontogeny of honey bee nestmate recognition. Anim Behav 55:13–20

Breed MD, Page RE, Hibbard RE, Bjostad LB (1995) Interfamily variation in comb wax hydrocarbons produced by honey bees. J Chem Ecol 21:1329–1338
Breed MD, Williams KR, Fewell JH (1988) Comb wax mediates the acquisition of nest-mate recognition cues in honey bees. Proc Nat Acad Sci 85:8766–8769
Buchwald R, Breed MD, Greenberg AR, Otis GW (2006) Interspecific variation in beeswax as a biological construction material. J Exp Biol 209:3984–3989
Bush GL (1975) Modes of animal speciation. Ann Rev Ecol Syst 6:339–364
Camazine S (1991) Self-organizing pattern formation on the combs of honey bee colonies. J Behav Ecol Sociobiol 28:61–76
Camazine S, Sneyd J, Jenkins MJ, Murray JD (1990) A mathematical model of self-organized pattern formation on the combs of honeybee colonies. J Theor Biol 147:553–571
Chapman NC, Nanork P, Gloag RS, Wattanachaiyingcharoen W, Beekman M, Oldroyd BP (2009) Queenless colonies of the Asian red dwarf honeybee (*Apis florea*) are infiltrated by workers from other queenless colonies. Behav Ecol 20:817–820
Cronin EW, Sherman PW (1976) A resource-based mating system: the orange rumped honeyguide. Living Bird 15:5–32
Darchen R (1954) Quelques regulations sociales dans les constructions chez les abeilles. Insectes Soc 1:219–228
de Guzman LI, Rinderer TE, Whiteside RR (1996) A scientific note on the occurrence of *Varroa* mites on adult worker bees of *Apis nuluensis* in Borneo. Apidologie 27:429–430
Detrain C, Deneubourg JL (2006) Self-organized structures in a superorganism: do ants 'behave' like molecules? Phys Life Rev 3:162–187
Diamond AW, Place AR (2008) Wax digestion by black-throated honey-guides, *Indicator indicator*. Ibis 130:558–561
Downs CT, van Dyk RJ, Iji P (2002) Wax digestion by the lesser honeyguide, *Indicator minor.* Comp Biochem Physiol A. Mol. Integ Physiol 133:125–134
Dutton RW, Free JB (1979) The present status of beekeeping in Oman. Bee Wld 60:176–185
Fröhlich B, Tautz J, Riederer M (2000a) Chemometric classification of comb and cuticular waxes of the honeybee *Apis mellifera carnica*. J Chem Ecol 26:123–137
Fröhlich B, Riederer M, Tautz J (2000b) Comb-wax discrimination by honeybees tested with the proboscis extension reflex. J Exp Biol 203:1581–1587
Hamilton RJ (ed) (1995) Waxes: chemistry, molecular biology and functions. The Oily Press, Dundee
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer-Verlag, Berlin
Hepburn HR (1994) Reproductive cycling and hierarchical competition in Cape honeybees, *Apis mellifera capensis* Esch. Apidologie 25:38–48
Hepburn HR (1998) Reciprocal interactions between honeybees and combs in the integration of some colony functions in *Apis mellifera* L. Apidologie 29:47–66
Hepburn HR (2010) Wax discrimination in the Lesser Honeyguide, *Indicator minor*. Ostrich 80:119–120
Hepburn HR (2011) Absconding, migration and swarming. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 133–158
Hepburn HR, Muller WJ (1988) Wax secretion in honeybees: thermal integrity of the festoon. Naturwissenschaften 75:628–629
Hepburn HR, Radloff SE (1998) Honeybees of Africa. Springer, Berlin
Hepburn HR, Whiffler LA (1991) Construction defects define pattern and method in comb-building by honeybees. Apidologie 22:381–388
Hepburn HR, Duangphakdee O, Phiancharoen M, Radloff SE (2010) Comb wax salvage by the red dwarf honeybee, *Apis florea* F. J Insect Behav 23:159–164
Hepburn HR, Hugo J, Mitchell D, Nijland MJM, Scrimgeour A (1984) On the energetic costs of wax production by the African honeybee, *Apis mellifera adansonii* (= *scutellata*). S Afr J Sci 80:363–368

Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax in honeybees. Apidologie 22:21–36
Hepburn HR, Radloff SE, Duangphakdee O, Phiancharoen M (2009) Interspecific utilisation of wax in comb-building by honeybees. Naturwissenschaften 96:719–723
Hepburn HR, Pirk CWW, Radloff SE (2011) Energetic aspects of flight. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 293–312
Jenkins MJ, Sneyd J, Camazine S, Murray JD (1992) On a simplified model for pattern formation in honey bee colonies. J Math Biol 30:281–306
Koeniger N, Koeniger G, Tingek S, Kelitu A (1996) Interspecific rearing and acceptance of queens between *Apis cerana* Fabricius, 1793 and *Apis koschevnikovi* Buttel-Reepen, 1906. Apidologie 27:371–380
Kolattukudy PE (1976) Chemistry and biochemistry of natural waxes. Elsevier, Amsterdam
Lindauer M (1956) Communication among the honeybees and stingless bees of India. Z Vergl Physiol 38:521–557 [in German]
Moritz RFA, Latorff HMG, Crous KL, Hepburn HR (2011) Social parasitism of queens and workers in the Cape honeybee (*Apis mellifera capensis*). Behav Ecol Sociobiol 65:735–740
Nanork P, Wongsiri S, Lim J, Gloag RS, Oldroyd BP (2006) The reproductive dilemmas of queenless red dwarf honeybee (*Apis florea*) workers. Behav Ecol Sociobiol 61:91–97
Oldroyd BP, Wongsiri S (2006) Asian honey bees: biology, conservation and human interactions. Harvard University Press, Cambridge
Oschmann H (1965) Can *Apis mellifica* bees rear brood of *A. cerana*? Die Biene, Giessen 101:150–151 [in German]
Pirk CWW, Hepburn HR, Radloff SE, Tautz J (2004) Honeybee combs: construction through a liquid equilibrium process? Naturwissenschaften 91:350–353
Pirk CWW, Crous KL, Duangphakdee O, Radloff SE, Hepburn HR (2011) Economics of comb wax salvage by the red dwarf honeybee, *Apis florea*. J Comp Physiol 181:353–359
Potichot S, Wongsiri S, Dietz A (1993) Attempts in queen rearing of *Apis cerana* larvae in *Apis mellifera* colonies and *Apis mellifera* larvae in *Apis cerana* colonies. In: Connor LJ, Rinderer TE, Sylvester HA, Wongsiri S (eds) Asian apiculture. Wicwas Press, Cheshire, pp 128–133
Pratt SC (2004) Collective control of the timing and type of comb construction by honey bees (*Apis mellifera*). Apidologie 35:193–205
Raffiudin R, Crozier RH (2007) Phylogenetic analysis of honey bee behavioral evolution. Mol Phylogenet Evol 43:543–552
Rinderer TE, Oldroyd BP, de Guzman LI, Wattanachaiyingcharoen W, Wongsiri S (2002) Spatial distribution of the dwarf honey bees in an agroecosystem in southeastern Thailand. Apidologie 33:539–543
Rösch GA (1927) Untersuchungen über die Arbeitsteilung und das Alter der Baubienen. Weiterer Bautrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 12:1–71
Roubik DW (1992) Ecology and natural history of tropical bees. Cambridge University Press, Cambridge
Ruttner F (1988) Biogeography and taxonomy of honeybees. Springer, New York
Ruttner F, Maul V (1983) Experimental analysis of reproductive interspecies isolation of *Apis mellifera* L and *Apis cerana Fabr*. Apidologie 14:309–327
Sackin BM (1998) Honey bee repellent composition comprising tea tree oil. US Patent 5738863
Sasaki M, Fujiwara M, Numaguchi Y (2000) Proboscis extension reflex (PER) for bee odour and non-volatile surface wax components: analysis of nestmate discrimination. In: Proceedings of 4th Asian Apicultural Association Conference, Kathmandu, p 37
Seeley TD (1995) The wisdom of hive: the social physiology of honey bee colonies. Harvard University Press, London
Su S, Cai F, Si A, Zhang S, Tautz J (2008) East learns from west: Asiatic honeybees can understand dance language of European honeybees. PLoS ONE 3:e2365. doi:10.1371/journal.pone.0002365
Tan K, Hepburn HR, He S, Radloff SE, Neumann P, Fang X (2006) Gigantism in honeybees: *Apis cerana* queens reared in mixed-species colonies. Naturwissenschaften 93:315–320

Tan K, Yang MX, Radloff SE, Hepburn HR, Zhang ZY, Luo LJ, Li H (2008) Dancing to different tunes: heterospecific deciphering of the honeybee waggle dance. Naturwissenschaften 95:1165–1168

Tan K, Yang MX, Radloff SE, Pirk CWW, Crewe RM, Phiancharoen M, Hepburn HR, Oldroyd BP (2009) Worker reproduction in mixed-species colonies of honey bees. J Behav Ecol 20:1106–1110

Tan K, Yang MX, Wang ZW, Li H, Zhang ZY, Radloff SE, Hepburn HR (2010) Cooperative wasp-killing by mixed-species colonies of honeybees, *Apis cerana* and *Apis mellifera*. Apidologie 43:195–200

Underwood BA (1992) Notes on the orange-rumped honeyguide *Indicator xanthonotus* and its association with the Himalayan honeybee *Apis laboriosa*. J Bombay Nat Hist Soc 89:290–295

Via S (2001) Sympatric speciation in animals: the ugly duckling grows up. Trends Ecol Evol 16:381–390

Wattanachaiyingcharoen W, Wongsiri S, Oldroyd BP (2008) Aggregations of unrelated *Apis florea* colonies. Apidologie 39:531–536

Wilde J, Woyke J, Wilde M (2001) Rearing honey bee queens on cups of *Apis mellifera* and *Apis dorsata* wax. In: Proceedings of 7th Asian Apic Assoc Conf, Chiang Mai, pp 207–209

Wilson EO (1971) The insect societies. Belknap Press, Harvard University Press, Cambridge

Winston ML (1987) The biology of the honey bee. Harvard University Press, London

Wongsiri S, Lekprayoon C, Thapa R, Thirakupt TE (1997) Comparative biology of *Apis andreniformis* and *Apis florea* in Thailand. Bee Wld 78:23–35

Yang MX, Tan K, Radloff SE, Pirk CWW, Hepburn HR (2010a) Hetero-specific queen retinue behavior of worker bees in mixed-species colonies of *Apis cerana* and *Apis mellifera*. Apidologie 41:54–61

Yang MX, Wang ZW, Li H, Zhang ZY, Tan K, Radloff SE, Hepburn HR (2010b) Thermoregulation in mixed-species colonies of honeybees (*Apis cerana* and *Apis mellifera*). J Insect Physiol 56:706–709

Yang MX, Hepburn HR, Duangphakdee O, Phiancharoen M (2010c) Comb construction in mixed-species colonies of honeybees, *Apis cerana* and *Apis mellifera*. J Exp Biol 213:1659–1664

Chapter 5
Communication by Vibrations and Scents in the Comb

Abstract Communication on honeybee combs includes both distance and direction in the case of waggle dances. Potential recruits attending a dancer emit vibrations which elicit a response from the dancer to give the emitter a sample of nectar. Tooting and quacking by queens are both airborne sounds and substrate vibrations which are carried mainly by the fundamental frequency component. Bees recognize these signals mainly by their temporal structure and comparisons of the threshold, emission level, and attenuation with distance, which suggests that they are used only within a restricted area of the comb. When waggle-dancing honeybees move on comb, they produce vibratory movements that indicate the location of the waggle dancer and the pulsed vibrations are increased during waggle phases, so amplifying the signals for remote dance followers. Because sound intensity decreases with the density of the medium and with distance, beeswax is a medium for sound transmission. Pheromones in comb serve as slow-release systems with long time constants and include transmissions of colony odour, queenrightness, cell capping, colony odour, kin recognition, footprint pheromones, wax-salvaging behaviour etc. The specific dance sites that occur on combs are due to chemical tagging. Masking colony odour occurs when receiver bees are conditioned to the same comb source as introduced bees, which are accepted. A series of only a few methyl esters produced by queens and workers are sufficient to induce capping of mature brood; but capping worker brood may depend on the depth of larvae in comb cells and not just ratios of ester emissions. Nonetheless, these results are not mutually exclusive in principle.

5.1 Introduction

The buzzing of honeybees has accompanied mankind through the ages and even highly-specialised sounds such as the piping of queens were known since ancient times (Free 1982; Crane 1999). Yet, formal studies on sounds produced by honeybees were first recorded only in 1609 by Charles Butler, polymath of his times,

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_5,

who transcribed the first musical notation of the piping of honeybee queens (Pirk et al. 2013). Some 300 years later, the morphology of the auditory senses of honeybees was described (Snodgrass 1910; McIndoo 1922). Subsequently, Hansson (1945) reported that honeybee sounds, inaudible to humans, can indeed be recorded with high amplification, following which, Wenner (1962a) observed that workers emit pulsed sounds of about 200 Hz during the waggle phase of the waggle dance. Wenner (1962b) also demonstrated that a caged honeybee queen, installed in an observation hive already containing a virgin queen, piped in response to artificial piping, which was played to it through the substrate. Then, Simpson (1964) showed that honeybee queens breathed continuously while piping, and were able to pipe with all spiracles except one blocked, so the sound could not have been produced by air entering or leaving the spiracles. He concluded that it is produced by operating the flight motor without spreading the wings, and sound is radiated partly by the substratum, to which the vibrations are communicated by pressing the thorax against it.

Charles Butler's work was more than two decades before any real headway into the acoustical analysis of sound, which only began in 1636, when Mersenne formulated principles, now laws, which mathematically described the frequencies of oscillations of stretched strings. (These laws still apply in the construction of pianos, harps etc.). Incidentally, this extraordinary man associated with Descartes, Pascal, de Roberval and Fabri de Peiresc in his day, all of whom worked before the publication of the first journals of the Royal Society in London and the Académie des Sciences in Paris (Bernstein 1996).

Returning to honeybees, sound reception finally took a quantum leap with the introduction of laser Doppler vibrometry in the last half century. In this short review, we trace the experimental development of honeybee communication using the comb as a substrate. Communication of sounds, signals or cues arising from honeybee combs include mechanical as well as chemical information. Indeed, following the discovery of dance language, its mode of actual transmission has long remained enigmatic (von Frisch 1967; Michelsen 2012). However, when potential honeybee recruits attend a dancing forager, they periodically emit vibrations against the combs which elicit a response from the dancer to give the emitter a sample of nectar (Michelsen et al. 1986a; Sandeman et al. 1996). When honeybee foragers perform waggle dances, they transmit both distance and direction as vibrations that are transmitted through the wax substrate of their combs.

The roles of pheromones and volatile odours are of extraordinary importance in the combs of bees where they mainly serve as reservoirs and slow-release systems. These encompass cell capping, colony odour, kin recognition, footprint pheromones, wax-salvaging behaviour etc. Of these, probably the oldest known of the systems of honeybee scents is that of colony odour, which was noted from at least since the 19th century (Bethe 1898) and has been more recently summarised in a paper by Kalmus and Ribbands (1952). Further contributions to the identification of nestmate recognition cues were demonstrated by Juska (1978); that queens secrete a substance from their tarsal glands (footprint pheromones) and as they

move about, they effectively tag regions of the comb which convey information that the colony is queenright. This is a parsimonious way to propagate a general, integrative signal. The real biological significance is that footprint pheromones inhibit the construction of queen cells at the bottom of the combs. The idea of 'colony odour' in honeybees has been developed particularly by Breed et al. (1988a, et seq.) who showed that colony odour is soon acquired by newly emerged worker bees. Some of the recognition cues are of genetic origin and therefore acquired prior to adult emergence. Nonetheless, genetic or relatedness differences between bees of different colonies can be completely masked as shown in cross-fostering experiments. Breed et al. (1988b) concluded that previous results showing that worker honeybees are recognized on the basis of environmentally acquired odours (= colony odour), do not actually contradict their results. Rather, bees probably use whatever cues are available for recognition. When environmental odour sources are carefully controlled, cues remain that allow for discrimination of nestmate versus non-nestmate individuals.

5.2 Vibrations

Not long after Hansson (1945) reported that honeybee sounds, inaudible to humans, can be recorded with high amplification, Wenner (1962a) observed that worker honeybees emit pulsed sounds of about 200 Hz during the straight run segment of the waggle dance. He then suggested that these pulsed sounds could well be the means by which the distance component is conveyed to other honeybee workers. He then demonstrated that both the sound production time and the number of pulses in the straight run phase of the waggle dance are also capable of carrying distance information. Since the ratio of the sound pulse rate to waggle rate is approximately 2.5:1, the sound is not an incidental result of the waggling of the abdomen by the dancing bees (Wenner 1962a). The straight run time and number of sound pulses present in the straight run portion of the waggle dance were found to be inseparable on the basis of available data. Either of these two components was found to be a better possibility for transmitting distance information than any of the other components of the dance (Wenner 1962a). He further concluded that in the waggle dances: (a) the time of waggling during the straight run; (b) the number of waggles produced during the straight run; (c) the time of sound production during the straight run, or, (d) the number of sound pulses produced during the straight run are the most likely possibilities for conveying information about distance of food sources from the nest.

Similar vibrations, the tremble or stop dance, are made by returning foragers who have difficulty in off-loading nectar to house bees (Kirchner et al. 1988, 2003). The signal here may be a means of negative feedback, reducing further forager recruitment (Seeley 1992; Kirchner 1993; Nieh 1993) and/or as a means of recruiting more nectar-receiver bees (Seeley 1992). Finally, queen piping is mediated through vibration of the combs (Michelsen et al. 1986b); this, together

with the wing vibrations of dancers, is perceived by the Johnston's organ of the workers. Other bee-comb interactions of a reciprocal nature involve the transmission of vibrations (sounds) for communication.

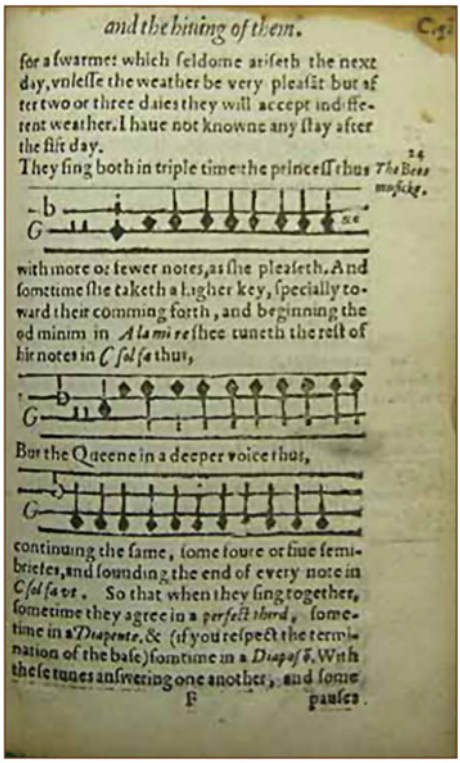
One of the events that occur among bees attending a dancing forager is that periodically a worker will press her thorax against the comb and vibrate. This often elicits a response from the dancer to give the emitter a sample of nectar (Michelsen et al. 1986a). The tremble or stop dance works acoustically in the same way (Kirchner 1993). A third example is that of queen piping, in which the quacking element emitted by virgin queens still in their cells is transmitted through the wax (Michelsen et al. 1986b). Indeed Wenner (1962b) was able to show that a caged honeybee queen installed in an observation hive which already contained a virgin queen, piped in response to artificial piping which was played to it through the substrate.

5.2.1 Queen Honeybees

As far as we can determine, the first attempts to record the piping (tooting and quacking) sounds of *A. mellifera* queen honeybees were those of Charles Butler, who transcribed these sounds into musical notation (Figs. 5.1 and 5.2). Charles Butler wrote of the swarming of bees and noted that sounds of 'piping' occur 7 to 11 days after swarming, which may be followed by another swarm (Butler 1609). Piping is a sound composed of two different signals made by queen bees; namely 'tooting' and 'quacking' (Michelsen et al. 1986c). A queen bee recently emerged from her cell will toot and this is followed by the quacking signal made by queens still confined in their cells (Michelsen et al. 1986a).

Butler (1609) transcribed these sounds onto a treble clef musical score, denoting high pitch, and noted that the most common result of tooting and quacking was the production of the musical harmonies of a major third or perfect fifth (Figs. 5.1 and 5.2). A major third is equal to an increase of four semitones from the starting note, while a perfect fifth is equal to an increase of seven semitones from the starting note—this is equal to four or seven frets on a guitar, respectively. This, Butler determined only by ear. Furthermore, he transcribed the sound in triple time in the tradition of the musical culture in the 17th century; all music was written in triple time as a testament to the Christian belief in a Holy Trinity (Stanford and Forsyth 1937).

The extraordinary musical notation provided by Butler (1609, 1634) finally became tested and verified some three and a half centuries later in the acoustical measurements of *A. mellifera* by Michelsen et al. (1986c). They examined the sonic structure of the piping sound and noted that tooting syllables begin at a frequency of 340 Hz and increase to 500 Hz, while the quacking (responsive) syllables remain relatively constant at 300 Hz. Using the quacking syllable (300 Hz) as a basis, the major third and perfect fifth harmonies for this syllable are 377.976 and 449.492 Hz respectively. This falls within the frequency range for


and the hining of them. Cap.

for a ſwarme: which ſeldome ariſeth the next day, vnleſſe the weather be very pleaſit but after two or three daies they will accept indifferent weather. I haue not knowne any ſtay after the firſt day.

They ſing both in triple time: the princeſſ thus 24 *The Bees muſicke.*

with more or fewer notes, as ſhe pleaſeth. And ſometime ſhe taketh a higher key, ſpecially toward their comming forth, and beginning the 2d minim in *A la mi re* ſhee tuneth the reſt of hir notes in *C ſol fa* thus,

But the Queene in a deeper voice thus,

continuing the ſame, ſome foure or fiue ſemibreues, and ſounding the end of every note in *C ſol fa vt*. So that when they ſing together, ſometime they agree in a *perfect third*, ſometime in a *Diapente*, & (if you reſpect the termination of the baſe) ſometime in a *Diapaſon*. With theſe tunes anſwering one another, and ſome

F pauſes.

Fig. 5.1 Musical score of an *A. mellifera* queen "shows a four line staff with the letter G on the second line from the *bottom* indicating that this is a treble clef. There are no bar lines but the two semibreve rests at the beginning of the staves indicate that we are in a triple metre, and indeed the text states that the bees 'sing' in triple time. The notation indicates that the two most common results of the simultaneous piping and quacking of rival queens are the musical intervals of either a perfect fifth or a major third"… "Quacking is the responsive sound of rival queens who have not yet emerged from their cells, and piping is the regal identification of a virgin queen soon after she has emerged from the cell in which she developed" (Butler 1609)—Figure and text commentary, the National Library of Scotland

tooting syllables of 340 to 500 Hz observed by Michelsen et al. (1986a). Furthermore, this range of fundamental frequencies corresponds to the pitch used by alto singers (147–659 Hz—Bozhidar 2006), thus supporting Butler's (1609) use of the treble clef in his transcription.

Michelsen et al. (1986a) recorded and measured tooting and quacking signals emitted by the queens as airborne sound and as substrate vibrations of the combs by means of a microphone and a laser vibrometer, respectively. The fundamental frequency component is larger than the harmonics when the signals are measured as vibration velocity, and they argued that the signals are carried mainly by the fundamental frequency component. The frequencies emitted depend on the queens' age, and the tooting syllables contain a frequency sweep. The fundamental

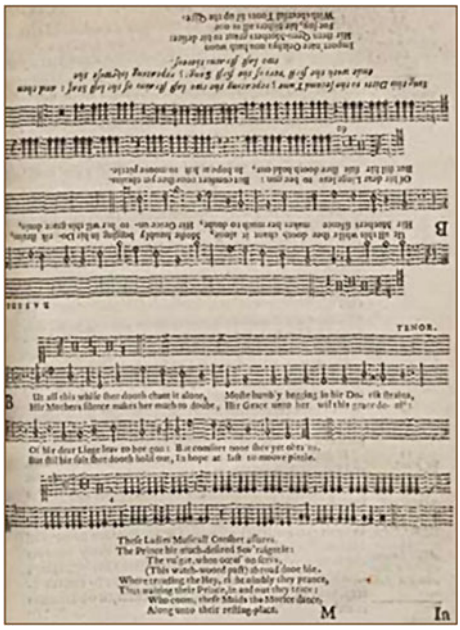


Fig. 5.2 Butler's (1634) edition presents a two-part madrigal for four voices incorporating melodic elements based on the actual sounds produced by *A. mellifera* bees. The music has been printed in the manner of a part-book to be read by the Mean (soprano) and Tenor sitting on one side of a table and by the Bassus and Countertenor facing them on the other side (Butler 1634)—Figure and text commentary, the National Library of Scotland

carrier frequencies of the toots and quacks overlap, but the tooting syllables have longer rise times than the quacking syllables.

Recordings of the vibrations of cells in which queens were confined allowed Michelsen et al. (1986a) to measure the threshold for the release of quacking in these queens evoked by artificial toots and by natural toots from emerged queens. Artificial toots with a long syllable rise time are more effective in releasing quacking responses than toots with a short syllable rise time. These observations suggest that the bees recognize these signals mainly by their temporal structure (Michelsen et al. 1986a). A comparison of the threshold, emission level, and attenuation with distance, suggests that these and other vibration signals are only used by honeybees for local communication within a restricted area of the comb. Therefore, we can conclude that, while not precisely correct, Butler's (1609) musical transcription three centuries ago on the piping sounds produced by rival queen bees was certainly very accurate (Pirk et al. 2013).

In the experiments of Michelsen et al. (1986b) the airborne sound emitted from the combs and the vibration velocity of the comb surfaces were recorded with a microphone and a laser vibrometer as shown in Fig. 5.3. The laser light was focused at a spot of highly reflective paint on the comb. Comb vibrations in the direction of the laser beam caused Doppler shifts in the frequency of the reflected light, and the output voltage of the instrument is linearly related to the instantaneous vibration velocity. The recorded sound and vibration signals were stored on an instrumentation tape recorder and on the disc drive of a computer which allowed a detailed control of the functions and internal memories of a frequency analyser. Graphs of

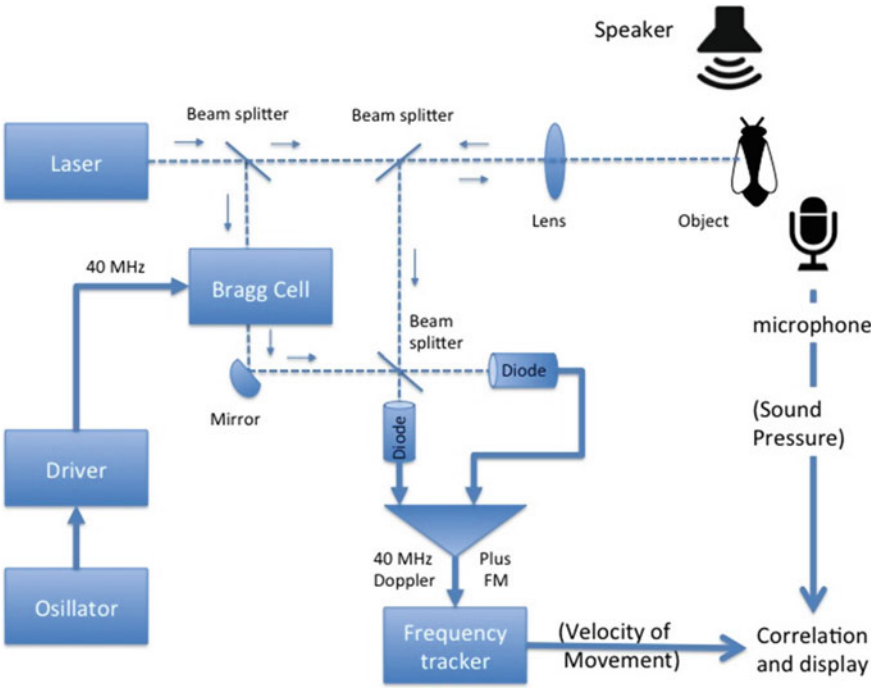


Fig. 5.3 Experimental set-up for measuring *A. mellifera* queen sounds by (Michelsen et al. 1986b), after Michelsen and Larsen (1978), for explanation see text

the signals were also made by transferring time samples from the memory of a digital oscilloscope through the computer to a plotter (Michelsen et al. 1986b).

When the signals were measured as vibration velocity, the fundamental frequency component was larger than the harmonics, so they concluded that the communication signals are mainly carried by the fundamental frequency component. The frequencies emitted varied with the ages of the queens (Fig. 5.4), and the tooting syllables contained a frequency sweep, which probably explain previous, diverse frequency values reported in the literature (Figs. 5.5 and 5.6).

5.2.2 Worker Vibrations

Considerable further progress in understanding the significance of the airborne elements associated with the waggle dance has been reported (Michelsen et al. 1987, 1988, 1992). More recently, these findings have been extended by Nieh and Tautz (2000) who noted that waggle dancing honeybees produce vibratory movements that may facilitate communication by indicating the location of the waggle dancer. Previously, an important component of these vibrations had not

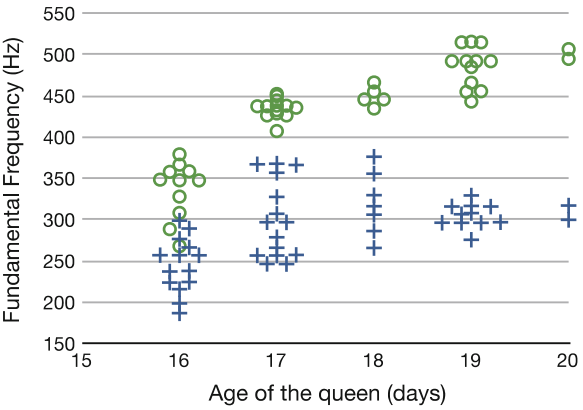


Fig. 5.4 Relationship between queen age and fundamental frequencies of tooting (o) and quacking (+) in *A. mellifera*. The frequency of tooting was computed from the last section of syllables (Michelsen et al. 1986a)

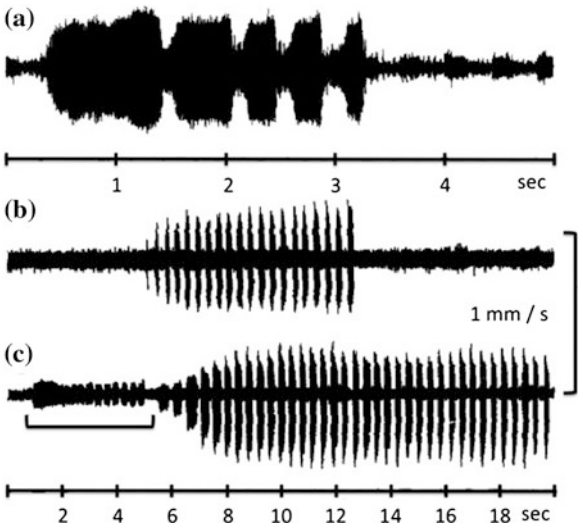


Fig. 5.5 Tooting and quacking recorded as vibration velocity on the comb surface in *A. mellifera*. **a** toot, **b** quack, **c** a quack released by a toot (*black bar*). Note the different time structures of the quacks in (**b**) and (**c**). The *upper* time scale applies only to (**a**), the *lower* to (**b**) and (**c**) (Michelsen et al. 1986a)

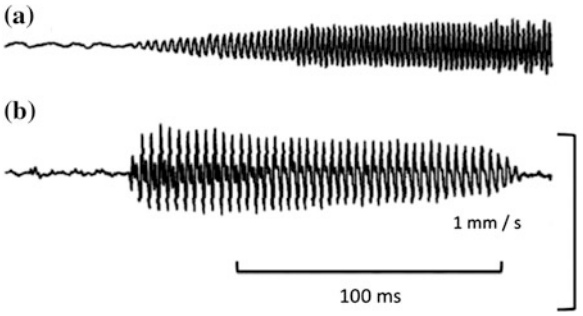


Fig. 5.6 Fine structure of tooting (**a**) and quacking (**b**) syllables in *A. mellifera*. **a** At the beginning of each tooting syllable the frequency increases. **b** Frequency is nearly constant in the quacking syllables. Note different rise times between (**a**) and (**b**) (Michelsen et al. 1986a)

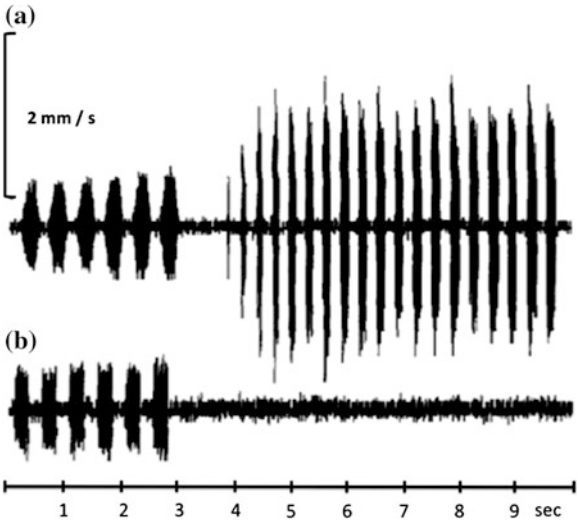


Fig. 5.7 Artificial tooting (450 Hz vibration of comb) releases quacking when the rise and delay is 100 ms (**a**) but not when it is 10 ms (**b**). Recordings were made from the surface of a confined *A. mellifera* queen's cell (Michelsen et al. 1986a)

been detected in the comb. They developed a highly sophisticated method of fine-scale behavioural analysis that allowed them to analyse separately the comb vibrations near a honeybee waggle dancer during the waggle and the return phases of her dance (Figs. 5.7 and 5.8).

Nieh and Tautz (2000) simultaneously recorded honeybee waggle dances using digital video and laser-Doppler vibrometry, and performed a behaviour-locked Fast Fourier Transform analysis on the vibrations in the substrate comb. Nieh and Tautz (2000) discovered significantly higher amplitude, 200–300 Hz, vibrations during the waggle phase than during the return phase, but no significant differences in the neighbouring frequency regions between 100–200 Hz and 300–400 Hz. They recorded peak waggle phase vibrations from 206 to 292 Hz. The maximum measured signal–noise level was +12.4 dB during the waggle phase (mean +5.8 ± 2.7 dB). The maximum vibrational velocity, calculated from a filtered signal, was 128 μm s^{-1} peak-to-peak, corresponding to a displacement of 0.09 μm s^{-1} peak-to-peak at 223 Hz. On average, they measured a vibrational velocity of 79 ± 28 μm s^{-2}) peak-to-peak from filtered signals. These signal amplitudes overlap with the detection threshold of the honeybee (Fig. 5.9).

Tautz et al. (2001) noted that *A. mellifera* foragers performing dances on the combs are apparently able to attract dance-followers from distances across the combs that are too remote for tactile or visual cues to play a role. An alternative signal could be the vibrations of the comb at 200–300 Hz generated by dancing bees but which, without amplification, may not be large enough to alert remote dance followers back to where the signal originated (Fig. 5.10). The phase reversal occurs across walls 2 and 3, walls 2b and 3b, walls and 2c and 3c (Tautz et al. 2001). Tautz et al. (1996) reported an unusual behaviour of waggle dancers in that they actually stride across the comb, which the authors interpreted as a mechanical means of increasing the pulsed vibrations that occur during waggle phases.

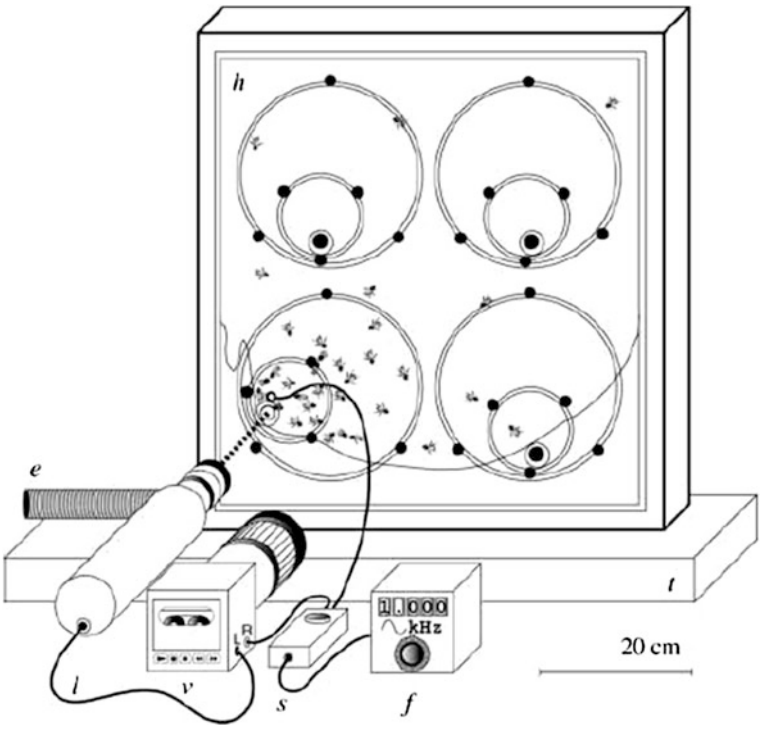


Fig. 5.8 Apparatus for measuring waggle dance vibrations by *A. mellifera* workers: (*h*) observation hive, (*e*) hive entrance, (*l*) laser vibrometer, (*v*) digital video camera, (*s*) switch for passing a 1 kHz sound pulse to the video camera and simultaneously illuminating a light-emitting diode, (*f*) function generator, and (*t*) vibration dampening table. The laser vibrometer head was mounted on the same vibration-dampening table (Nieh and Tautz 2000)

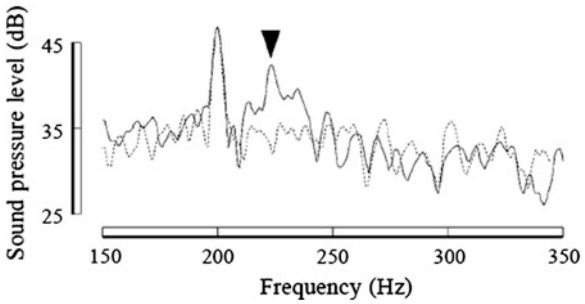


Fig. 5.9 Fast Fourier Transform spectra of comb vibrations produced by an *A. mellifera* waggle dancer 1 cm from the edge of the comb. Waggle phase spectrum, *solid line*; return phase spectrum, *dotted line*. The laser was positioned at 30°, 18 mm away from waggle dancer. *Arrow* indicates peak waggle dance frequency between 200 and 300 Hz. The 200 Hz is an equipment-generated frequency peak used to calibrate signal levels (Nieh and Tautz 2000)

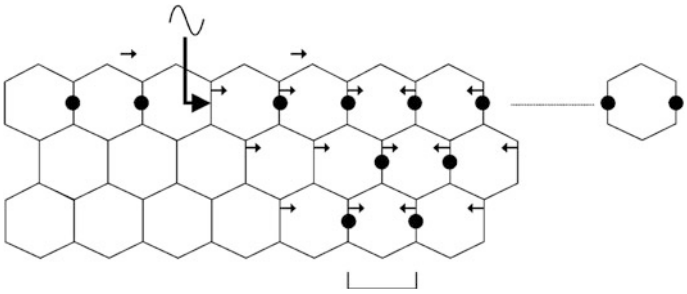


Fig. 5.10 Diagram on *A. mellifera* comb of showing the location of the stimulus probe and the three rows of cell walls from which measurements were taken. The cell rows are oriented along the same horizontal axis as they would be in the hive. The *large arrow* shows the point of application of the lateral sinusoidal displacement to the top rim of a cell wall. The power stroke of the stimulus is the direction of the arrow, thus 'pulling' the cell walls on the *left* and 'pushing' the cell walls on the *right*. The return stroke of the probe allows the comb to move. No phase reversals were found to occur across cells on the 'pull' side of the stimulus (Tautz et al. 2001)

Subsequently, Tautz et al. (2001) reported an equally unexpected property of the comb when subjected to vibrations of about 200 Hz in that it effectively amplifies vibratory signals to remote dance followers. They found that at a specific distance from the origin of an imposed vibration, the walls across a single comb cell abruptly reverse the phase of their displacement and move in opposite directions to one another.

Behavioural measurements showed that the distance from which the majority of remote dance followers are recruited coincides with the location of this phase-reversal phenomenon relative to the signal source. They reasonably proposed that effective amplification of the signal by a phase-reversal phenomenon occurs when bees straddle a cell across which the phase reversal is expressed. Such a bee would be subjected to a situation in which the legs were moving towards and away from one another instead of in the same direction. In this manner, remote dance followers could be alerted to a dancer performing in their vicinity (Fig. 5.11). (As an aside it is worth noting that Seeley et al. (2005) recently tested plastic combs and beeswax foundation and found the former markedly poorer in transmitting the 250 Hz vibrations produced by dancing bees; but, nevertheless comb built with plastic foundation proved a suitable substrate for waggle dance communication).

The possible roles of vibrations in the economy of honeybee colonies also include the recent proposal by Bergman and Ishay (2007) that social hornets and honeybees exploit ultrasonic acoustic resonance properties of cells to achieve accurate structures in combs; however, their experimental data do not extend to honeybee combs. It is evident that the analyses of the vibrations and sound signals of dancing bees are highly variable, so that from this perspective, dancers are only providing a rough indication as to where the goal is situated.

Using and entirely different approach, Tsujiuchi et al. (2007) investigated the pedicel of the antenna to determine the mechanical and neural response characteristics of antennae and Johnston's organ to acoustic stimuli. Their results

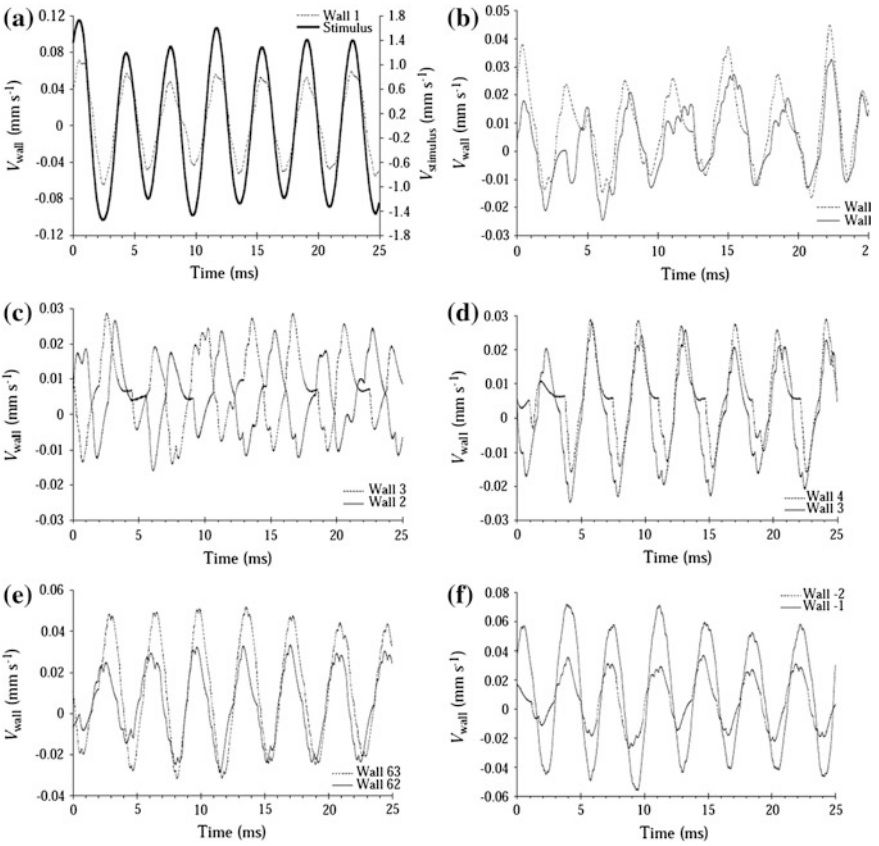


Fig. 5.11 Comparisons of *A. mellifera* combs measured for displacement velocities—Vwall of cell walls at different distances from the stimulus. **a** Time course of the stimulus and movement of the wall opposite (Wall 1). There is no phase lag but the wave form is already distorted. Vstimulus = velocity of stimulus. **b** A sequence of Wall 1 excursions compared with those of Wall 2. The displacement of Wall 2 (*dotted line*) exhibits a small phase lead over that of Wall 1 (*solid line*). **c** The small phase lead of Wall 2 over Wall 1 advances suddenly so that the displacements of Walls 2 and 3 are about 180° out of phase. **d** One cell further along the line from the stimulus (Walls 3 and 4) the cell wall displacements are again in phase with one another. **e** The small phase lag between Walls 62 and 63 is introduced by the finite conduction velocity of the signal across the comb. **f** Displacement velocities in the "pull" direction between Wall-1 and -2. No phase reversal was found in this direction at any distance from the stimulus (Tautz et al. 2001)

indicated that the neurons in the antennae and Johnston's organ of mature honeybees are best attuned to detect 250–300 Hz sounds generated from a distance during waggle dances. Furthermore, the Johnston's organ neurons can preserve both frequency and temporal information of acoustic stimuli including the sounds generated during waggle dances.

Because Tsujiuchi et al. (2007) found that the responses of the Johnston's organ neurons were found to be age-dependent they concluded that dance communication is only possible between aged foragers. The waggle dances of honeybees roughly encode the distance and direction to a food source in the duration and the body angle during the waggle phase. The recent results of measurements by Ai and Hagio (2013) showed that there is indeed a neurophysiological connection between the cervical setae of a waggle dancer, via three thoracic ganglia that feeds back to Johnston's organ, providing independent evidence supporting the interpretations of Tsujiuchi et al. (2007).

Michelsen (2012) summarized his thoughts in a recent essay on how honeybees obtain information about direction by following dances. Several sensory modalities including touch, vision, hearing, substrate vibrations and air flow have been proposed and experimentally analyzed to establish their possible roles in this regard. Michelsen himself invested a quarter of a century in studying the ways in which sounds and air flow generated by dancing bees could influence other worker bees. When foragers dance, they vibrate their wings and act as dipoles and the unexpectedly large sound pressures and air flow that they generate decreases rapidly with distance from their source, clearly reducing their range in effective communication.

In an earlier study, Michelsen (2003) produced a robotic honeybee dancer that could recruit foragers to certain positions in the field, but it was less effective in attracting recruits than normal bees, probably because the oscillating air flow caused by the wings' vibrations and wagging movements are too complicated to transmit the information. Subsequent measurements with lasers and anemometers showed that the vibrating wings cause an air jet behind the dancer's abdomen which is in a narrow plane, parallel to the comb, and might convey information about direction to bees behind the dancer. He also reported that the narrow air jets may co-occur with a broad flow of air, which seems ideally suited for transporting dance pheromones. A particularly telling point here is that both narrow and broad flows can be switched on and off by the dancer.

There are many other isolated instances of vibrations emitted by workers, the significance of which is not entirely clear. Among these interesting cases are observations on sound production as a means of defense in *Apis cerana* (Koeniger and Fuchs 1972; Fuchs and Koeniger 1974), dorso-ventral abdominal vibrations among *A. mellifera* honeybees Fletcher (1975), vibratory activities of successful *A. mellifera* foragers (Schneider 1986), and studies of vibration signals as a form of modulatory communication in the vibratory activities of *A. mellifera* honeybee colonies (Hyland et al. 2007).

There are also some observations on worker piping to relate. While worker piping had previously been associated with queenlessness and disturbances to colonies, Pratt et al. (1996) reported that *A. mellifera* workers pipe by pressing the thorax to the comb, spreading the wings slightly and lifting the abdomen towards the wings, which vibrated noticeably as the bee emitted an audible wail. Pipers wandered throughout the hive for up to 2.5 h, stopping every few seconds to pipe, which lasted about 1 s. The sound showed little frequency modulation, and a

fundamental frequency of 330–430 Hz. It appeared to be produced by wing muscle vibrations and to be transferred onto the comb by pressing the thorax against the comb. Piping in this context may serve as a foraging-related signal, although its receivers and the information it transmits remain unknown.

An equally interesting set of observations are those of Sen Sarma (2002), who described a novel defence response by *A. florea* in which the emission of an initial warning signal from one individual ('piping') is followed by a general response from a large number of bees ('hissing'). Piping is audible to the human ear, with a fundamental frequency of 384 ± 31 Hz and lasting for 0.82 ± 0.35 s. Hissing is a clearly audible, broad-band, noisy signal, produced by slight but visible movements of the bees' wings. Hissing begins in individuals close to the piping bee, spreads rapidly to neighbours and results in an impressive coordinated crescendo occasionally involving the entire colony. Piping and hissing are accompanied by a marked decrease, or even cessation, of worker activities, such as forager dancing and departures from the colony. Whereas hissing of the colony can be elicited without piping, the sequential and correlated piping and hissing response is specific to the presence of potential predators close to the colony (Sen Sarma 2002). It is suggested that the combined audio-visual effect of the hissing might deter small predators, while the cessation of flight activity could decrease the risk of predation by birds and insects which prey selectively on flying bees.

These observations complement those of Kastberger et al. (2013) who recently showed that the giant honeybees, *A. dorsata,* utilize a 'Mexican wave-like' shimmering behaviour in coordinated cascades across the nest surface. While the time–space properties of these emergent waves are response patterns which have become of adaptive significance for repelling enemies visually. They further showed that the mechanical impulse of these patterns, measured by laser Doppler vibrometry, generate vibrations at the central comb of the nest at a natural frequency of 2.156 ± 0.042 Hz, which is twice the average repetition rate of the shimmering waves. Kastberger et al. (2013) then analyzed the Fourier spectra of the comb vibrations and proposed two possible explanations for the compound physical system of the bee nest: (1) in an 'elastic oscillatory plate model', the comb vibrations set off supra-threshold cues to other bees situated close to the comb; or (2) in the 'mechanical pendulum model', the comb vibrations are sensed by bees throughout the whole curtain enabling mechanoreceptive signalling across the nest and also through the comb itself.

The results of Kastberger et al. (2013) showed that weak forces, (general quiescence or diffuse mass flight activity) cause a harmonic frequency spectrum of the comb, driving the comb as an elastic plate. However, the shimmering waves provide sufficiently strong forces to move the nest as a mechanical pendulum. Finally Kastberger et al. (2013) concluded that this vibratory behaviour may support the colony-intrinsic information hypothesis, that the mechanical vibrations of the comb provoked by shimmering have the potential to facilitate immediate communication of the momentary defensive state of the honeybee nest to the

majority of its members. In closing this section, it seems reasonable to assume that other instances of communication by vibrations will be discovered in other Asian species of honeybees (Kirchner 1993).

5.3 Scents

In all of the above examples, transmission of vibrations through a medium depends on the density, elasticity and temperature of the substrate. For practical purposes, temperature and density can be taken as constants in the honeybee nest. Because elasticity is not a constant means that the greater the stiffness, the higher the speed of transmission. Because sound intensity decreases with the density of the medium and with distance, an essentially anhydrous, low density wax is an ideal solid biological medium for sound transmission. Kirchner (1993) indicated some important differences in communication by sounds and pheromones. Vibrational signals allow for the speedier transmission of a signal, a temporal coding element, and localization of the message through rapid attenuation of the signal. Pheromones and other chemical signals are pervasive in distribution and, because they follow the laws of diffusion, there is a long time constant for the life of the signal (Kirchner 1993). Perfect examples of these effects lie in colony odour and intranest transmission of queenrightness.

A classic illustration of this paradigm was exemplified by Seeley (1979) who showed that when worker bees come into contact with the queen they acquire queen substance which is subsequently transferred to other workers. The queen facilitates dispersal of the pheromone by remaining stationary, during which time workers come into close contact. Workers that have made extensive (>30 s) queen contact function as 'messengers' dispersing queen substance, walking among and antennating with their nestmates and receiving frequent inspections by them. To paraphrase Seeley (1979), the bee-to-bee surface transport model for queen substance transmission by workers is supported by: (1) the higher frequency of antennations and inspections by nestmates of messenger bees relative to control bees; (2) the close correlation of messenger bees between the duration of their contact with the queen and the number of inspections by nestmates; and (3) the low frequency of food donations compared to nestmate antennations by messenger bees in the 30 min following their contact with the queen. Messenger bees, analyzed by gas chromatography, lacked any detectable trace of (E)-9-oxodec-2-enoic acid. Naumann et al. (1992) confirmed Seeley's interpretations by showing that some of the queen's mandibular gland pheromonal components secreted on her body are acquired by workers in her retinue. Previously unexposed, naïve workers having contact with retinue bees acquire the signal themselves. However the signal is also spread in footprints from the passage of the queen and workers onto the combs in which it diffuses and is slowly but eventually lost from the system (Naumann et al. 1992).

Table 5.1 **D**ances performed by 20 *A. mellifera* marked dancers on the combs of a two-frame observation hive[a]

Date/Time		Dances on upper frame	Dances on lower frame
11 September 1995			
14:00–14:10		0	*10*
14:10–14:20	Exchange of frames		
14:20–14:30		*15*	2
14:30–14:40	Exchange of frames		
14:40–15:10		3	*29*
15:10–15:20	Exchange of frames		
15:20–15:50		*42*	7
15:50–16:00	Exchange of frames		
16:00–16:30		4	*32*
12 September 1995	Both sides of each frame accessible to the bees	*26* (front)	11 (front)
10:20–10:50		0 (rear)	*2* (rear)
10:50–11:00	Exchange of frames		
11:00–11:30		13 (front) *5* (rear)	*34* (front) 0 (rear)
11:30–13:30	No exchange		
13:30–14:00		1	*22*
14:00–14:10	Exchange of frames		
14:10–14:40		*21*	6
12 September 1995			
11.30–11:40		*17*	0
11:40–11:50	Exchange of frames		
11:50–12:20		2	*61*

[a] Columns show the dates and durations of the observations and numbers of dances observed on upper and lower combs. On 12.09.95, bees were allowed access to both sides of the combs. At 11:30 on the same day the bees were removed from the hives and then allowed to return but the combs were not exchanged (Tautz and Lindauer 1997)

5.3.1 Waggle Dance Scent-marking: Probable Cause?

One of the most curious and interesting discoveries in the dance communication field of honeybees in the past decade or so is the work of Tautz (1996) and Tautz and Lindauer (1997). Having seen that the delivery of forage from the field and the further recruitment of new foragers are associated with transmission of sound through the comb, they asked if there were specific sites for waggle dances. Tautz discovered that foragers which danced on empty areas of comb recruited three times as many dance followers as bees that danced on areas of capped brood. Clearly the recruitment process could be enhanced if there were particular dance sites that were mutually recognizable to both active dancers and dance followers, especially because dancing bees are commonly found on the lower comb near the entrance of observation hives (Tautz 1996).

The existence of specific dance sites on combs was elegantly demonstrated by Tautz and Lindauer (1997) using a two-frame observation hive with the entrance half way up the side. After the bees had been trained to an unscented sugar solution in a nearby feeder, the locations of dancing bees were noted for several days. Foragers were then marked and allowed to visit the feeders and return to dance in the hive. After a while the feeders were closed and all of the bees were shaken out of the hive and the positions of the two combs were switched; the former upper comb became the bottom comb and vice versa. The bees were then allowed back into their hive, the feeders were reopened and the sites of the dancers on the combs recorded. All dances performed by the 20 marked dancers were recorded before and after switching the positions of the upper and lower combs during seven such switches in subsequent days. From the total series of seven comb position switches in nearly 90 % of the 365 dancing episodes, the dancers went to the exact site on the specific comb on which the initial dance had first been performed, irrespective of the position (upper or bottom) of the comb in the hive after being switched around (Table 5.1).

Tautz and Lindauer (1997) considered the possibility that the bees might use spatial cues, but noted that even after combs were switched around, the bees walked over the combs until they located the site of the initial dance before recommencing dancing. They suggested that the rediscovery of a specific dance site would be most parsimoniously explained by a chemical marker (such as a queen's footprint pheromone), that would allow site reinforcement throughout the day but which would fade at night, so that new locations for new dances sites might change with changing conditions in the nest. By the same token, many of the recently discovered volatiles (cf. mechano-chemical changes for brood capping) could serve as cues for dance recruits, if they too seek the same dance site after switching the combs.

This could hold important implications in the analysis of patriline performance in the division of labour. The possibility of comb scent-marking needs be only part of the system for the recruitment of new foragers, because shortly after, Tautz et al. (2001) showed that an alternative signal could be the vibrations of the comb generated by dancing bees, but which, without amplification, may not carry far enough to alert remote dance followers. They then described that an unexpected property of honeycomb subjected to vibrations at around 200 Hz could represent an effective amplification of the vibratory signals for remote dance followers (Fig. 5.12).

5.3.2 Comb and Scents

The mechanism for colony odour masking was pursued in an experiment by Breed et al. (1988b), when he introduced previously comb-conditioned (acquired home colony odour) bees into related colonies of comb-naïve bees, the former were rejected (Fig. 5.12). However when the receiver bees were conditioned to the same

Odour in Recipient Group

Odour in donor group	Wax only		Anise and wax	
Wax only	64.9	(57)	45.6	(57)
Anise & wax	52.1	(48)	89.4	(47)

	Anise only		Sugar only	
Anise only	62.8	(43)	63.4	(41)
Sugar only	73.9	(46)	73.7	(38)

Fig. 5.12 Comparisons of odour effects on *A. mellifera* workers in the presence of wax (same versus different odour) were significant; odour effects without wax were not significant. Data expressed as percentages and sample size given in brackets (Breed et al. 1988a)

comb source as the introduced bees, the latter were accepted (this principle of odour conditioning has been common practice in beekeeping (Dyer 1781; Newby 1832; Wighton 1842; Cale 1946) as well as in swine husbandry for centuries (Coburn 1890). Whatever their origin, bees that were conditioned to the same comb source were more readily acceptable than bees from between comb type transfers. Although Breed did not specifically control for individual worker bee odours, perhaps owing to genetic differences, it is tacitly assumed that any such scent would have been over-ridden and masked when bees from different colonies were conditioned to the same comb, hence becoming mutually acceptable. This interpretation is indirectly but strongly supported by the fact that Michelsen et al. (1989) always had to 'condition' their robotic dancing bees to stave off attacks. Moreover, Breed et al. (1998) repeated the experiments of Kirchner et al. (1989) with neutral paraffin wax and other paraffins to which he added scent to some and others not, and they obtained the same results as in the first experiment.

It remains uncertain if the observed effects derive in part from genetically-based differences in the chemistry of the source combs and/or the possible passive adsorption of environmental odours into the waxes (remembering that waxes are virtually sponges for the adsorption of volatiles). Nonetheless the two possibilities are not mutually exclusive. A common mechanism could well be an interaction between shared pools of adsorbed chemical volatiles from the epicuticle of the bees as well as from the combs. This would explain how flower scent volatiles alter 'colony odour' after different foraging plant species availability changes through the year (Ribbands 1953). Similarly in an interesting recent study,

Couvillion et al. (2013) performed experiments which clearly showed that recognition errors in nestmate discrimination behaviour are context-dependent in *A. mellifera*. The significance of 'context' also occurs in the context of interspecific wax discrimination (cf. Chap. 4).

Comb waxes mediate several other kinds of behaviours as well (Free 1987). For example, Fergusson and Free (1981) found that the odour of comb alone was a sufficient stimulus for worker bees to release scent from the Nasanov gland, a source of a cluster-inducing pheromone (Free 1987). This is consistent with the fact that honeybees prefer old combs to new ones, possibly because they have been doped with footprint pheromones (Free 1987). Comb volatiles also stimulate increased foraging (Rinderer and Hagstad 1984); but bioassays on the possible stimulatory effects of oxygenated comb volatiles proved equivocal (Blum et al. 1988).

5.3.3 Capping Brood Cells

The case of capping brood also involves a particularly interesting scenario as seen from two different but not incompatible perspectives; that of pheromones and/or mechanical stimuli. Le Conte et al. (1990) established that there is a series of only a few methyl esters (methyl palmitate, ethyl palmitate and methyl linolenate), which are normal constituents of pheromones produced by queens and workers (Free 1987), that alone are sufficient to induce the capping of brood cells containing mature larvae (Figs. 5.13, 5.14, 5.15). The compounds are actually multifunctional and differing ratios both modulate the feeding behaviour of worker bees or lead to capping their cells (Trouiller et al. 1991; Trouiller 1993; Le Conte et al. 1995a).

In the case of queen cells, the developmental progress of the larvae is constantly signalled by the ester ratios. This was confirmed unequivocally by using substitute 'dummy' larvae doped with pheromone (Le Conte et al. 1995a). Following the capping of the doped dummy queens, their continued acceptance in the colony depends on their ability to emit the 'correct' esters (Fig. 5.16). The results recall those on the inhibition of queen cell construction in the experiments of Boch and Morse (1979).

Nonetheless, signal specificity remains a vexing concern, because at any given time a variable percentage of the uncapped larval population could be emitting capping signals of varying ester ratios. These might only indicate that there is an area of brood comb requiring capping. Goetz and Koeniger (1992) argued that if capping depends entirely on pheromones then brood cells should be capped according to larval age. Alternatively, if capping can be advanced in time by artificially decreasing the depth of the cells, or delayed by increasing it, then the size of larvae in relation to the depth of their cells could be important in triggering capping behaviour. Experimentally they artificially modified the distance of larvae from their cell openings by increasing or decreasing the depth of cells (Figs. 5.17

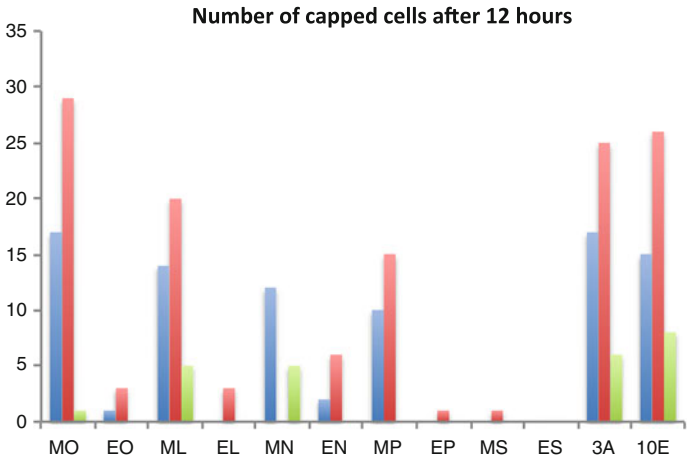


Fig. 5.13 Capping of *A. mellifera* brood cells containing lures. Lures were doped with an ester or mixture of esters at concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 0 (controls). Cells were observed 12 h (**a**) and 36 h (**b**). Experiment was repeated 10 times with 3 colonies at concentrations of 10^{-2} (*black bars*), 10^{-3} (*white bars*) and 10^{-4} (*grey bars*). MO = methyl oleate; EO = ethyl oleate; ML = methyl linoleate; EL = ethyl linoleate; MN = methyl linolenate; EN = ethyl linoleate; MP = methyl palmitate; EP = ethyl palmitate; MS = methyl stearate; ES = ethyl stearate; while 3A and 10E are mixtures of the compounds placed in empty cells (Le Conte et al. 1990)

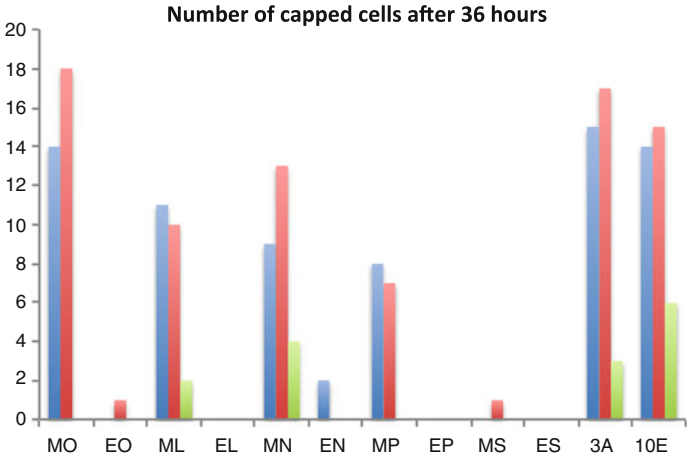


Fig. 5.14 Brood cell capping in *A. mellifera* following the application of a mixture of 10 esters on worker larvae of the same age. An amount of 0.25 µl was spread on each larva and the experiment was repeated 5 times with 5 different colonies. The results were significantly different except at 20 h. MO = methyl oleate; EO = ethyl oleate; ML = methyl linoleate; EL = ethyl linoleate; MN = methyl linolenate; EN = ethyl linoleate; MP = methyl palmitate; EP = ethyl palmitate; MS = methyl stearate; ES = ethyl stearate; while 3A and 10E are mixtures of the compounds placed in empty cells (Le Conte et al. 1990)

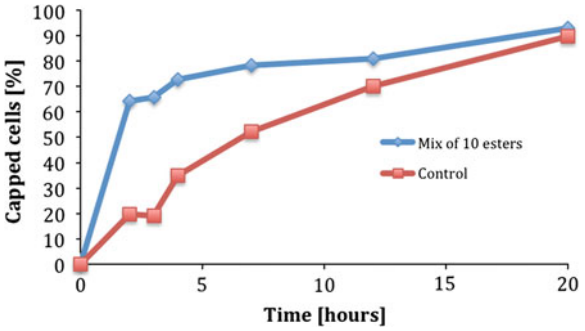


Fig. 5.15 Amounts of the ten fatty acids found on young and old *A. mellifera* worker larvae (Le Conte et al. 1994)

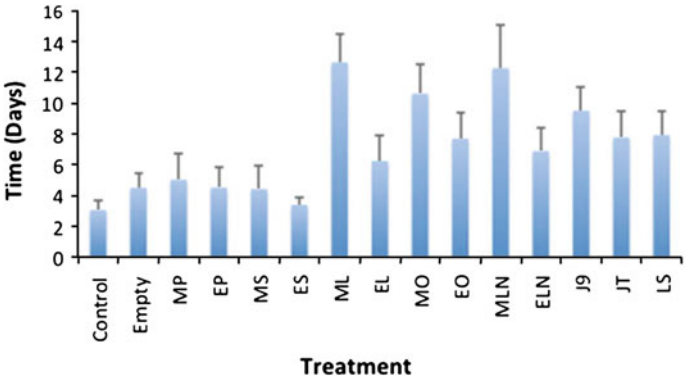


Fig. 5.16 Acceptance of *A. mellifera* queen cells including paraffin lures with fatty acid esters at concentrations of 10^{-2} and 10^{-3} (w/w). MP = methyl palmitate; EP = ethyl palmitate; MS = methyl stearate; ES = ethyl stearate; ML = methyl linoleate; EL = ethyl linoleate; MO = methyl oleate; EO = ethyl oleate; MLN = methyl linolenate; ELN = ethyl linoleate; J9 = a mixture of all compounds found on workers; JT = mixture of all compounds on queen pupae; and LS = mixture of all compounds on queen pupae (Le Conte et al. 1995b)

and 5.18). Their results suggested that worker bees might respond by capping cells according to distance and not age, hence ratios of ester emissions.

Subsequently, Le Conte et al. (1994) revisited this apparent contradiction. They had previously shown that worker bees will cap cells containing paraffin dummies doped with an ester blend of mature larvae (Le Conte et al. 1994). They then proceeded to a more thorough analysis of ester concentration, the position of the larvae in the cells, the effects of ester blends on worker capping and of queen cells. Their results showed that capping activity depended on the ester concentrations, which again suggested that the presence of larvae is mediated pheromonally. The position of the larvae (distance from cell opening) could still influence worker capping behaviour because by changing the distance of the larva from the cell

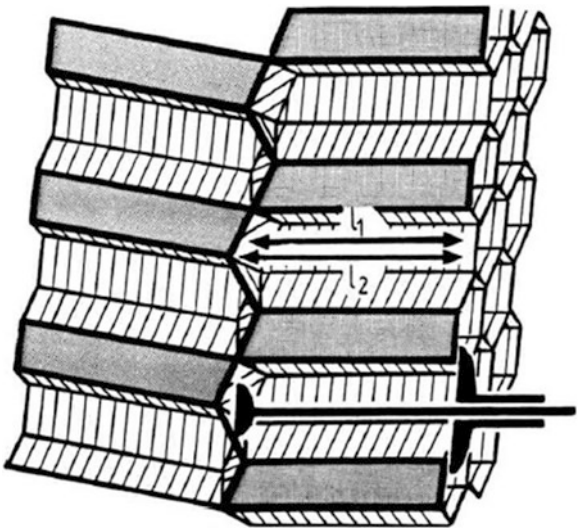


Fig. 5.17 Longitudinal section of an *A. mellifera* comb where: 1 = measurement obtained with a probe; and 2 = after correction for depth of pyramidal cell base (Goetz and Koeniger 1992)

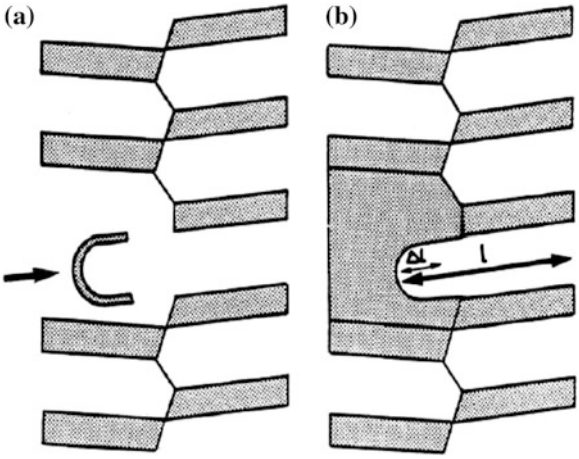


Fig. 5.18 Longitudinal sections of an *A. mellifera* comb: **a** the base of a cell is removed; and **b** the cavity is filled with wax to the desired depth. L = cell depth, Δl = amount of elongation (Goetz and Koeniger 1992)

opening, the available head-space of pheromone could be altered. In tests of blend discrimination, dummies doped with the young larval blend of ethyl esters were not capped, dummies with the mature blend of methyl esters induced capping in *A. mellifera*. These results were also confirmed in *A. cerana* (Zeng et al. 2010).

Le Conte et al. (1994) conducted further experiments on queen cell construction and capping in dequeened colonies, in which the effects of both larval position in the cells and pheromonal blends were measured. More queen cell construction occurred when dummies were doped with the young larval blend and positioned at the bottom of the cells. Thus, the results from both the capping of worker cells and queen cell construction experiments indicated that worker bees can, and do, discriminate between young and mature larvae by scent, the bouquets of the ester

blends as well as the position of larvae in their cells. The combined results of Goetz and Koeniger (1992) and those of Le Conte et al. (1990, 1994) are not mutually exclusive in principle, but suggest that additional experiments are needed to investigate the possible interrelationships of the variables.

References

Ai H, Hagio H (2013) Morphological analysis of the primary center receiving spatial information transferred by the waggle dance of honeybees. J Comp Neurol 521:2570–2584

Bergman DJ, Ishay JS (2007) Do bees and hornets use acoustic resonance in order to monitor and coordinate comb construction? Bull Math Biol 69:1777–1790

Bethe A (1898) Dürfen wir den Ameisen und Bienen psychische Qualitäten zuschreiben?. Strauss, Bonn

Bernstein PL (1996) Against the gods: the remarkable story of risk. Wiley, New York

Blum MS, Jones TH, Rinderer TE, Sylvester HA (1988) Oxygenated compounds in beeswax: identification and possible significance. Comp Biochem Physiol 91B:581–583

Boch R, Morse RA (1979) Individual recognition of queens by honey bee swarms. Ann Ent Soc Am 72:51–53

Bozhidar A (2006) The illustrated encylopedia of musical instruments. Konemann, New York

Breed MD, Williams KR, Fewell JH (1988a) Comb wax mediates the acquisition of nestmate recognition cues in honey bees. Proc Natl Acad Sci USA 85:8766–8769

Breed MD, Stiller TM, Moor MJ (1988b) The ontogeny of kin discrimination cues in the honey bee, *Apis mellifera*. Behav Genet 18:439–448

Breed MD, Leger EA, Pearce AN, Wang YJ (1998) Comb wax effects on the ontogeny of honey bee nestmate recognition. Anim Behav 55:13–20

Butler C (1609) The feminine monarchie or a treatise concerning bees, and the due ordering of them. Joseph Barnes, Oxford

Butler C (1634) The feminine monarchie or a treatise concerning bees, and the due ordering of them. Joseph Barnes, Oxford

Cale GH (1946) Management for honey production. In: Grout RA (ed) The hive and the honeybee. Dadant & Sons, Hamilton, pp 313–383

Coburn FD (1890) Swine husbandry. A practical manual for the breeding, rearing and management of swine. Orange Judd Co, New York

Couvillon MJ, Segers FHID, Cooper-Bowman R, Truslove G, Nascimento DL, Nascimento FS, Ratnieks FLW (2013) Context affects nestmate recognition errors in honey bees and stingless bees. J Exp Biol. doi:10.1242/jeb.085324

Crane E (1999) The world history of beekeeping and honey hunting. Taylor & Francis, London

Dyer W (1781) The apiary laid open. Shipton Olliffe, UK

Fergusson LA, Free JB (1981) Factors determining the release of Nasanov pheromones at the hive entrance. Physiol Entomol 6:15–19

Fletcher DJ (1975) Significance of dorsoventral abdominal vibration among honey-bees (*Apis mellifera* L.). Nature 256:721–723

Free JB (1982) Bees and mankind. Harper Collins Publishers Ltd, New York

Free JB (1987) Pheromones of social bees. Chapman and Hall, London

Fuchs S, Koeniger N (1974) Sound production as a means of defense of the honeybee colony (*Apis cerana*). Apidologie 5:271–287

Goetz B, Koeniger N (1992) Structural features trigger capping of brood cells in honey bees. Apidologie 23:211–216

Hansson A (1945) Lauterzeugung und Lautaufassungvermogen der Bienen. Opusc Ent 6:1–122

Hyland KM, Cao TT, Malechuk AM, Lewis LA, Schneider SS (2007) Vibration signal behaviour and the use of modulatory communication in established and newly founded honeybee colonies. Anim Behav 73:541–551

Juska A (1978) Temporal decline in attractiveness of honeybee queen tracks. Nature 276:261

Kalmus H, Ribbands CR (1952) The origin of odors by which honey bees distinguish their companions. Proc R Soc B140:50–59

Kastberger G, Weihmann F, Hoetzl T (2013) Social waves in giant honeybees (*Apis dorsata*) elicit nest vibrations. Naturwissenschaften 100:595–609

Kirchner WH, Lindauer M, Michelsen A (1988) Honeybee dance communication. Naturwissenschaften 75:629–630

Kirchner WH (1993) Vibrational signals in the tremble dance of the honeybee, *Apis mellifera*. Behav Ecol Sociobiol 33:169–172

Kirchner WH, Lindauer M, Michelsen A (2003) Signals and flexibility in the dance communication of honeybees. J Comp Physiol A189:165–174

Koeniger N, Fuchs S (1972) Communication by sound production in the *Apis cerana* colony. Naturwissenschaften 59:169

Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B (1990) Identification of a brood pheromone in honeybees. Naturwissenschaften 77:334–336

Le Conte Y, Sreng L, Trouiller J (1994) The recognition of larvae by worker honeybees. Naturwissenschaften 81:462–465

Le Conte Y, Sreng L, Poitout SH (1995a) Brood pheromone can modulate the feeding behaviour of *Apis mellifera* workers. J Econ Ent 88:798–804

Le Conte Y, Sreng L, Sacher N, Trouiller J, Dusticier G, Poitout SH (1995b) Chemical recognition of queen cells by honey bee workers *Apis mellifera* (Hymenoptera: Apidae). Chemoecology 5(6):6–12

McIndoo NE (1922) The auditory sense of the honey-bee. J Comp Neurol 34:173–199

Michelsen A (2003) Signals and flexibility in the dance communication of honeybees. J Comp Physiol A189:165–174

Michelsen A (2012) How do honey bees obtain information about direction by following dances? In: Galizia CG, Eisenhardt D, Giurfa M (eds) Honeybee neurobiology and behavior. Springer, Berlin, pp 65–76

Michelsen A, Larsen ON (1978) Biophysics of the ensiferan ear. I. tympanal vibrations in bushcrickets (Tettigoniidae) studied with laser vibrometry. J Comp Physiol 123:193–203

Michelsen A, Kirchner WH, Andersen BB, Lindauer M (1986a) The tooting and quacking vibration signals of honeybee queens: a quantitative analysis. J Comp Physiol A158:605–611

Michelsen A, Kirchner WH, Lindauer M (1986b) Sound and vibrational signals in the dance language of the honeybee, *Apis mellifera*. Behav Ecol Sociobiol 18:207–212

Michelsen A, Kirchner WH, Lindauer M (1986c) Sound and vibrational signals in the dance language of the honeybee, *Apis mellifera*. Behav Ecoll Sociobiol 11:269–28l

Michelsen A, Towne WF, Kirchner WH, Kryger P (1987) The acoustic near field of a dancing honeybee. J Comp Physiol 161:633–643

Michelsen A, Andersen B, Kirchner WH, Lindauer M (1989) Honeybees can be recruited by a mechanical model of a dancing bee. Naturwissenschaften 76:277–280

Michelsen A, Andersen BB, Storm J (1992) How honeybees perceive communication dances, studied by means of a mechanical model. Behav Ecol Sociobiol 18:207–212

Naumann K, Winston ML, Slessor KN, Prestwich GD, Latli B (1992) Intra-nest transmission of aromatic honey bee queen mandibular gland pheromone components as a unit. Can Entomol 124:917–934

Newby TC (1832) The cottager's guide for the management of his bees. TC Newby, Bury St Edmunds', UK

Nieh JC (1993) The stop signal of honey bees: reconsidering its message. Behav Ecol Sociobiol 33:51–56

Nieh JC, Tautz J (2000) Behaviour-locked signal analysis reveals weak 200–300 Hz comb vibrations during the honeybee waggle dance. J Exp Biol 203:1573–1579

Pirk CWW, Hepburn HR, Bakkes DK (2013) Queen bees sing in alto: musical notation from 1609 for piping by queen honeybees are in line with laser vibrometry over 350 years later. Interface (In ms.)
Pratt SC, Kuhnholz S, Seeley TD, Weidenmuller A (1996) Worker piping associated with foraging in undisturbed queenright colonies of honey bees. Apidologie 27:13–20
Ribbands CR (1953) The behaviour and social life of honeybees. Bee Research Association, London
Rinderer TER, Hagstad WA (1984) The effect of empty comb on the proportion of foraging honeybees collecting nectar. J Apic Res 23:80–81
Sandeman DC, Tautz J, Lindauer M (1996) Transmission of vibration across honeycombs and its detection by bee leg receptors. J Exp Biol 199:2585–2594
Schneider SS (1986) The vibration dance activity of successful foragers of the honeybee *Apis mellifera* (Hymenoptera: Apidae). J Kans Ent Soc 59:699–705
Seeley TD (1979) Queen substance dispersal by messenger workers in honeybee colonies. Behav Ecol 5:391–415
Seeley TD (1992) The temble dance of the honeybee: message and meaning. Behav Ecol Sociobiol 31:375–383
Seeley TD, Reich AM, Tautz J (2005) Does plastic comb foundation hinder waggle dance communication? Apidologie 36:513–521
Sen Sarma M, Fuchs S, Werber C, Tautz (2002) Worker piping triggers hissing for coordinated colony defence in the dwarf honeybee *Apis florea*. Zoology 105:215–223
Simpson J (1964) The mechanism of honey-bee queen piping. Z Vergl Physiol 48:277–282
Snodgrass RE (1910) The anatomy of the honeybee. USDA Bur Entomol Tech Ser 18:l–162
Stanford CV, Forsyth C (1937) A history of music. The Macmillan Company, London
Tautz J (1996) Honey bee waggle dance: recruitment success depends on the dance floor. J Exp Biol 199:1375–1381
Tautz J, Rohrseitz K, Sandeman DC (1996) One-strided waggle dance in bees. Nature 382:32
Tautz J, Lindauer M (1997) Honeybees establish specific sites on the comb for their waggle dances. J Comp Physiol A180:537–539
Tautz J, Casas J, Sandeman D (2001) Phase reversal of vibratory signals in honeycomb may assist dancing honeybees to attract their audience. J Exp Biol 204:3737–3746
Trouiller J (1993) La communication chimique inter- et intra-spécifique chez l'abeille. Thesis, Université de Paris VII, Paris
Trouiller J, Arnold G, Le Conte Y, Masson C, Chappe B (1991) Temporal pheromonal and kairomonal secretion in the brood of honeybees. Naturwissenschaften 78:368–370
Tsujiuchi S, Sivan-Loukianova E, Eberl DF, Kitagawa Y, Kadowaki T (2007) Dynamic range compression in the honey bee auditory system toward waggle dance sounds. PLoS ONE. doi:10.1371/journal.pone.0000234
von Frisch K (1967) The dance language and language and orientation of bees. Oxford University Press, London
Wenner AM (1962a) Sound production during the waggle dance of the honey bee. Anim Behav 10:79–95
Wenner AM (1962b) Communication with queen honey bees by substrate sound. Science 138:446–448
Wighton J (1842) The history and management of bees. Longman, London
Zeng YF, Zeng ZJ, Yan WY, Wu XB (2010) Effects of three aliphatic esters of brood pheromone on worker feeding and capping behavior and queen development of *Apis cerana cerana* and *A. mellifera ligustica.* Acta Ent Sinica 2010-01

Chapter 6
Wax Secretion, Comb Construction and the Queen

Abstract Discovery of queen substance led to the experimental dissection of the importance of these chemical signals in comb construction, especially because more combs are produced in the presence of mated queens than with virgin queens, whose pheromonal bouquets substantially differ. In a series of experiments, Whiffler and Hepburn (1991a) showed that bees secrete the same amount of wax whether queenless or queenright, with either mated or virgin queens, and living or dead. Moreover, removing mandibular glands or restricting workers access to the pheromones of queens has no effect on wax secretion. Similarly, wax secretion does not significantly differ among colonies with caged or division board queens, with intact mandibular and abdominal tergite glands or not. The actual secretion of wax is independent of queen status. However, comb-building differs because colonies headed by mated queens construct significantly more comb than queenless colonies, results consistent with other studies on *A. mellifera* and *A. cerana*. Collectively, these results indicate that the bouquet of the queen's mandibular gland cannot alone fully explain enhanced comb-building by queenright workers. Whatever the source of the comb-building stimulus, its effect requires direct contact with the queen because most comb is always built when workers have full access to a mated free-running, physical and chemical queen; and, little comb is built when the colony has access only to the 'chemical' or 'physical' queen. The independence of wax secretion, as opposed to comb-building, from the pheromonal influence of the queen (Whiffler and Hepburn 1991a) was subsequently confirmed in experiments by Ledoux et al. (2001).

6.1 Introduction

That the queen may have a specific relationship to the synthesis and secretion of wax as well as to comb-building has been moot for a few centuries. Indeed, de Réaumur (1740) was the first to note that a caged colony of queenless bees constructed comb after 2 days confinement. However, he had given the bees some

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queen cells and, unfortunately, we do not know how long the bees had been queenless prior to their incarceration or whether they had been given any other brood. A far more instructive experiment was performed by Schirach (1770), who observed that on the loss or removal of a queen, a colony would construct emergency queen cells over some of the worker cells containing eggs or larvae, and new queens would be reared from them. This important result was confirmed in numerous experiments by Huber (1814), and is the basis for the queen-rearing industry of today. Huber also knew that queenright colonies normally construct queen cells in the spring, as a prelude to reproductive swarming. So queen cells may be constructed in the presence or absence of a queen. These somewhat ambivalent results led Huber (1814) to another experiment, in which a hive was so divided that about half of the bees were in direct contact with their queen, while the other half had access only to the odour and sounds of the queenright half of the colony. In this situation, the 'queenless' half of the colony began the construction of queen cells, the other half did not.

Turning to comb construction, the first recorded observations relating the queen to comb construction are those of Huber (1814), who noted that queenless bees construct little comb, a point confirmed often since for *A. mellifera* (Gundelach 1842; Dreischer 1956; Goetze and Bessling 1959; Darchen 1968) and *A. cerana* (Rajashekharappa and Channabasavanna 1979). These observations, coupled to the demonstration that pheromones of the queen also suppress ovarial development in workers (Butler 1954; Pain 1955), suggest that the queen and/or her pheromones may well affect the secretions of wax and the construction of combs by worker bees (Darchen 1956a, b, 1957; Hepburn 1986). Suppression of queen cell construction and ovarial development require direct physical contact between workers and queen (Müssbichler 1952; Butler 1954; de Groot and Voogd 1954; Pain 1954; Verheijen-Voogd 1959), effects that have been attributed to her pheromones (cf. Free 1987; Slessor et al. 1988, 1990). Furthermore, the bouquets of mated and virgin queens differ within and between honeybee races of *A. mellifera* (Crewe and Velthuis 1980; Crewe 1982, 1988), and comb construction varies with queen quality in both *A. mellifera* (Darchen 1956a, b, 1957; Verheijen-Voogd 1959) and *A. cerana* (Rajashekharappa and Channabasavanna 1979).

While the production of queen cells is of obvious importance to the honeybee colony, so too is the regulated construction of comb cells in which to rear brood and to hold stores. For example, Gundelach (1842) observed that when queenless bees were caged and given honey, they secreted wax scales within 2 days, but did not construct combs in the absence suitable young larvae from which to rear queens. Against this, Dreischer (1956) found that a queenright and a queenless colony of the same size both produced comb, but the former was fourfold greater than the latter. The same kind of experiment has been performed several times on various races of *A. mellifera* with the same results (Darchen 1956a, 1957; Free 1967; Jay and Jay 1983; Hepburn et al. 1984). This provides the historical background from the 18th to the mid-20th century. It can be noted that virtually all of this research pertains to *A. mellifera,* as does most of the discussion in this chapter, with an occasional reference to *A. cerana*. This is simply the state of play with

respect to queens and comb-building in *Apis*. However, it is worth noting that the mandibular gland pheromones *A. andreniformis, A. florea* and *A. dorsata* queens have now been identified (Plettner et al. 1997), so hopefully further progress with field experiments on queens and comb-building with these species will soon be conducted.

6.2 The Queen: A Necessary Stimulus for Comb-Building?

Following the discovery of queen substance (Butler 1954; de Groot and Voogd 1954; Pain 1954), the slow experimental dissection of the importance of these chemical signals in comb construction began. For example, because of the ever-present group effects which influence the behaviour of workers, it was desirable to know how the relative size of a colony might relate to comb production by *A. mellifera*. Darchen (1956b, 1957) investigated how comb production was related to different stocking rates under different queening conditions. He formed colonies of 6-day-old bees and to some he gave normal, mated and laying queens, to others virgin or dead queens and, finally, some remained queenless. The results of these experiments are given in Table 7.1. They show that a queenright colony of only 50 bees is just sufficient for comb construction, given a live queen. Even a dead queen could stimulate some construction by 200 bees, but 1000 queenless bees produced no comb at all (the latter point being confirmed by Frichot-Riera 1961) (Table 6.1).

In terms of the expected efficacy of the pheromones, it is not obvious why fewer than 200 bees headed by the corpse of a queen would not construct combs, while more than this number did. In any event, this led Darchen (1956b, 1957) to attempt to separate the signals of the queen from her physical presence. He encased a queen in such a way that the workers of a small colony could smell but not touch her (a technique used to great advantage by Müssbichler 1952). In this experiment, like that of Huber (1814), the bees did not construct combs. In a different experiment, Darchen confined a queen so that her head was accessible to one group of bees and the rest of her body accessible to a different group of bees. Those bees having access to the head of the queen (and to the queen substance of the mandibular gland) began to construct combs, while the bees lacking such access did not.

Comb-building in relation to the queen has also been studied in the Asian honeybee, *A. cerana,* by Rajarhekharappa and Channabasavanna (1979) who established replicate colonies (size not stated) from queenright stock. One pair of these colonies was made queenless, each of a second pair was given a virgin queen, and each of a third pair was given a mated, laying queen. Performance was measured as the area of comb built over 10 days. On final examination, the queenless bees and those headed by virgin queens had both produced the same amount of comb, 89 cm^2/colony; those headed by mated queens had produced, on average, 341 cm^2/colony: nearly four times as much. On the l0th day of their experiment, Rajarhekharappa and Channabasavanna gave mated queens to those

Table 6.1 Comb construction by *A. mellifera* of different colony sizes given mated, virgin, dead queens or under queenless conditions (Darchen 1956b, 1957)

Colony size (no. of bees)	Queenless	Dead queen	Virgin queen	Mated queen
Queen conditions[a]				
0–25	None	None	None	None
26–50	None	None	None	None
51–75	None	None	Construction	Construction
76–100	None	None	Construction	–
101–200	None	None	–	Construction
201–300	None	Construction	Construction	
301–400	None	Construction		Construction
401–500	None	–	–	–
501–600	None	–	–	–
601–700	None	–	–	–
701–800	None	–	–	–
801–900	None	–	–	Construction + egg laying
901–1000	None	–	–	–

[a] From Darchen (1956b, 1957). *Dashes* indicate that no tests were made for conditions stated

colonies which had previously been queenless and to those with virgin queens. The colonies formerly headed by mated queens were now made queenless. After 2 days, the now queenless bees (which had constructed a great deal of comb when queenright), had not constructed comb while the now queenright colonies both produced about the same amount of comb.

In these experiments more combs were always produced in the presence of a mated queen than was obtained under virgin queens, possibly implying pheromonal differences between virgin and mated queens. In a recent study of the pheromones of queens, Crewe (1982) showed that the pheromonal bouquets substantially differ between virgin and mated queens in three races of *A. mellifera,* so there is good reason to believe that large scale comb construction in *A. cerana,* like *A. mellifera,* depends upon the full pheromonal bouquet such as is obtained from mated queens or from egg-laying workers that have become pheromonally false queens.

6.3 Comb-Building Experiments by Whiffler and Hepburn (1991a)

6.3.1 Queenright and Queenless Colonies

Whiffler and Hepburn (1991a) reported the results of experiments to further investigate the relationship of queen state and source of pheromones (head or abdomen) in the secretion of wax and the building of combs by worker honeybees. All experiments were performed with queenright honeybee colonies (*A. m. capensis*

Table 6.2 Festoon bees with wax scales, weights of the scales per bee, comb construction and colony size of *A. m. capensis* and *A. m. scutellata* colonies with differing queenship status (Whiffler and Hepburn 1991a)

Treatment[a]	*n*	Colony size	% Bees bearing wax	Wax/bee (μg)	Comb weight (μg)
Queenship status in experiment $\dot{X} \pm SD$					
A. cap/*A. cap*	4	9355 ± 3622	67 ± 15	473 ± 66	2745 ± 591
A. cap/Q'less	4	3793 ± 1481	55 ± 14	576 ± 250	325 ± 402
A. scut/*A.scut*	9	9205 ± 3441	75 ± 13	576 ± 128	3494 ± 1562
A. scut/Q'less	4	7399 ± 3842	73 ± 5	622 ± 70	1129 ± 721
A. cap/*A. scut*	4	6371 ± 3025	61 ± 18	470 ± 68	3240 ± 1061
A. scut/*A. cap*	4	6992 ± 3058	77 ± 3	639 ± 52	3925 ± 668
A. cap/Virgin Q	4	6464 ± 4664	47 ± 1	358 ± 75	1039 ± 870

[a] *A. cap*/*A. cap* = *A. capensis* colonies with mated *A. capensis* queen; *A. cap*/Q'less = queenless *A. capensis* colonies; *A. scut*/*A. scut* = *A. scutellata* colonies with mated *A. scutellata* queen; *A. scut*/Q'less = queenless *A. scutellata* colonies; *A. cap*/*A. scut* = *A. capensis* colonies with mated *A. scutellata* queen; *A. scut*/*A. cap* = *A. scutellata* colonies with mated *A. capensis* queen; *A. cap*/Virgin Q = *A. capensis* colonies with virgin *A. capensis* queen

and *A. m. scutellata*) in five-frame nucleus hives containing: 1 frame of brood, 2 of honey and pollen and 2 empty frames for comb construction. Colonies were routinely dequeened early in the morning and requeened in the evening. Each colony had access to feeders. Each treatment ran for 1 week after which colony size was estimated by weighing the bees, newly constructed combs and samples of festoon bees were collected from each colony. The percentages of bees bearing wax scales were recorded and the wax scales of individual bees and constructed combs were weighed. Queens were decapitated and their heads analyzed using standard gas chromatographic techniques (Crewe 1982), and identification of compounds of the secretions was made by comparison of the retention times with those of authentic standards.

The experimental methodology largely consisted of comparing free-running queens with other queens in cages or division boards, a well established technique (Müssbichler 1952; Ribbands 1953; Free 1987; Hepburn 1986). In the first experiment, queenright colonies were dequeened. After a week all samples were collected and reciprocal exchanges of queens were performed for both subspecies. A week later, the queenright *A. m. capensis* colonies were requeened with virgin *A. m. capensis* queens. This experiment showed that the percentage of festoon bees with wax scales and the weights of the scales did not significantly differ among the queenright and queenless colonies or between races (Table 6.2). The amount of raw wax available for comb-building was essentially the same for queenright and queenless bees. In the reciprocal transfer of queens, *A. m. capensis* colonies with *A. m. scutellata* queens, there was significantly less wax/bee than colonies in the reverse arrangement (Table 6.2). The queenless colonies constructed significantly less comb than queenright ones in both *A. m. capensis* and *A. m. scutellata* colonies (Table 6.2). No differences in the amount of comb constructed arose in the

reciprocal transfer of queens between races (Table 6.2). Colonies headed by *A. m. capensis* virgin queens constructed comb equivalent to that of queenless colonies (Table 6.2). The amounts of 9-ODA, 9-HDA and 10-HDA in the mandibular glands did not significantly differ within or among queens for both races. There were no correlations between the amounts or ratios of pheromones and any of the other variables measured.

6.3.2 Free-Running and Confined Queens

In another experiment, Whiffler and Hepburn (1991a) tested queenright *A. m. capensis* colonies to compare the effects of free-running queens, queens confined in either single or double layered gauze cages (as in Müssbichler 1952; Butler 1954), dead queens and queenless colonies. Here the percentage of festoon bees with wax scales and the weights of wax scales did not differ significantly among the colonies (Table 6.3). Colonies with dead queens, caged queens (single or double-layered cages) and queenless colonies constructed significantly less comb than those headed by free-running, mated queens (Table 6.3). There were no significant differences in mandibular gland acids of the queens (Table 6.4), nor were the pheromones correlated with any of the construction variables measured. Variations in colony size were not significant. The percentage of festoon bees with wax scales and the weight of the scales did not significantly differ among queenright and queenless colonies or between races.

6.3.3 Division Board Experiments

In an experiment using division boards, Whiffler and Hepburn (1991a) placed queenright colonies of *A. m. capensis* in five-frame nucleus hives (in pairs), but with their entrances in opposite directions. Each colony was transferred to one side of a ten-frame Langstroth hive divided in half using a division board with a hole near its top. Each half of the hive had separate and opposite entrances. Queens were placed in the hole, giving one colony access to the head and thorax, and the other colony only the abdomen. After dequeening, the paired colonies were given (never their own) living or dead queens of all permutations of living queens with/without mandibular glands and with/without occluded abdominal tergite glands. Mandibular glands were surgically excised after anaesthesure on ice (Gary 1961), and abdominal tergites occluded with varnish (Velthuis 1970). Four other completely different colonies were each given a queen in a division board that only extended downwards one-third of the distance between the top and bottom of the Langstroth hive, such that all the worker bees had access to both the head and abdomen of the queen.

Table 6.3 Percentages of festoon bees with scale wax, weights of scales comb construction and colony size in caged queen experiments with *A. m. capensis* queens (Whiffler and Hepburn 1991b)

Treatment	*n*	Colony size	% Bees bearing wax	Wax/bee (μg)	Comb weight (μg)
1. Double caged queen	3	5255 ± 2188	78 ± 18	696 ± 462	0
Free queen	3	4142 ± 6165	54 ± 10	819 ± 264	312 ± 448
2. Single caged queen	5	3876 ± 2307	64 ± 20	636 ± 245	0.3 ± 0.6
Free queen	5	4946 ± 2992	73 ± 17	491 ± 210	189 ± 336
Dead queen	3	3571 ± 1803	54 ± 11	869 ± 103	125 ± 109
3. Free queen	3	1761 ± 1655	58 ± 10	801 ± 463	572 ± 189
Queenless	3	4487 ± 2757	63 ± 25	564 ± 329	0
Total of free queens	19	5127 ± 3770	65 ± 15	630 ± 457	609 ± 1183

Table 6.4 Distribution of mandibular gland components of mated *A. m. capensis* and *A. m. scutellata* queens (Whiffler and Hepburn 1991b)

Queen	*n*	% Composition of the components present[a]			Total (μg/head)
		9-ODA	9-HDA	10-HDA	
Experiment 1					
A. capensis	4	73 ± 26	16 ± 27	11 ± 21	70 ± 12
A. scutellata	9	51 ± 49	43 ± 55	15 ± 11	143 ± 68
Experiment 2					
A. capensis					
Double caged queens	3	46 ± 3	54 ± 3	0.5 ± 0.1	61 ± 0
Single caged queens	5	59 ± 14	29 ± 18	12 ± 17	103 ± 124
Free queens	3	55 ± 1	44 ± 14	0.8 ± 0.1	60 ± 11

[a] *9-ODA* 9-oxo-2-decenoic acid; *10-HDA* 10-hydroxy-2-decenoic acid; *9-HDA* 9-hydroxy-2-decenoic acid

In this experiment, the percentage of festoon bees with wax scales did not significantly differ among colonies having access to the whole live queen, or only to her head or abdomen (Table 6.5). The amount of wax/bee did not significantly differ among colonies with free-running queens (Table 6.5). Given access only to the head of a queen, colonies with intact queens bore more wax/bee than those with queens that lacked mandibular glands (Table 6.5). Those bees with access only to the abdomen of the queen were equivalent to those with normal, whole queens (Table 6.5). Despite large variations, there were no significant differences in comb constructed in the various permutations of queens (with/without mandibular glands and with/without abdominal tergal glands) when the workers had access to the whole queen (Table 6.5). Among division board colonies, those bees with access to only the abdomen of the queen (intact or not) constructed no comb, while those with access to only the head (mandibular glands present or not) did not

Table 6.5 Festoon bees with wax scales, scale weights and comb constructed by *A. m. capensis* where mated queens were in division boards (Whiffler and Hepburn 1991b)

Treatment	% Bees bearing wax						Wax/bee (μg)						Comb weight (μg)					
	Free moving queen		Queens in division boards				Free moving queen		Queens in division boards				Free moving queen		Queens in division boards			
	n	Whole queen	n	Head of queen	n	Abdomen of queen	n	Whole queen	n	Head of queen	n	Abdomen of queen	n	Whole queen	n	Head of queen	n	Abdomen of queen
+m/+t	9	68 ± 16	3	57 ± 15	3	61 ± 6	9	357 ± 213	3	554 ± 245	3	278 ± 151	9	1304 ± 1241	3	117 ± 202	3	0
−m/+t	7	62 ± 11	3	53 ± 13	3	65 ± 6	7	273 ± 97	3	242 ± 114	3	382 ± 190	7	380 ± 615	3	409 ± 376	3	0
+m/−t	7	78 ± 11	3	68 ± 13	3	65 ± 1	7	376 ± 125	3	478 ± 207	3	344 ± 95	7	1334 ± 1112	3	1023 ± 978	3	0
−m/−t	6	64 ± 11	3	63 ± 1	3	62 ± 21	6	357 ± 71	3	371 ± 161	3	494 ± 443	6	1236 ± 1805	3	582 ± 1008	3	0
+m/+t (dead)	4	63 ± 10	3	64 ± 16	3	80 ± 4	4	322 ± 167	3	236 ± 72	3	420 ± 68	4	264 ± 591	3	0	3	0
+m/+t controls	3	64 ± 6			3	57 ± 21	3	361 ± 179					3	439 ± 360				

+m/+t queen with mandibular and abdominal tergal glands intact;*−m/+t* queen without mandibular gland and with tergal gland; *+m/−t* queen with mandibular but without tergal glands; *−m/−t* queen without mandibular and tergal glands; *+m/+t (dead)* dead but intact queen; *+m/+t controls* intact live queen

Table 6.6 Distribution of mandibular gland components of *A. m. capensis* queens in division board experiments (Whiffler and Hepburn 1991b)

Treatment[a]	*n*	Percentage distribution of the components measured				Total (μg/head)
		9-ODA	9-HDA	10-HDA	10-HHDA	
Free queens						
+m/+t	9	69 ± 32	23 ± 28	6 ± 10	2 ± 5	150 ± 376
−m/+t	7	28 ± 20	33 ± 34	14 ± 16	25 ± 29	6 ± 7
+m/−t	7	66 ± 31	21 ± 19	10 ± 17	3 ± 4	14 ± 14
−m/−t	6	78 ± 5	4 ± 5	11 ± 8	7 ± 2	0.1 ± 0.1
Queens in division boards						
+m/+t	3	72 ± 48	26 ± 45	1 ± 1	1 ± 2	12 ± 18
−m/+t	3	32 ± 29	26 ± 45	2 ± 4	40 ± 33	5 ± 7
+m/−t	3	83 ± 12	9 ± 14	2 ± 2	5 ± 4	9 ± 12
−m/−t	3	71 ± 14	9 ± 10	16 ± 10	5 ± 5	2 ± 3

[a] Symbols for the free queen entries as in Table 6.5. Division board queens as follows: *+m/+t* colonies with access to only head and thorax but head and tergal glands intact; *−m/+t* colonies with access to head (without mandibular gland) and thorax only but tergal glands intact; *+m/−t* colonies with access to intact head and thorax only but tergite occluded; *−m/−t* colonies with access to head (without mandibular gland) and thorax only, and tergite occluded. *9-ODA* 9-oxo-2-decenoic acid; *9-HAD* 9-hydroxy-2-decenoic acid; *10-HDA* 10-hydroxy-2-decenoic acid; *10-HHDA* 10-hydroxy-decanoic acid

differ significantly in the amount of comb constructed (Table 6.5). Finally, significantly more comb was constructed by colonies having access to a whole queen (glands present or not) than by any colony having access to only part of the queen (Table 6.6).

Queens with intact mandibular glands tended to have more total queen substance acids than queens whose mandibular glands had been extirpated (Table 6.6). Although the queens lacking both mandibular and tergal glands tended to have relatively higher percentages of 9-ODA than intact queens, the individual titres are actually quite small in terms of total acid recovered (Table 6.6). The amounts of 9-HDA and 10-HDA did not differ significantly among the various queens. However, more 10-HHDA and 10-HDA were associated with the abdomen than with the heads of queens (Table 6.6). In the division board part of the experiment, queens with intact mandibular glands tended to have more 9-ODA than queens without mandibular glands. Queens without mandibular and tergal glands had significantly more 10-HDA than the other division board queens (Table 6.6). There were no significant differences between the colonies for 9-HDA (Table 6.6). Queens with intact mandibular glands (with/without tergal glands) had significantly more 9-ODA than queens without mandibular glands (Table 6.6). The percentage composition of these pheromones in the abdomens of the division board queens was also measured, but only small amounts of queen substance substance acids were found (Table 6.6). No correlations between any of the pheromones (from the head or abdomen) and the comb construction variables were found.

Table 6.7 Festoon bees with wax scales, scale weight, comb constructed and colony size in colonies headed by *A. m. capensis* virgin queens (Whiffler and Hepburn 1991b)

Treatment[a]	*n*	Colony size	% Bees bearing wax	Wax/bee (μg)	Comb weight (μg)
+m/+t control	6	10909 ± 4103	70 ± 20	426 ± 130	3947 ± 2753
+m/+t	6	9221 ± 5316	80 ± 10	379 ± 99	1653 ± 1660
−m/+t	5	7574 ± 3619	81 ± 15	480 ± 89	3211 ± 4646
+m/−t	3	5489 ± 3631	81 ± 10	392 ± 136	958 ± 1659
−m/−t	3	9443 ± 8100	–	–	0
dq	13	8479 ± 3902	71 ± 5	298 ± 50	291 ± 665
q−	2	10541 ± 8512	70	351	33 ± 47
+m	4	3379 ± 2823	73 ± 24	2540 ± 45	0
+t	4	3777	64	494	0

[a] *+m/+t control* mated queen, with intact mandibular and tergite glands control; *+m/+t* mated queen with intact mandibular and tergite glands; *−m/+t* virgin queen, with mandibular and abdominal tergite glands present; *+m/−t* mated queen, with mandibular and abdominal tergite glands absent; *−m/−t* virgin queen, mandibular and abdominal tergite glands absent; *dq* dead virgin queen, glands intact; *q−* queenless; *+m* virgin queen, colonies with access only to intact head and thorax; *+t* virgin queen, colonies with access only to intact abdomen

The percentages of festoon bees bearing wax and the amounts of wax borne by these bees did not differ significantly among groups with the exception of colonies led by virgin queens without mandibular glands and whose abdominal tergal glands were occluded (Table 6.7). The colonies with mated queens constructed significantly more comb than the other colonies. Although colonies headed by intact virgin queens constructed more comb than was constructed in almost all of the permutations of the virgin queen colonies, there were no significant differences among any of the virgin queen colonies (Table 6.7). No comb was constructed by colonies having virgin queens in division boards (Table 6.7). All of the queens with intact mandibular glands had significantly greater amounts of pheromones than queens without mandibular glands; but there were no significant differences in the percentages of pheromones among the various queens (Table 6.8). There were no colony size effects.

6.3.4 General Conclusions from the Experiments of Whiffler and Hepburn (1991a, b)

The results from many field colonies showed that festoon bees bore the same amount of wax scales, whether queenless or queenright, with either mated or virgin queens, living or dead (Whiffler and Hepburn 1991a). Moreover, removing glands or restricting worker access to the pheromonal sources of the queen had no effect on the amount of wax scales on the festoon bees (Figs. 4.5 and 4.7). Similarly, the amount of wax recovered from individual festoon bees was the same in colonies

Table 6.8 Distribution of mandibular gland components of *A. m. capensis* virgin queens (Whiffler and Hepburn 1991b)

Treatment[a]	Percentage of the components measured				Total (μg/head)
	n	9-ODA	9-HDA	10-HDA	
+m/+t control	6	71 ± 29	28 ± 28	0.8 ± 1	34 ± 33
+m/+t	6	81 ± 21	0	19 ± 21	70 ± 110
−m/+t	5	87 ± 22	0	13 ± 22	8 ± 4
+m/−t	3	65 ± 5	14 ± 16	21 ± 12	116 ± 153
−m/−t	3	64 ± 32	0	36 ± 32	2 ± 2
dq	13	72 ± 26	1 ± 2	27 ± 24	10 ± 9
+m	4	43 ± 51	0	24 ± 41	4 ± 4

[a] Symbols under treatment are the same as those in legend for Table 6.7. *9-ODA* 9-oxo-2-decenoic acid; *9-HAD* hydroxyl-2decenoic acid; *10-HAD* 10-hydroxy-2-decenoic acid

preparing to swarm and in moving swarms (Hepburn 1988; Hepburn and Whiffler 1988). That *A. m. scutellata* workers with an *A. m. capensis* queen bore significantly more wax/bee than was recorded in the reverse arrangement is regarded as anomalous. The data overwhelmingly support the conclusion that the actual secretion of wax by workers is not influenced by queen status. The percentage of bees bearing wax scales was the same whether the bees were queenright or queenless, and whether headed by their own queen or one of a different race (Table 6.3). Similarly, the percentage of bees bearing wax did not significantly differ among colonies whose queens were caged or held in division boards, whether the mandibular and abdominal tergal glands were intact or not (Tables 6.2, 6.4, 6.5).

While the percentage of festoon bees bearing wax and the mass of wax actually borne by bees are independent of queen status, comb-building is entirely different. Colonies headed by mated queens constructed significantly more comb than did queenless colonies of both *A. m. capensis* and *A. m. scutellata* (Table 6.3). These results are entirely consistent with those of all other similar studies on *A. mellifera* (Gundelach 1842; Darchen 1957; Goetze and Bessling 1959; Verheijen-Voogd 1959; Jay and Jay 1983), and *A. cerana* (Rajashekharappa and Channabasavanna1979). Because the mandibular gland bouquets of *A. m. capensis* virgin queens, unlike those of other races, approximate those of mated queens (Crewe 1988; and Tables 6.5 and 6.7), colonies headed by mated and virgin queens may construct similar amounts of comb. However, colonies headed by virgin *A. m. capensis* queens constructed as little comb as did queenless *A. m. capensis* colonies (Tables 6.1, 6.2, 6.6). This result is the same as that obtained for both *A. mellifera* (Verheijen-Voogd 1959) and *A. cerana* (Rajashekharappa and Channabasavanna1979). Collectively, these results indicate that the bouquet of the mandibular gland of the queen cannot alone fully explain enhanced comb-building by qeenright workers.

Whatever the source of the comb-building stimulus, its effect requires direct contact with the queen (Table 6.2) because more combs are built when workers have full access to a free-running, mated queen (both a *physical* and *chemical*—pheromonal—queen). Indeed, a queen moving around the nest maybe essential to

its means of chemical communication (Velthuis 1976, 1985, 1990). Little comb is built when the colony has access to only the 'chemical' queen (double-layered caged queen) or the 'physical' queen (=dead queen). The limitations of the 'chemical' queen are further indicated by the fact that there were no significant differences in the percentage composition of mandibular gland secretions of the various queens.

In summary, the results indicate the likelihood that pheromones of the queen, whether from the mandibular glands or elsewhere in the head, acquired through contact chemoreception, stimulate comb construction in honeybees. Chemoreception is as important for comb-building as it is for the inhibition of ovarial development in worker bees (Verheijen-Voogd 1959; Velthuis 1970) and for emergency queen cell construction (Butler 1960). It is also evident that the queen has little effect on wax secretion, a physiological process aptly described in the 19th century as the 'involuntary' secretion of wax (Gundelach 1842). The independence of wax secretion, as opposed to comb-building, from the pheromonal influence of the queen (Whiffler and Hepburn 1991a) was also confirmed in a different set of experiments by Ledoux et al. (2001).

6.4 Comb-Building Experiments of Ledoux et al. (2001)

Apparently unaware of the earlier work by Whiffler and Hepburn (1991a, b), Ledoux et al. (2001) reinvestigated the role of the queen in comb-building. They investigated the influence of the queen and her pheromonal signals on comb-building using four groups of *A. mellifera* colonies as follows: (1) 8 colonies with mated queens; (2) 8 others with virgin queens; (3) 8 others queenless but containing a synthetic queen substance pheromone dispenser and finally (4) 8 colonies lacking queens and pheromone dispensers. After 10 days the combs produced and the sizes of the wax scales were measured. Ledoux et al. (2001) estimated mean wax scale size per *A. mellifera* worker bee for colonies with mated queens, others with synthetic queen substance dispensers, others with virgin queens as well as queenless colonies (Fig. 6.1) which again confirm the results of Whiffler and Hepburn (1991a) on the physiological independence of wax secretion from any pheromonal effects of the queen. Their results clearly show that the colonies with mated queens constructed significantly more comb by area (Fig. 6.2) and weight (Fig. 6.3) than the other colonies (Fig. 6.4). Ledoux et al. (2001) also reconfirmed that queenless workers build substantially less comb and usually drone size cells indicating that both cell size and the quantity of comb built are mediated through the queen. These results have been observed many times previously for *A. mellifera* (Dreischer 1956, Goetze and Bessling 1959, Darchen 1968, Whiffler and Hepburn 1991a).

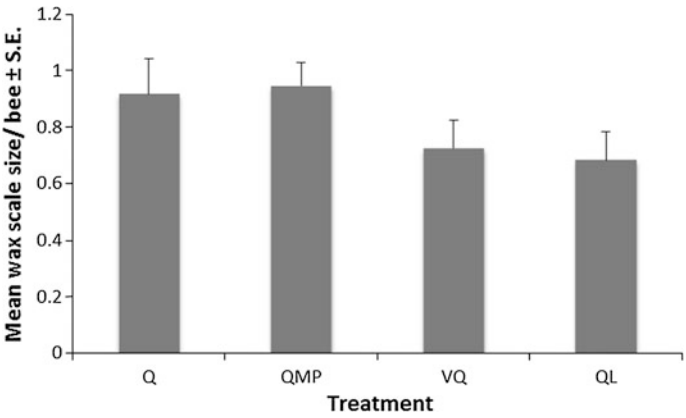

Fig. 6.1 Mean wax scale size/bee obtained from colonies headed by mated queens (*Q*), virgin queens (*VQ*), queen pheromone dispenser (*QMP*) and queenless (*QL*) *A. mellifera* colonies (Ledoux et al. 2001)

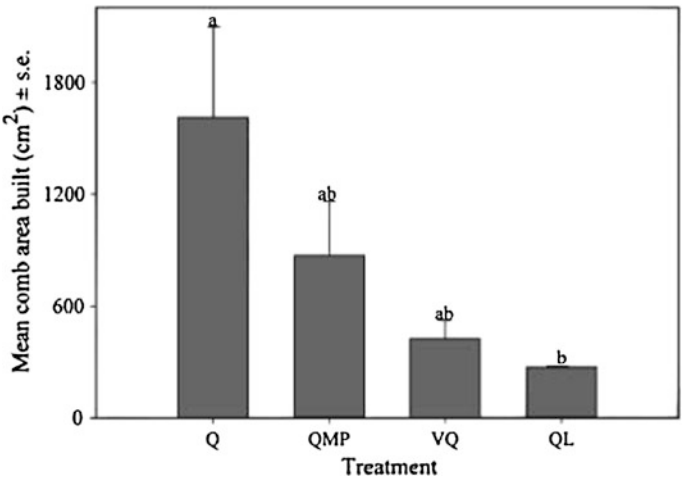

Fig. 6.2 Mean comb area constructed by colonies headed by mated queens (*Q*), virgin queens (*VQ*), queen pheromone dispenser (*QMP*), and queenless (*QL*) *A. mellifera* colonies (Ledoux et al. 2001)

6.5 Perception of Queenrightness

The construction and the repairs of combs is the very last step in the elaboration of wax by bees. Clearly manipulations of wax must be preceded by the entrainment and development of the wax gland system itself, and then by the actual secretion of wax. In the various experiments described above, attempts were made to assay the

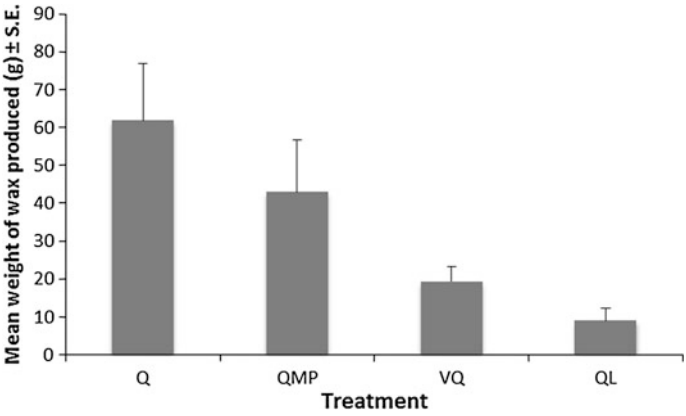

Fig. 6.3 Mean weight of comb constructed by colonies headed by mated queens (*Q*), virgin queens (*VQ*), queen pheromone dispenser (*QMP*), and queenless (*QL*) *A. mellifera* colonies (Ledoux et al. 2001)

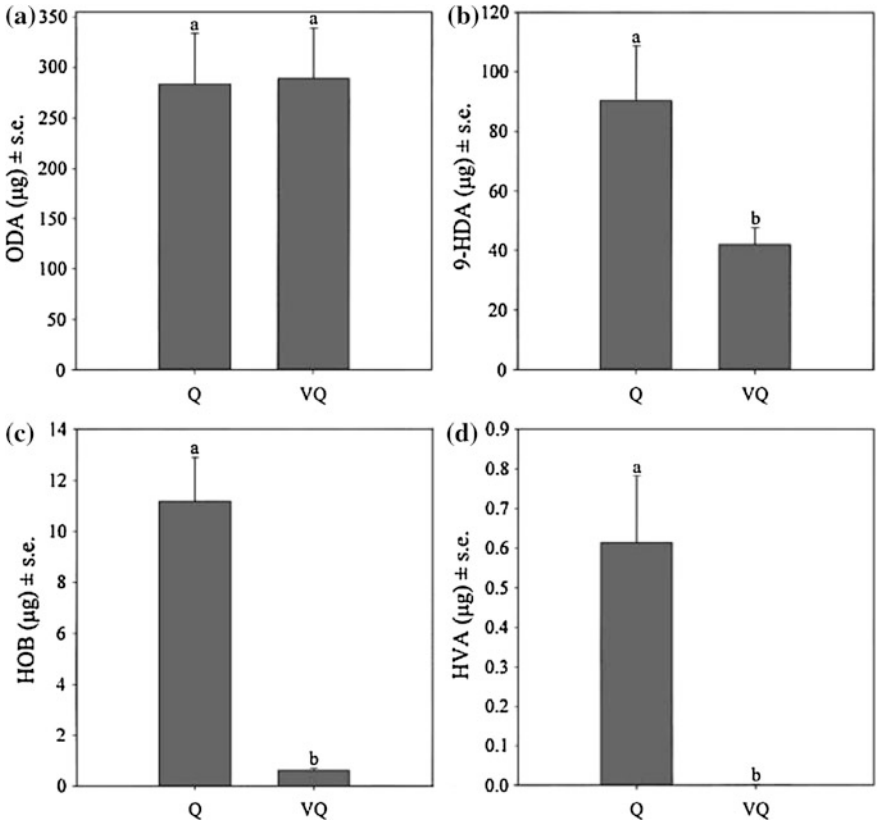

Fig. 6.4 Comparison of queen substance components between mated and virgin *A. mellifera* queens. *Q* mated queen, *VQ* virgin queen, *A* 9-ODA, *B* 9-HDA, *C* HOB, *D* HVA (Ledoux et al. 2001)

role of the queen or of queen-like odours in the separate development of each of the three phases. Knowledge in these areas is fragmentary. Although Dreischer (1956) did not mention the size of her colonies (they must have been smallish to have been kept in observation hives), she found that the histological development of the wax glands (measured as the height of the epidermis) were more or less the same in bees from either queenright or queenless colonies. The more precise experiments and measurements of Goetze and Bessling (1959) also showed that there were no significant differences in the extent of wax gland development in small (100) queenright or queenless colonies of bees. Dreischer, Darchen, Goetze and Bessling and Free all worked in apiaries, so that queenless bees were never physically far removed from normal queenright colonies. Thus the possibility of shared pheromones was not entirely precluded (an effect well known in pallet beekeeping.

How a honeybee queen is perceived by the workers of her colony has long been a question of considerable interest. In one such study on wax glands, Hepburn et al. (1984) compared the development of the wax glands of 12-day-old bees taken from queenright and queenless colonies of 500 bees each. The queenless colonies were of two kinds: some, although kept in their own hives, shared an environmental chamber with queenright colonies; the other queenless bees were kept well isolated from queenright bees in a room in which bees had never been kept. Under these conditions, there was no significant difference in the development of the wax glands between the queenright bees and those queenless bees sharing the compartment; however, the wax glands were significantly less well-developed in those queenless bees which had been kept apart. The matter of the actual secretion of wax, after development of the glands and before comb construction, has always been extremely difficult to assess in a direct experimental way.

If a given bee of suitable age is examined and found to lack wax scales, their absence does not necessarily indicate that the bee is not actively secreting wax—it may have just contributed wax scales to the building effort. Nonetheless, Goetze and Bessling (1959) tried to assess secretory activity by measuring the standing crop of scales in 6-, 12- and 20-day-old bees taken from queenright and queenless colonies. The queenright bees bore a 40 % greater mass of wax than did the queenless ones. More convincingly, on the 20th day of the experiment, the queenright bees had constructed about 20 % more comb (713 mg) than the queenless bees (586 mg). In the absence of any analysis of the pheromones of these bees, one would most likely conclude that the rates of secretion of wax, as well as comb construction, are modulated by queen pheromones. It would appear, then, that young bees are capable of developing their wax glands in the absence of the queen bouquet, but that the extent of this development might be slightly enhanced by her presence. Given developed wax glands, the same would be true for the rate of secretion. Comb construction itself depends greatly upon the quality or ‘state’ of the queen. All things being equal, some egg-laying-workers and virgin queens stimulate comb construction, but not to the same extent as mated queens, which strongly indicates the importance of the relative composition of the queen-like scent as the driving force in comb construction.

The perception of "queenness" by bees had led Darchen (1956b, 1957) to believe that there is a construction pheromone which lingers on after the death of a queen. He and his colleagues (Chauvin et a1. 1961) and another worker (Frichot-Riera 1961) prepared crude ether/acetone extracts of queens and were able to induce comb construction in the absence of a queen by giving bees these compounds on filter paper. That the perception of these compounds is by smell is supported by two observations. When the extracts were combined in a candy or, indeed, if the queens themselves were added to a candy paste and fed to the bees, the bees did not construct combs. Nonetheless, the possible significance of the tactile properties of the queen cannot be ignored, as was shown in the experiments of Müssbichler (1952) and other workers.

Finally, Darchen (1956b, 1957) extended his experiments to comb-building by queenless bees; he used 5,000 and 15,000 workers in two different colonies. His results are quite interesting (Table 6.9). The 5,000 queenless bees began to construct combs after two weeks had passed and the presence of laying-workers had been confirmed. In the case of the 15,000 bees, virtually the obverse result was obtained (Table 6.9). These seemingly anomalous results can now be satisfactorily interpreted with respect to queen substance or pheromones, following a brief digression on laying-workers.

Although Riem (1770) was apparently the first to observe that worker bees sometimes lay eggs, it was the redoubtable Huber (1814) who established time and again that some workers lay eggs in the absence of a queen. Moreover, he had shown that the ovaries of such bees were more developed than those of ordinary workers which did not lay eggs. This has been confirmed many times and it has also been shown that ovarian development, in the absence of a queen, proceeds independently of age (Perepelova 1928), but is certainly subject in some way to group effects (Hess 1942). Nonetheless, laying-workers certainly occur in perfectly normal queenright colonies of the Cape honeybee, *A. m. capensis* (Onions 1912). An historical account of the research related to the origin of laying-workers is beyond our present needs and the subject has been adequately reviewed over the years (Ribbands 1953; Velthuis et al. 1965; Visscher 1989; Hepburn 1994).

Following the discovery that 'queen substance' is actually a collection of several different compounds (Boch et al. 1979), Crewe and Velthuis (1980) were able to recover these same chemicals from worker bees. Moreover, they were able to recognise two kinds of laying-workers in pheromonal terms: those that develop all the components of a queen-like bouquet and thus function as false queens, and those that retain the characteristic aroma of worker bees (Table 6.10). These classes cannot yet be readily resolved with the 'anatomical' and 'physiological' laying worker classes mooted by Perepelova (1926), but actually occur in a graded spectrum of such bees (Hepburn 1994). These important results of Crewe and Velthuis (1980) allow us some latitude in explaining Darchen's (1956b, 1957) final experiments on comb construction by queenless bees. We note, referring to Table 6.10 that the appearance of laying-workers coincided with comb construction in the one case, which would be consistent with the development, in at least one of those laying-workers, of a queen-like complement of chemical signals.

Table 6.9 Comb construction by queenless *A. mellifera* colonies (Darchen 1956b, 1957)

A. Colony of 5,000 bees													
Experimental days	1–13	15	16	17	18–19	20–21	22–23	24	25				
Comb (cm^2)	0	11	46	60	124	226	76	32	25				
B. Colony of 15,000 bees													
Experimental days	1–2	3–4	5	6	7	8	9	10–11	12	13	14	15	16
Comb (cm^2)	270	89	126	78	44	28	62	96	12	5	13	5	3

Table 6.10 Mandibular gland substances in *A. mellifera* workers and queens and workers in relation to activation of the ovaries (Crewe and Velthuis 1980)

Group [a]		Total acids (μg/head)	Components present (%)						
			1	2	3	4	5	6	7
A	4 individuals	1.5	100.0						
	10 individuals	3.0	87.0	13.0					
	1 individuals	4.3	78.4	15.7	5.9				
	2 laying workers	4.5	81.4	13.6	5.1				
B	3 laying workers	5.4	65.2	34.8					
	7 laying workers	6.0	80.3	12.2	7.5				
	1 laying worker	22.5	52.5	7.8	12.0		18.3	9.4	
C	17 *A.cap*/*A.cap*	61.5	42.4	3.0	6.8		33.9	14.0	
	3 *A.cap*/*A.mell*	22.4	5.5	0.5	7.7		76.2	10.0	
D	7 1-day queens	136.7	61.8	1.8	7.0		26.5	2.3	0.6
	5 mated, laying queens	197.2	12.1	7.9	32.2	2.4	36.1	6.9	2.3

[a] Groups are as follows: *A* 2 laying-workers and 15 other workers (chosen at random) from an isolated group of 50 *A. m. mellifera* workers; *B* 11 laying-workers from a colony of queenless *A. m. mellifera* workers; *C* individual *A. m. capensis* workers from groups of either 5 *A. m. capensis* workers (*A. cap*/*A. cap*) or one *A. m. capensis* and 4 *A. m. mellifera* workers (*A. cap*/*A. mell*); *D A. m. mellifera* queens of different ages. Abbreviations for the compounds present are as follows: 1 = (E)-10-hydroxy-2-decenoic acid; 2 = 10-hydroxydecanoic acid; 3 = (E)-9-hydroxy-2-decenoic; 4 = (E)-9-oxo-2-decenoic acid; 6 = 8-hydroxyoctanoic acid; 7 = methyl *p*-hydroxybenzoate (tentative) (Crewe and Velthuis 1980)

On the other hand, in the queenless colony of 15,000 bees that initially produced combs and then curtailed their operations, we would surmise for the sake of consistency that a pheromonally acceptable false queen was present over the first 10 days, following which its queen-like bouquet waned—just as is thought to be the case in queen cell construction under supersedure conditions.

In yet another study of comb-building, Yang et al. (2010) reported the results of studies on comb-building in mixed-species, *A. cerana* and *A. mellifera*, colonies of honeybees in which three colonies of mixed workers in one group were given *A. cerana* queens, and three others *A. mellifera* queens. Three additional colonies of each species headed by their own queens served as controls. Although they were

interested in the nature of comb-building (numbers of each species of workers in festoons, cell sizes, etc.) under the different treatments (which is discussed elsewhere (cf. Chap. 3). What is germane here is that both species of workers engaged in comb-building whether headed by conspecific or heterospecific queens. This was despite some obvious differences in the relative composition of the mandibular glands of *A. cerana* and *A. mellifera* queens.

6.6 Comb-Building Experiments of Maisonnasse et al. (2010)

Maisonnasse et al. (2010) reinvestigated the role of queen substance in comb-building in *A. mellifera,* and noted that although pleiotropic effects on colony regulation are accredited to queen substance, it does not elicit the same range of worker response observed in the presence of a queen, suggesting that yet other compounds may come into play. They tested the hypothesis of a pheromone redundancy in honeybee queens by comparing the effects of queens with and without mandibular glands on a variety of worker behaviours, of which here we only consider the comb-building experiments. The experiments by Maisonnasse et al. (2010) confirmed that 9-ODA is uniquely produced in the queen mandibular glands and suggested the existence of another source of the production of HOB and 9-HAD, as found in *A. m. capensis* and *A. m. scutellata* queens by Whiffler and Hepburn (1991a).

Maisonnasse et al. (2010) asked whether queens lacking mandibular glands were as effective as normal queens in regulating ovary activation, comb construction and retinue behaviour. Maisonnasse et al. (2010) tested the effects of queens lacking mandibular glands and normal queens on comb construction in cage experiments. Three different groups were tested: cages with normal queens (MG+: positive control); queenless cages (QL: negative control); and cages with demandibulated queens (MG−). After 2 weeks, the combs from each cage were collected and the number of cells counted. The mean diameter of 20 cells per treatment cage was determined, and divided into two categories according to their size. In addition, the number of queen cells was counted in the different groups. Three replicates were performed giving a total of 125 data sets. Maisonnasse et al. (2010) found that comb size significantly increased in the presence of queens (MG+, MG−) compared to QL, however no differences were detected between the two types of queens. Workers reared with MG+ and MG− queens built worker-sized cells that did not differ, but QL workers built drone-sized cells. No queen cells were constructed in either MG+ or MG− groups; however, QL workers constructed one to three queen cells per cage.

These results clearly show that demandibulated queens retain their full regulatory functions, which is in agreement with the studies of Velthuis and Van Es (1964) and Velthuis (1970). The data of Maisonnasse et al. (2010) suggest that

queen substance is not solely responsible for the regulation of colony function by the queen. In addition, by testing the effect of mandibular gland removal on the composition of 9-ODA, 9-HDA and HOB, they showed that demandibulated virgin queens were as effective as normal virgin queens in regulating colony function. Interestingly, workers in MG− group produced worker-sized cells, and built a large number of cells, as in the MG+ group, in contrast to the QL group in which workers constructed a small number of drone-sized cells. Thus, their results indicate that comb construction is also regulated by queen chemicals other than 'classical' queen substance.

In the absence of queens, *A. m. capensis* workers that reproduce via thelytokous parthenogenesis , and *A. m. scutellata* that reproduce via arrhenotokous parthenogenesis, build only worker or drone cells respectively, but queenless hybrid colonies produce both or only worker cells (Neumann et al. 2000). This would support the idea that comb construction can be regulated by chemicals other than queen-derived substances that are also produced by some workers. However, since *A. m. capensis* workers develop queen-like pheromonal bouquets high in 9-ODA (Simon et al. 2001), the construction of worker cells in those queenless colonies could also be due to this pheromone.

All the experiments on comb construction (excluding queen cells) lead us to believe that the presence of certain chemicals, formerly designated as 'queen substance', result in the construction of considerably more comb than is produced in their absence, whether the chemicals come from a queen or a false queen (laying-worker). Nonetheless, one still observes that some bees will secrete wax and construct comb whether these signals are present or not. A pheromonal basis for construction was suggested by Darchen (1956b, 1957). The subsequent identification of these substances by Boch et al. (1979) and their recognition in both queens and workers (Crewe and Velthuis 1980) provide a platform for a further analysis of these compounds in relation to wax synthesis and comb-building. The pheromonal quality of a queen is obviously important and it seems inescapable that some vehicle, in addition to olfactory perception of a 'queen', is required. The construction of emergency queen cells appears to work in exactly the same way (Huber 1814; Müssbichler 1952; Verheijen-Voogd 1959).

6.7 The Construction of Queen Cells

At first sight it may appear that secretions from the mandibular glands in living queens are necessary to stimulate comb-building, because significantly more combs are constructed by bees with access to whole queens having intact mandibular glands than bees whose queens lacked them (Fig. 6.4). The results of the division board experiments do not fully support this idea because workers having access to only the head of a queen constructed similar amounts of comb whether mandibular glands were present or not (Table 6.4). It is possible that some pheromonal secretions of the queen's head other than those of the mandibular glands, provide a

comb-building stimulus. Any contribution from possible secretions from their abdomens is doubtful because no combs were built when workers only had access to the abdomens of queens (Table 6.4). This interpretation would be consistent with observations that queens without mandibular glands still maintain control of colony behaviour (Gary and Morse 1962; Velthuis and van Es 1964; Velthuis 1970; Butler et al. 1974; Free 1987), and with others that challenge the role of the queen's mandibular gland (Slessor et al. 1990).

That the queen may have a specific relationship to the synthesis and secretion of wax as well as to comb-building has been moot for several centuries. Indeed, de Réaumur (1740) was the first to note that a caged colony of queenless bees had constructed comb after 2 days confinement. However, he had given the bees some queen cells and, unfortunately, we do not know how long the bees had been queenless prior to their incarceration, or whether they had been given any other brood. A far more instructive experiment was performed by Schirach (1770) who observed that, on the loss or removal of a queen, a colony would construct emergency queen cells over some of the worker cells containing eggs or larvae, and new queens would be reared from them. This important result was confirmed in numerous experiments by Huber (1814) and is the basis for the queen-rearing industry of today. Huber also knew that queenright colonies normally construct queen cells in spring in preparation for reproductive swarming. So queen cells may be constructed in the presence or absence of a queen.

These somewhat ambivalent results led Huber (1814) to another experiment in which a hive was so divided that about half the bees were in direct contact with their queen, while the other half had access only to the odour and sounds of the queenright half of the colony. In this situation, the 'queenless' half of the colony began to construct queen cells, the other half did not. In a slightly different experiment, Huber (1814) simply placed a queen in a cage and inserted it in a colony of bees. All the workers were able, in theory, to feel the queen through the cage with their antennae. In this case, no queen cells were constructed. Huber's results have subsequently been confirmed by Lehnart (1935) and their complexity extended by the work of Müssbichler (1952). The latter author added the refinement of dividing the colony with a screen through which the queenless half of the colony could reach the queen with their antennae. Nonetheless, these bees began to construct queen cells. If, as in Huber's experiment, the queen was simply caged among the bees, no queen cells were built. However, if a caged queen was placed on one side of a screen, thus dividing a colony in half, those bees on the opposite side of the screen, away from the queen, began the construction of queen cells.

That the absence of a queen may provoke the building of queen cells (Schirach 1770; Huber 1814), coupled with the results of Müssbichler (1952), suggest a restriction in the flow of some material from the queen. This inevitably leads us to consider: (1) the means by which workers are aware of their queen; (2) how the presence or absence of a queen affects the behaviour of workers; and (3) what precisely do the worker bees do with respect to wax secretion or the building of emergency cells. Within a few hours after the loss of a queen, a general agitation spreads amongst the workers (Huber 1814; Fell and Morse 1984; Skirkevicius

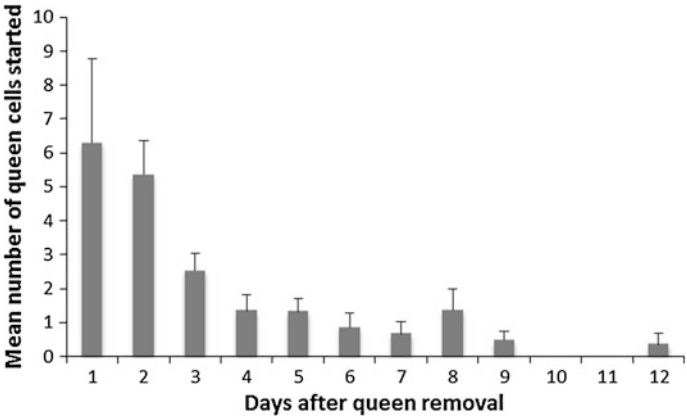

Fig. 6.5 Mean number of queen cells begun each day after removal of an *A. mellifera* queen, but before the emergence of a new queen. N = 13 for days 1–9, n = 12 on day 110, n = 11 for day 11, and n = 9 for day 14 (Fell and Morse 1984)

2004), the rate at which this happens being apparently related to the size of the colony. When disturbed, such bees make a noise by vibrating their wings and they also release a scent. All this kind of behaviour is however suppressed if such queenless bees are given a dead queen. Fell and Morse (1984) quantified some of the changes associated with the removal of a queen. The rate of queen cell construction is at first high and then rapidly declines (Fig. 6.1). Similar results were obtained with *A. m. capensis* (Hepburn et al. 1988) (Fig. 6.5).

Queen cell construction is inversely related to scenting behaviour (an index of the degree of agitation) of the queenless colony (Skirkevicius 2004). All these observations on the construction of queen cells in the face of apparent emergency (and others on the suppression of ovarial development in workers) finally led to experimental confirmation of the increasingly pervasive idea that a queen secretes substances, the presence or absence of which modifies the behaviour of the worker bees (Butler 1954; Pain 1954; Voogd 1955). The importance of queen substance to building is dramatically illustrated in the experiment by Darchen (1960) in which a small colony of several hundred bees, which had been queenless for 3 months, began the construction of comb only 2 days after the introduction of a dead queen.

In an entirely different set of experiments, Lensky and Darchen (1962) introduced two caged queens (presumably having twice the amount of queen substance) into a small colony, but the workers soon began the construction of queen cells. In a second experiment they placed three queens (2 old and 1 young one) into such a colony and again queen cells were constructed. Finally, three young caged queens did not inhibit the construction of queen cells. Earlier experiments with *A. mellifera* similar to these were performed by Melnik (1951) and another by Kovtvn (1949) with the opposite result. Finally, it has been shown that certain anxiolytic drugs enhance queen cell construction in queenless bees (Leonard and Darchen 1978).

The interpretation of all these results is difficult in the absence of any measurements of queen substance. Such signals might have come from laying workers (discussed below), and then there are the possible synergistic effects of workers in the even distribution of queen substance. Even though it has been shown that the presence of a queen sometimes inhibits queen cell construction, an effect specifically attributed to 9-ODA secreted by the queen (Butler and Callow 1968; Boch and Lensky 1976; Lensky and Slabezki 1981), an explanation for switching on or off queen cell production under emergency conditions or otherwise, based solely on the constituents of queen substance, is inadequate (Winston et al. 1990, 1991). Nonetheless, Grozinger et al. (2003) demonstrated that queen mandibular pheromone caused changes in gene expression in the brain of adult worker honeybees, and that these changes can be correlated to downstream behavioural responses induced by queen mandibular pheromone. Their data demonstrate that queen substance regulates expression of several hundred genes either transiently or chronically. Clearly, pheromone-mediated gene expression could be expected to modulate worker behaviour in the inhibition of queen cell construction.

Other queen cell constructions, probably more common in nature, have received less attention than has the emergency queen cell. The little concrete information available on the construction of queen cells for the purposes of reproductive swarming or supersedure has been summarised by Ribbands (1953) and Butler (1957, 1974). Of the former, we know that comb construction abates and that queen cell construction begins despite the fact that one of the queen substances, 9-ODA, appears in quantities that are indistinguishable in swarming and non-swarming queens (Seeley and Fell 1981). Obviously the presence of a single component of the queen signal is neither necessary nor sufficient to explain the commencement or cessation of either comb construction or the building of queen cells. As to queen cell construction for supersedure, it is commonly believed to be related to a decline in the production of essential queen substances, but there is no experimental evidence to support this idea. Moreover, were this true, it is likely that supersedure is only quantitatively different from queen cell construction under emergency conditions (Ruttner 1983).

It has long been supposed that new queens are not produced by workers if adequate amounts of queen mandibular gland pheromone are present and circulating among the bees (Butler 1954; Butler and Simpson 1958; Winston et al. 1989, 1990, 1991). However, only relatively recently, Naumann et al. (1993) explored the possible transfer and dissemination of queen pheromone by comparing populous colonies with other less populous ones. They used synthetic queen substance containing tritiated 9-keto-2(E)-decenoic acid, as a marker component of queen substance. Considering their results, we think that colony crowding does not significantly affect the dissemination and transportation of queen substance and that their data better support their alternate sub-hypothesis: that queen-rearing could be associated with changes in the thresholds of the worker bees at swarming time.

References

Boch R, Lensky Y (1976) Pheromonal control of queen-rearing in honeybee colonies. J Apic Res 15:59–62

Boch R, Shearer DA, Shuel RW (1979) Octanoic and other volatile acids in the mandibular glands of the honeybee and in royal jelly. J Apic Res 18:250–252

Butler CG (1954) The method and importance of the recognition by a colony of honey-bees (*A. mellifera* L.) of the presence of its queen. Trans R Entomol Soc Lond 105:11–29

Butler CG (1957) The process of queen supersedure in colonies of honeybees (*Apis mellifera* L.). Insectes Soc 4:211–223

Butler CG (1960) Queen recognition by worker honeybees (*Apis mellifera* L.). Experientia 16:424–426

Butler CG (1974) The world of the honeybee. Collins, London

Butler CG, Callow RK (1968) Pheromones of the honeybee (*Apis mellifera* L): the "inhibitory scent" of the queen. Proc R Entomol Soc Lond 43:62–65

Butler CG, Simpson J (1958) The source of the queen substance of the honey-bee (*Apis mellifera* L.). Proc R Entomol Soc Lond 33:120–122

Butler CG, Callow RK, Greenway AR, Simpson J (1974) Movement of the pheromone 9-oxodec-2-enoic acid, applied to the body surfaces of honeybees (*Apis mellifera*). Entomol Exp Appl 17:112–116

Chauvin R, Darchen R, Pain J (1961) Sur l'existence d'une hormone de construction chez les Abeilles. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 253:1135–1136

Crewe RM (1982) Compositional variability: the key to the social signals produced by honeybee mandibular glands. In: Breed MD, Michener CD, Evans HE (eds) The biology of social insects. Westview, Boulder, pp 318–322

Crewe RM (1988) Natural history of honey-bee mandibular gland secretions: development of analytical techniques and the emergence of complexity. In: Needham GR, Page RE, Delfinado-Baker M, Bowman CE (eds) Africanized honey bees and bee mites. Ellis Horwood Limited, Chichester, pp 149–156

Crewe RM, Velthuis HHW (1980) False queens: a consequence of mandibular gland signals in worker honeybees. Naturwissenschaften 67:467–469

Darchen R (1956a) La construction sociale chez *Apis mellifera*. Insectes Soc 3:293–301

Darchen R (1956b) La reine d'*Apis mellifica* et les constructions cirières. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 243:439–441

Darchen R (1957) La reine d'*Apis mellifica*, les ouvrieres pondeuses et les constructions cirières. Insectes Soc 4:321–325

Darchen R (1960) Les regulations neurohormonales de l'instinct constructeur d' *Apis mellifera*. Ann Abeille (Paris) 3:329–333

Darchen R (1968) Le rôle de la reine dans la construction cirière. In: Chauvin R (ed) Traité de biologie de l'abeille. Masson, Paris, pp 305–324

de Groot AP, Voogd S (1954) On the ovary development in queenless worker bees (*Apis mellifera* L.). Experientia 10:384

de Réaumur RAF (1740) Memoires pour servir a l'histoire des insects, vol 5. L'Imprimerie Royale, Paris

Dreischer H (1956) Untersuchungen über die Arbeitstätigkeit und Drüsenentwicklung altersbestimmter Bienen im weisellosen Volk. Zool Jb Allg Zool Physiol Tiere 66:429–472

Fell RD, Morse RA (1984) Emergency queen cell production in the honey bee colony. Insectes Soc 31:221–237

Free JB (1967) The production of drone comb by honeybee colonies. J Apic Res 6:29–36

Free JB (1987) Pheromones of social bees. Chapman and Hall, London

Frichot-Riera M (1961) Le test de Darchen sur la construction de la cire. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 263:334–336

Gary NE (1961) Mandibular gland extirpation in living queens and worker honey bees *Apis mellifera* L. Ann Entomol Soc Am 54:529–531
Gary NE, Morse RA (1962) Queen cell construction in honeybee (*Apis mellifera* L.) colonies headed by queens without mandibular glands. Proc R Entomol Soc Lond A 37:76–78
Goetze G, Bessling BK (1959) Die Wirkung verschiedener Fütterung der Honigbiene auf Wachserzeugung und Bautätigkeit. Zeit Bienenforsch 4:202–209
Grozinger CM, Sharabash NM, Whitfield CW, Robinson GE (2003) Pheromone-mediated gene expression in the honey bee brain. Proc Natl Acad Sci 100(Suppl 2):14519–14525
Gundelach FW (1842) Die Naturgeschichte der Honigbienen. Cassel, Bohne
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR (1988) Absconding in the African honeybee: the queen, engorgement and wax secretion. J Apic Res 27:95–102
Hepburn HR (1994) Reproductive cycling and hierarchical competition in Cape honeybees, *Apis mellifera capensi*s Esch. Apidologie 25:38–48
Hepburn HR, Whiffler LA (1988) Dwarfs and queenballs in swarms of the Cape honeybee. S Afr J Sci 84:851–852
Hepburn HR, Hugo JJ, Mitchell D, Nijland MJM, Scrimgeour AG (1984) On the energetic costs of wax production by the African honeybee, *Apis mellifera adansonii* (=*scutellata*). S Afr J Sci 80:363–368
Hepburn HR, Nefdt RJC, Whiffler LA (1988) Queen loss in the Cape honeybee: the interactions of brood, laying workers (false queens) and queen cells. S Afr J Sci 84:778–780
Hess G (1942) Ueber den Einfluss der Weisellösigkeit and des Fruchtbarkkeitsvitamine E auf die Ovarien der Bienenarbeiterin. Schweiz Bienenztg 1:33–109
Huber F (1814) Nouvelles observations sur les abeilles. Barde, Manget, Genèvre (English translation, 1926) Dadant, Hamilton
Jay SC, Jay DH (1983) Wax production by caged worker honey bees of European and tropical African origin. J Apic Res 22:229–231
Kovtun FN (1949) How to make and use multiple-queen colonies. Pchelovodstvo 26:413–414 (in Russian)
Ledoux MN, Winston ML, Higo H, Keeling CI, Slessor KN, Le Conte Y (2001) Queen and pheromonal factors influencing comb construction by simulated honey bee (*Apis mellifera* L.) swarms. Insectes Soc 48:14–20
Lehnart A (1935) Die Schwarmsache. Rhien Bienenztg 66:143–145
Lensky J, Drachen R (1962) Queslques observations sur la construction des royales en presence de plusiers reines d'abeilles (*Apis mellifica*, var. *ligustica*). C R Hebd Seances Acad Sci Ser D Sci Nat Paris 9:1778–1780
Lensky Y, Slabezki Y (1981) The inhibiting effect of the queen bee (*Apis mellifera* L.) foot-print pheromone on the construction of swarming queen cups. J Insect Physiol 27:313–323
Leonard G, Darchen R (1978) Essais de quelques substances sur le comportment constructeur de cellules royales d'abeilles orphelines (*Apis mellifica* L.). C R Hebd Seances Acad Sci Ser D Sci Nat Paris 286D:1899–1902
Maisonnasse A, Alaux C, Beslay D, Crauser1 D, Gines D, Plettner E, Le Conte Y (2010) New insights into honey bee (Apis mellifera) pheromone communication. Is the queen mandibular pheromone alone in colony regulation? Front Zool 7:18–25
Melnik (1951) Managing multiple-queen colonies. Pchelavodstvo 28:36–37
Müssbichler A (1952) Die Bedeutung äusserer Einflüsse und der Corpora allata bei der Afterweiselentstehung von *Apis mellifica*. Z Vergl Physiol 34:207–221
Naumann K, Winston ML, Slessor KN (1993) Movement of honey bee (*Apis mellifera* L.) queen mandibular gland pheromone in populous and unpopulous colonies. J Insect Behav 6:211–223
Neumann P, Hepburn HR, Radloff SE (2000) Modes of worker reproduction, reproductive dominance and brood cell construction in queenless honeybee (*Apis mellifera* L.) colonies. Apidologie 31:479–486
Onions GW (1912) South African 'fertile worker bees'. Agric J Un S Afr 3:720–728

Pain J (1954) Sur l'ectohormone des reines d'abeilles. CR Hebd Seances Acad Sci Ser B Sci Nat Paris 239:1869–1870
Pain J (1955) Dosage biologique et spectrographique de l'ectohormone des reines d'abeilles. CR Hebd Seances Acad Sci Ser B Sci Nat Paris 240:670–672
Perepelova LI (1926) Biology of laying workers. Opyt Pas 1:8–10 (in Russian)
Perepelova LI (1928) Biology of laying workers. Opyt Pas 3(6–10):59–61 (in Russian)
Plettner E, Otis GW, Wimalaratne PDC, Winston ML, Slessor KN, Pankiw T, Punchihewa PWK (1997) Species- and caste-determined mandibular gland signals in honeybees (*Apis*). J Chem Ecol 23:363–377
Rajashekharappa BJ, Channabasavanna GP (1979) Importance of mated queen in comb expansion of the honeybee *Apis cerana*. Mysore J Agric Sci 13:51–54
Ribbands CR (1953) The behaviour and social life of honeybees. Bee Research Association, London
Riem J (1770) Fundamentalgesetze zu einer perennierenden Kolonie-Bienenpflege (cited from von Buttel-Reepen, 1915)
Ruttner F (1983) The events which take place during the natural replacement of the queen in a colony of bees. In: Ruttner F (ed) Queen-rearing. Apimondia, Bucharest
Schirach AG (1770) Ausführliche Erläterung der unschätzbaren Kunst junge Bienenschwärme oder Ableger zu erziehen. Budissa, Drachstadt
Seeley TD, Fell RD (1981) Queen substance production in honey bee (Apis mellifera) colonies preparing to swarm (Hymenoptera: Apidae). J Kans Ent Soc 54:192–196
Simon UE, Moritz RFA, Crewe RM (2001) The ontogenetic pattern of mandibular gland components in queenless worker bees (*Apis mellifera capensis* Esch). J Insect Physiol 47:735–738
Skirkevicius A (2004) First symptoms of queen loss in a honeybee colony (*Apis mellifera*). Apidologie 35:565–573
Slessor KN, Kaminski LA, King GGS, Borden JH, Winston ML (1988) Semiochemical basis of the retinue response to queen honeybees. Nature 332:354–356
Slessor KN, Kaminski LA, King GGS, Winston ML (1990) Semiochemicals of the honeybee queen mandibular glands. J Chem Ecol 16:851–860
Velthuis HHW (1970) Queen substance from the abdomen of the honeybee queen. Z Vergl Physiol 70:210–222
Velthuis HHW (1976) Egg laying, aggression and dominance in bees. Proc XV Congr Entomol, Washington, pp 436–449
Velthuis HHW (1985) The honeybee queen and the social organization of her colony. Fortschr Zool 31:343–357
Velthuis HHW (1990) Chemical signals and dominance communication in the honeybee *Apis mellifera* (Hymenoptera: Apidae). Entomol Gen 15:83–90
Velthuis HHW, van Es J (1964) Some functional aspects of mandibular glands of the queen honeybee. J Apic Res 3:11–16
Velthuis HHW, Verheijen FJ, Gottenbos AJ (1965) Laying worker honey bee: similarities to the queen. Nature 207:1314
Verheijen-Voogd C (1959) How workers perceive the presence of their queens. Z Vergl Physiol 41:527–582
Visscher PK (1989) A quantitative study of worker reproduction in honey bee colonies. Behav Ecol Sociobiol 25:247–254
Voogd S (1955) Inhibition of ovary development in worker bees by extraction fluid of the quwn. Experientia 11:181–182
Whiffler LA, Hepburn HR (1991a) Inhibition of queen cell construction in the Cape honeybee, *Apis mellifera capensis*. Apidologie 22:229–236
Whiffler LA, Hepburn HR (1991b) The queen in relation to wax secretion and comb-building in honeybees. J Comp Physiol A169:209–214

Winston ML, Slessor KN, Willis LG, Naumann K, Higo HA, Wyborn MH, Kaminski LA (1989) The influence of queen mandibular pheromones on worker attraction to swarm clusters and inhibition of queen rearing in the honey bee (*Apis mellifera* L.). Insectes Soc 36:15–27

Winston ML, Higo HA, Slessor KN (1990) Effect of various dosages of queen mandibular gland pheromone on the inhibition of queen-rearing in the honey bee (Hymenoptera: Apidae). Ann Ent Soc Am 83:234–238

Winston ML, Higo HA, Colley SJ, Pankiw T, Slessor KN (1991) The role of queen mandibular pheromone and colony congestion in honey bee (*Apis mellifera* L.) reproductive swarming (Hymenoptera: Apidae). J Insect Behav 4:649–660

Yang M, Ken T, Radloff SE, Phiancharoen M, Hepburn HR (2010) Comb construction in mixed-species colonies of honeybees, *Apis cerana* and *Apis mellifera*. J Exp Biol 213:1659–1664

Chapter 7
The Significance of Brood

Abstract Differences in colony size among *Apis* species are not equated to the ratio of drones to workers or associated comb construction. Oviposition-related cell inspections reveal that a queen's decision to lay a fertilized egg or not, is determined by a specific stimulus generated on cell inspection. Uncapped or sealed queen cells are correlated to a reduction in the number of new cell constructions, possibly pheromonally mediated. Relative increases in the physiological activity of the wax glands in queenright bees are related to the age of the workers. Capped brood and broodlessness dampen the development of wax glands, while the presence of open brood stimulates their development as under queenright conditions. Queenright bees produce much more comb than queenless bees; while queenless, broodright bees construct more comb than queenless, broodless bees. The amount of wax produced is a linear function of the number of young bees in a colony, but the greatest amount of wax produced/bee, relative to colony size, occurs in small colonies. Bees prevented from brood-rearing produce the same amount of wax as those engaged in both comb-building and brood-rearing. Colonies precluded from comb construction rear no more brood than those engaged both in brood-rearing and comb-building. The proportion of drone comb depends on the amount of drone comb present and the number of adult drones present in a colony, and is positively correlated to the number of workers. The combination of queenright and broodright colonies appears to be a more powerful stimulus than any other for comb-building.

7.1 Introduction

Honeybee brood nests of are obviously essential to the existence and propagation of honeybee species. Firstly, there is the matter of choosing a suitable site for both open-air and cavity-nesting species (cf. Chap. 2), the subsequent organization of the contents of nests on a cyclical and seasonal basis in which sex ratios of queens, drones and workers vary enormously (cf. Chap. 3). As noted by Koeniger (2011),

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_7,

it may be energetically cheaper and more profitable to produce cheap, expendable drones than more expensive queens, although the cost of rearing queens and drones and maintaining them is only slightly different. In *Apis*, the ratio of queens to drones ranges in *A. florea* from 1:57 and about 1:500 in *A. dorsata* (Koeniger 2011), and raises questions, perhaps unanswerable, about a 'conflict over sex ratios'.

In any event, there are large variations in colony size among *Apis* species, differences which are not equalized by a colony's decision on its ratio of drones to workers and the corresponding necessary modifications in comb construction. There are two notable exceptions where there are no differences in cell diameter between worker and drone cells, the giant honeybees, *A. dorsata* and *A. laboriosa.* The construction of cells is discussed elsewhere (cf. Chap. 12), but here we review the basic biological traits which go hand-in-hand with the development of brood in relation to cell types: parthenogenesis, the meaning of brood, efficacy of brood, drone brood and brood-rearing in relation to honey storage.

7.2 Parthenogenesis

That queen honeybees lay eggs in worker-sized cells from which female worker adults emerge; and, conversely, that queens lay eggs in drone-sized cells from which drones eventually emerge, was for many centuries an incomprehensible puzzle, a conundrum of great proportions. The eventual solution to this problem involved two important discoveries: (1) honeybee queens were capable of parthenogenesis, and (2) queens could control the release of fertilized or unfertilized eggs during oviposition. The immediate history of these discoveries is a fascinating story of claims and counterclaims that ran through the pages of Eichstädt Bienenzeitung for a decade or so in the mid-19th century. In 1845, Jan Dżierzon (Fig. 7.1) published the results of his careful studies on the eggs of honeybees and how they arose. In short, he discovered parthenogenesis. This idea finally became accepted as a reality following collaboration between Dżierzon and von Siebold, a university Professor of Zoology at the Maximilians-Universität in Munich, who unequivocally confirmed the occurrence of parthenogenesis in some butterflies and honeybee queens (Dżierzon 1847; von Siebold 1856).

The above account of parthenogenesis in honeybee queens is based on research on European *A. mellifera,* but applies to all the other species of honeybees, with the exception of Cape honeybee, *A. m. capensis* workers, in which thelytokous parthenogenesis occurs in both queenless and queenright colonies. Aside from the many peculiar traits in the Cape honeybee (Hepburn and Crewe 1991), a cytological analysis of this phenomenon was conducted by Verma and Ruttner (1983), who demonstrated that egg diploidy is restored by the fusion of the two central meiotic products. Thelytoky has been shown to be controlled by a single major gene (Lattorff et al. 2005).

Fig. 7.1 Jan Dżierzon (16 January 1811–26 October 1906), was a pioneering Polish apiarist who discovered parthenogenesis in honeybees, *A. m. mellifera*, and also designed the first, successful movable-frame hive before von Berlepsch and Langstroth developed their hives

The significance of this on comb-building by *A. m. capensis* was the discovery of the following experimental results. Neumann et al. (2000) dequeened and removed all brood from 26 *A. m. capensis* and *A. m. scutellata* colonies and their natural hybrids. Neumann et al. (2000) found that *A. m. capensis* laying workers were thelytokous, and all *A. m. scutellata* arrhenotokous. Of the hybrid colonies 42.1 % produced only female offspring while none produced only male offspring. *A. m. capensis* colonies built only worker cells and *A. m. scutellata* only drone cells. Hybrid colonies produced either both cell types or only worker cells, according to the mode of laying worker reproduction. These results unequivocally demonstrate that the mode of worker reproduction in queenless, broodless colonies holds important consequences for cell construction, even if the mechanisms producing these effects have not yet been demonstrated.

7.3 Oviposition by Queens

The second remaining problem was an explanation for how a queen could control the sex of egg she laid and in which kind of cells. Reaching the answer to this problem was also a protracted one, which has been clearly described and discussed by Gessner and Ruttner (1977) who demonstrated that the spermathecal pump musculature controls the release of spermatozoa or not. Nonetheless it still remained to determine how queens could measure cell sizes. In extended observations on the behaviour of egg-laying queens, Koeniger (1970) noted that during the course of cell inspection, before actually depositing an egg in a cell queens introduce their two forelegs and head into the cells. Under the not entirely natural conditions of an observation hive, the queen lays one egg following two such inspections. No differences in this behaviour were observed between drone or worker cells. It appears that the decision to lay an egg in a cell or not is predicated

on an inspection of the cell. It is equally probable at such a moment that the size of cell (worker or drone) is taken into account by the queen. A method to interfere with these inspections was developed by placing small, square pieces of adhesive tape around the tibiae of the queen's the forelegs (Koeniger 1970).

The behaviour of the queen, even with the tape appendages appeared normal in every respect; she continued to oviposit and was fed and groomed by the workers. To assess the affects this method had on the queens' ability to distinguish cells, Koeniger (1970) presented drone cells to three *A. mellifera* control queens, in which drone eggs were laid. Only drone pupae were recovered from the drone cells. Subsequently, adhesive tape squares were attached to the tibiae of the queens' forelegs, and she was given a new set of drone cells. When these drone cells were examined only worker pupae were found. When the tape was removed, the queens laid only drone eggs in the drone-sized cells.

Following this, the role of the possible use of the queen's forelegs in drone cell recognition was further investigated by again taping the tibiae of the forelegs and allowing the queens access to drone cells. In this test 89 % of the eggs laid were worker eggs. Koeniger (1970) then performed a series of gradual amputations of both leg segments through trochanters, femora and tibiae, and found that 78, 18 and 3 % respectively of the pupae found in drone cells were workers. After amputation of one foreleg, only 0.3 % of the pupae developing in drone cells were workers. Koeniger concluded that the queen's decision to lay a fertilized or non-fertilized egg is determined by a specific stimulus generated when the queen inspects the drone cells with her forelegs. So, it is highly probable that queens use their forelegs essentially as a pair of inside calipers and actually measure cell size (Figs. 7.2, 7.3, 7.4).

7.4 The Meaning of Brood

As in many other areas of animal husbandry, the ancient truths of apiculture collect like clichés that grow into aphorisms. Thus, it is widely known that honeybees expand their nests at the onset of spring with warmer temperatures and the abundance of nectar and fresh pollen for brood-rearing (Butler 1609; Koch 1957, 1959, 1961). Unfortunately, this 'old truth' hides a horrible conundrum in which the role of brood as a stimulus for wax production lies hidden among many other complex and interdependent factors. Hence, bees build in response to the queens' need for available cells in which to lay eggs (Huber 1814). Bees never build combs if they lack a queen; or, if queenless, they lack brood from which to rear a new one (Gundelach 1842). Finally, the great Dzierzon (1848, 1861) tells us that as soon as breeding commences, the bees also produce wax; if breeding is interrupted, wax production is discontinued immediately, even under the most favourable conditions.

Against all of these claims, De Layens (1887) actually recommended that comb construction can be enhanced by the removal of brood from colonies for which comb construction is require so as to obviate the shunting of honey and the rearing

Fig. 7.2 An *A. mellifera* queen, having inspected a cell, has placed her abdomen into a recurved position to enter the cell and oviposit (photo courtesy of Niko Koeniger from Koeniger 1970)

Fig. 7.3 An *A. mellifera* queen inspecting a cell with head and forelegs (photo courtesy of Niko Koeniger, from Koeniger 1970)

Fig. 7.4 An *A. mellifera* queen ovipositing in a worker cell (photo courtesy of Niko Koeniger, from Koeniger 1970)

of larvae. It merely implies that 'brood' has different meanings in differing contexts, and that the stimulatory efficacy of brood may well vary with circumstances. This is amply demonstrated, as it so happens, in at least three ways in the experiments on comb construction by Dreischer (1956), Taranov (1958, 1959) and Free (1967), and in an entirely different way, by Fell and Morse (1984) with respect to queen cell construction.

In a study by Fell and Morse (1984) quite clearly there was a rapid decline in the rate of new queen cell construction of (cf. Fig. 6.4). In earlier work on the construction of queen cells, under both swarming and emergency conditions, Fell and Morse (1984) had shown that the presence of queen cells, containing uncapped larvae as well as sealed queen cells, is correlated with a reduction in the number of new cells or comb construction. To this we can add that, while worker bees cut away the apex of a queen cell before her emergence, the cappings of drone or worker cells are left intact. These authors suggested that both a reduction in new queen cell construction and the absence of re-working capped brood cells might both be mediated through a negative feed-back system driven by pheromones. Unfortunately, these intriguing ideas have not been further experimentally investigated.

Dreischer (1956) compared queenright and queenless *A. mellifera* colonies in late summer, usually a time of sparse comb-building. She introduced some marked, newly emerged bees each day into her colonies and subsequently sampled these bees of known age throughout her experiment. The progression of life in a queenless colony was divided into periods of differing social conditions as follows: (1) with both open and closed brood, then, as the brood became capped; (2) with sealed brood only, after the emergence of that brood; (3) entirely broodless; and finally (4) the presence of laying workers coupled with the open brood which they had produced. The daily addition of newly emerged, marked bees allowed for the appearance of bees of comparable age to occur in each of the different social situations defined for queenless bees. The queenright colony contained both open and sealed brood but presumably lacked laying workers. Dreischer then measured the course and extent of development of the ovaries, hypopharyngeal glands, wax gland epithelium and corpora allata for each condition of the colonies.

She found that the relative increase in height (a morphological indication of physiological activity, hence function—Rösch 1927, Boehm 1961, 1965), of the wax gland epithelium in bees of the queenright colony was related to the ages of the bees in just the same way as had been previously shown by Rösch (1927). Considering those bees from the queenless hive, in all four different social conditions, the initial increase in the epithelium progressed just as it did in the queenright bees and there was no significant difference in the height of the epithelium for the 11- to 15-day-old age group (encompassing the normal peak of wax secretion—cf. Chap. 15). However, following the peak height of the epithelium (at roughly 2 weeks of age), two entirely different patterns emerged among the queenless bees during the ensuing 2 weeks of a worker's life.

In the cases where queenless workers had uncapped brood, there was a decline in the epithelium of the wax gland, but at a slower rate of decrease than in the queenright bees (Fig. 7.5a, b). In the queenless bees with only sealed brood, or were

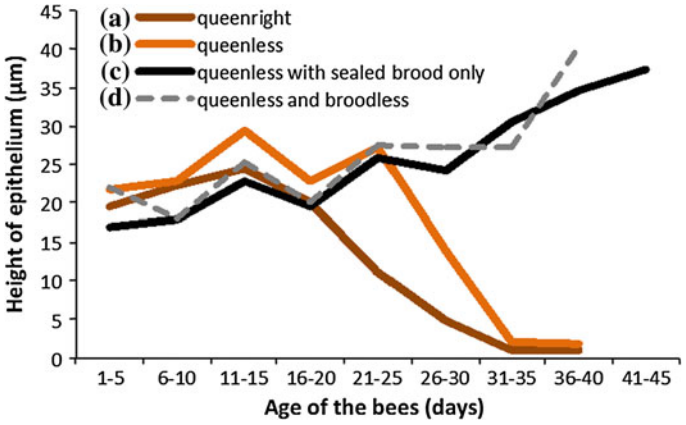

Fig. 7.5 Changes in height of the wax gland epithelium with age in *A. mellifera*: **a** queenright colony; **b** queenless colony with open and capped brood; **c** queenless colony with only capped brood; and **d** queenless and broodless colony (Dreischer 1956)

entirely broodless, the height of the wax gland epithelium did not regress at all; in fact, it slowly increased over the next fortnight to higher levels than those obtained at the normal 11–15-day peak in the queenright bees (Fig. 7.5c, d), the glands possibly remaining active. The same was true of the hypopharyngeal glands; the development of which normally precedes that of the wax glands (Rösch 1927).

A comparison of the bees of the queenless colony with open brood and those of the queenright one, showed the same general trend; the wax gland epithelium of both developed more or less apace and declined in the same way (Fig. 7.5). The same results were obtained in other experiments on queenright but broodless *A. m. scutellata*, colonies (Hepburn et al. 1984). While the significance of a queen is fairly obvious, one is not required for gland development in workers, given open brood. The role of worker brood in the isolated case is shown by comparing curves b, c and d in Fig. 7.5. Capped brood and broodlessness have precisely the same effects on the development of the wax gland (and also on the ovaries, hypopharyngeal gland, and corpora allata), while the presence of open brood stimulates the development of the wax glands in a pattern similar to that obtained under queenright conditions. The discovery of a brood pheromone that is chemically distinct from any of those elaborated by queens (Koeniger and Veith 1984; Le Conte et al. 1990) adds interest, if not clarity, to the observations.

7.5 Efficacy of Open Brood

The efficacy of open brood as a stimulus for wax production has been shown in a different way in some experiments by Free (1967), although his actual intention was to study drone cell production. During a late English summer, Free established

Table 7.1 Effects of the presence or absence of a queen and brood on comb cell production in *A. mellifera* (Free 1967)

	Experiment 1					
	Colony 1		Colony2		Colony3	
	Treatment	Cells built	Treatment	Cells built	Treatment	Cells built
26–28 Aug	Queenright	1921	Queenright	2250	Queenright	4206
28–31 Aug	Queenless	0	Queenless	635	Queenright	3316
31 Aug–3 Sept	Larvae added	1646	Larvae added	4750	Queenless	3737
3–7 Sept					Larvae added	4213
	Experiment 1		Experiment 2			
	Treatment	Cells built	Treatment	Cells built	Treatment	Cells built
26–28 Aug	Queenright	4206	Queenright	2042	Queenright	2440
28–31 Aug	Queenright	3827	Queenless	1367	Queenless	1921
31 Aug–3 Sept	Queenless	0	Larvae added	1812	Larvae added	3120

an apiary with six colonies, from which all combs had been removed. Each colony was given four test frames as building sites. Every few days the nests were examined and the combs built were photographed so that the number of cells constructed could be counted. Free (1967) managed the colonies as queenright for a few days, then queenless and broodless for a few days, and finally queenless and broodright for a few more days. The results of his experiment are shown in Table 7.1. When queenright, the bees produced more than twice as much comb, on average, as they did when they were queenless. Subsequently, when queenless but broodright the same colonies also constructed more than twice as much comb as they had done when they were queenless and broodless.

In an earlier experiment, Taranov (1959) had shown that the amount of wax produced was a linear function of the number of young bees present in a colony, at least for colonies of less than about 2.5 kg (Fig. 7.6). He also showed that the greatest amount of wax produced, relative to colony size, occurred in small colonies in which wax production went hand-in-hand with brood care (Fig. 7.7). In view of these results, Taranov questioned the inter-dependency of brood-rearing and wax production as competitive activities; does the increased work-load of wax production interfere with the nursing of young larvae, or does an increase in one function go hand-in-hand with the other?

Taranov explored these relationships by setting up three queenright colonies, each with about 10,000 young bees of the same age. The first colony was given empty frames, the second frames of drawn combs, and the third frames of drawn combs from which about one-third of the combs had been cut away. The first colony was kept broodless and could ‘concentrate’ on comb production; the second colony had no space to build additional comb, but enjoyed ample space for brood-rearing; and the third colony had some space for comb construction as well as brood-rearing. The colonies were supplied with pollen and were fed a 60 %

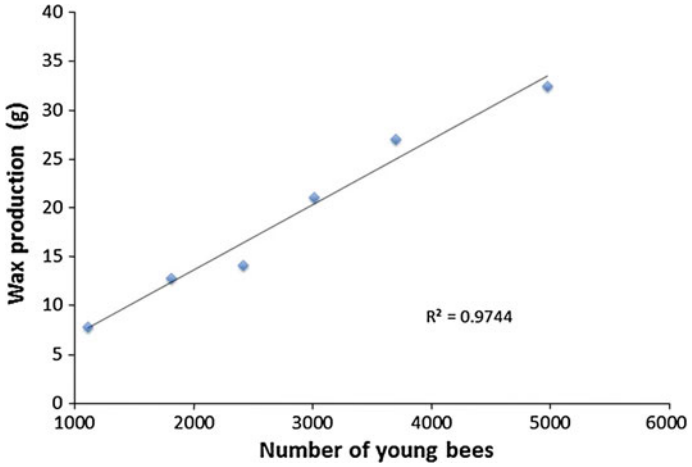

Fig. 7.6 Wax production as a function of the number of young bees in an *A. mellifera* colony is linear for colonies up to 2.5 kg (Taranov 1959)

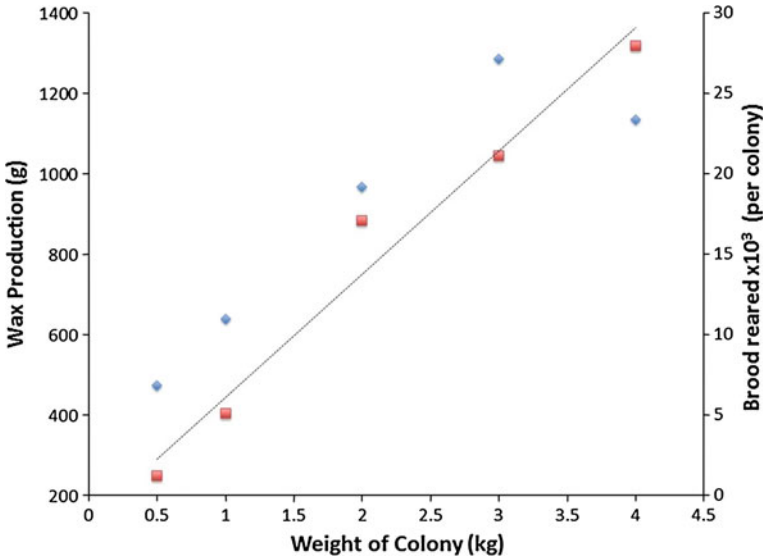

Fig. 7.7 Wax production by *A. mellifera* colonies of different sizes but all brood-rearing. *Solid line* = wax production linear for colonies of up to 3 kg ($r^2 = 0.97$); *broken line* = brood production as a function of colony size (replotted from data as published by Taranov 1959)

honey solution for 2 months. The results of this experiment were quite striking. Those bees prevented from brood-rearing produced the same amount of wax as did the colony engaged in both comb-building and brood-rearing (Table 7.2). Similarly, the colony precluded from comb construction reared no more brood than did

Table 7.2 Wax production and brood-rearing in *A. mellifera* (Taranov 1959)

Colony	Main work of the bees	Experiment 1	Experiment 2	Total
		Wax production (g)		
1	Produced wax only	333.2	387.4	711.6
2	Reared brood only	—	—	—
3	Produced wax + reared brood	465.2	336.9	802.1
		Brood rearing (No. larvae)		
1	Produced wax only	—	—	—
2	Reared brood only	26,525	1,261	39,135
3	Produced wax + reared brood	25,740	12,675	38,415

Table 7.3 Development of the wax gland epithelium in colonies of *A. mellifera* with 12-day-old bees under differing nest conditions (Taranov 1959)

Nest conditions	Wax gland epithelium height (μm)
1. Comb building	85
2. Brood rearing	76
3. Comb buildingand brood rearing	103
4. No building, nor brood rearing	40

the bees engaged in both brood-rearing and comb construction. Exclusion of either function did not lead to the accelerated development of the other one.

Taranov also measured the height of the wax gland epithelium in 12-day-old bees from these three colonies and from a fourth which produced neither wax nor had brood to care for, with the results shown in Table 7.3.

7.6 Drone Brood

Some years ago, Allen (1958, 1963), Free (1967) and Free and Williams (1975) investigated factors that determine, at least in part, the rearing and rejection of drones by *A. mellifera* honeybee colonies. The proportion of drone cells built was greatest in May, June and July although colonies continued to build drone comb long after they had ceased to rear drones. The proportion of drone comb built by a colony also depended on the amount of drone comb already present. The amount of drone brood and the number of adult drones present in a colony was positively correlated to the number of workers. Removing drone brood from colonies encouraged drone production; adding drone brood diminished drone production. A large percentage of eggs were sometimes laid in drone cells before the end of April, although few were reared. The proportion of drone brood was at its maximum in May and June. A colony could be forced to evict its drones by preventing the workers from foraging, and in autumn eviction could be greatly delayed by providing additional forage or removing the queen.

7.7 Brood-Rearing and Honey Storage

A final set of experiments elucidates our understanding of brood and wax production, and comes to us somewhat serendipitously. Cerimagic (1969) investigated the possibility of swarm prevention through the elimination of comb-building. He tested ten sister-queenright colonies of about 30,000 bees each over two successive spring seasons in Yugoslavia. In converting Cerimagic's original data into a wax-brood experiment, we have re-designated his 'controls' as the experimental group and vice versa. He gave 12 frames of foundation to one group of five colonies; to another control group of five colonies he gave fully drawn, old combs. In each case, as the brood chambers became filled, he supplied an empty box atop the brood chamber. Thus the experimental colonies were able to both construct combs and rear brood, the controls only the latter. All the colonies were able to forage during the nectar flow.

The trend in the results he obtained was very similar to those of Taranov (Table 7.2). There were no significant differences between the experimental and control colonies with respect to the amount of brood reared or honey stored. However, the experimental colonies produced nearly a kilogram of wax in each season, while the controls constructed no combs. Because all the colonies were headed by sister-queens (nature of matings unknown), genetic variation ought to have been minimal, in which case one would not have expected any large differences in the foraging abilities of the two groups. The combined measurements and experimental observations of Dreischer (1956), Taranov (1959), Free (1967) and Cerimagic (1969) revealed that the role of brood as a stimulus for the development of wax glands and subsequent secretion and comb construction, all juxtaposed against the queen as a stimulus. The data show, rather convincingly, that 'brood' means different things, depending upon the presence of a queen and, if there is no queen, upon whether or not the brood is uncapped or sealed. Similarly, the combination of a colony being queenright and broodright appears to be a more powerful stimulus than any of the other conditions investigated to date. What remains most puzzling about all this experimental data is the likely fate of energy that comes into these various situations.

Brood is a spectacular instance of how a wax production stimulus varies in duration, intensity and quality. The amount of time required for the development of a particular cycle of brood has been experimentally shown to vary with temperature (Milum 1930; Haydak 1970) and, by inference, with season as well. The availability of food is in part a function of season, with the effects that more brood (Nolan 1925) and heavier bees are associated with large influxes of pollen into the summer nest (Levin et al. 1954), but at a lower intensity than in spring (Todd and Bishop 1941). Against this, bees may be heavier in fall than in summer (De Groot 1953), owing to a change in the ratio between those that feed and those that are fed.

The ratio of brood to the adult population varies throughout the year. The production of brood is enhanced during nectar flow (Nelson and Sturtevant 1924), by the quality of the queen (Nolan 1925) and, of course, by the honeybee race.

This seemingly endless flow of variables forms a web of interactions that are not easily encompassed in feed-back loops and which do not clearly explain how the development of brood is related to the activities of the adult work force. Nonetheless, a understanding for this problem emerges from Ribbands (1953) who noted that changes in the proportions of brood and foraging bees are likely to have two combined effects: firstly, the proportion of foragers may be expected to vary inversely with brood, and secondly, brood consumes a substantial quantity of food. When these effects are considered, nectar influx increases sharply with colony size. Both effects undoubtedly influence the secretion of wax and the building of combs, but the ways in which they do so have not as yet been measured.

References

Allen MD (1958) Drone brood in honey bee colonies. J Econ Entomol 51:46–48
Allen MD (1963) Drone production in honey-bee colonies (*Apis mellifera* L.). Nature 199:789–790
Boehm B (1961) Beziehungen zwischen Fettkörper, Oenocyten und Wachsdrüsen Entwicklung bei *Apis mellifica* L. Natrurwissenschaften 21:1–3
Boehm B (1965) Beziehungen zwischen Fettkörper, Oenocyten und Wachsdrüsen Entwicklung bei *Apis mellifica* L. Z Zellforsch Mikrosk Anat 65:74–115
Butler C (1609) The feminine monarchie or a treatise concerning bees, and the due ordering of them. Joseph Barnes, Oxford
Cerimagic H (1969) Activity of the bee colony and relation thereof to intensity of wax production of the honey bee (*Apis mellifica* L.). In: 22nd international beekeeping congress, Apimonidia, Bucharest, pp 389–391
De Groot AP (1953) Protein and amino acid requirements of the honeybee (*Apis mellifica* L). Physiol Comp Oecol 3:197–285
De Layens G (1887) New experiments to determine the proportion of honey used by bees in the production of wax. Glean Bee Cult 15:171–172
Dreischer H (1956) Untersuchungen über Arbeistatigkeit und Drüsenentwicklung Alterbestimmter Bienen im weisellosen Volk. Zool Jahrb Abt Allg Anat Ont Tiere 22:429–472
Dżierzon J (1845) Gutachen über die von Hrn. Direktor Stöhr im ersten und zweiten Kapitel des General-Gutachens aufgestellten Fragen. Bienenzeitung Eichstädt 1:109–113, 119–121
Dżierzon J (1847) Neue verbesserte Bienenzucht. Ernst, Quedlinburg
Dżierzon J (1848) Theorie und Praxis des neuen Bienenfreundes. Brieg, Germany
Dżierzon J (1861) Rationelle Bienenzucht oder Theorie und Praxis. Falch'sche Buchdrukererei, Brieg
Fell RD, Morse RA (1984) Emergency queen cell production in the honey bee colony. Insectes Soc 31:221–237
Free J (1967) The production of drone comb by honeybee colonies. J Apic Res 6:29–36
Free JB, Williams IH (1975) Factors determining the rearing and rejection of drones by the honeybee colony. Anim Behav 23:650–675
Gessner B, Ruttner F (1977) Transfer der Spermatozoen in die Spermatheka der Bienenkönigin. Apidologie 8:1–18
Gundelach FW (1842) Die Naturgeschichte der Honigbbienen. JJ Bohne, Cassel
Haydak MH (1970) Honey bee nutrition. Ann Rev Entomol 15:143–156
Hepburn HR, Crewe RM (1991) Portrait of the Cape honeybee, *Apis mellifera capensis*. Apidologie 22:567–580

Hepburn HR, Hugo J, Mitchell D, Nijland MJM, Scrimgeour A (1984) On the energetic costs of wax production by the African honeybee, *Apis mellifera adansonii* (= *scutellata*). S Afr J Sci 80:363–368

Huber F (1814) Nouvelles observations sur les abeilles. English trans: (1926). Dadant, Hamilton

Koch HG (1957) Zur Phänologie der Bienenvölker. Z Angew Met 3:33–43

Koch HG (1959) Witterung und Nektartracht der Bienenvölker. Z Angew Met 3:278–292

Koch HG (1961) Der Baubeginn der Bienenvölker als phänologisches Ereignis. Z Angew Met 4:69–82

Koeniger N (1970) Über die Fähigkeit der Bienenkonigin (*Apis mellifica* L.) zwischen Arbeiterinnen- und Drohnenzelle zu Unterscheiden. Apidologie 1:115–142

Koeniger N, Koeniger G, Smith DR (2011) Phylogeny of the genus *Apis*. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 23–50

Koeniger N, Veith HJ (1984) Spezifitat eines Brutpheromones und Brutterkennung be der Honihbiene (*Apis mellifera* L.). Apidologie 15:205–210

Lattorff HMG, Moritz RFA, Fuchs S (2005) A single locus determines thelytokous parthenogenesis of laying honeybee workers (*Apis mellifera capensis*). Heredity 94:533–537

Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B (1990) Identification of a brood pheromone in honeybees. Naturwissenschaften 77:334–336

Levin MD, Nye WP, Knowlton GF (1954) Feeding pollen supplement and pollen substitute to honey bees. Utah Agrc Ext Bull 237

Milum VG (1930) Variations in time of developememt of the honeybee. J Econ Entomol 23:441–447

Nelson JA, Sturtevant AP (1924) The rate of growth of the honeybee larva. Bull USDA 1222:1–24

Neumann P, Hepburn HR, Radloff SE (2000) Modes of worker reproduction, reproductive dominance and brood cell construction in queenless honeybee (*Apis mellifera* L.) colonies. Apidologie 31:479–486

Nolan WJ (1925) The brood-rearing cycle of the honeybee. Bull USDA 1349:1–56

Ribbands CR (1953) The behaviour and social life of honeybees. Bee Research Association, London

Rosch GA (1927) Über die Bautätigkeit im Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 8:265–298

Taranov GF (1958) Fundamental laws governing wax secretion by a honeybee colony. In: 17th international beekeeping congress, Bologna, vol 408, pp 1–6

Taranov GF (1959) The production of wax in the honeybee colony. Bee Wld 40:113–123

Todd FE, Bishop RK (1941) The role of pollen in the economy of the hive. US Bur Entomol Circ E536:1–9

Verma S, Ruttner F (1983) Cytological analysis of the thelytokous parthenogenesis in the Cape honeybees (*Apis mellifera capensis* Escholtz). Apidologie 14:41–57

von Siebold CT (1856) Wahre Parthenogenesis bei Schmetterlingen und Bienen. Engelmann, Leipzig

Chapter 8
The Role of Pollen in Honeybee Colonies

Abstract The nutrients that workers derive from pollen provide all the proteins, lipids, vitamins, and minerals required for brood-rearing; the primary consumers of pollen are nurse bees which feed the brood. The greatest net increase in nitrogen content of bees is obtained when bees are fed their normal diet based on pollen. The most rapid rates of growth in young workers occur during the first week after eclosion and pollen must be available for the normal development of the wax glands, and subsequently comb construction. Under temperate zone conditions the relative abundance of pollen-rich flowers in spring drives brood-rearing. Likewise, increased access to pollen or protein resources is positively correlated with worker longevity. The amount of pollen required increases proportionately with the quantity of brood. Pollen-fed bees produce more comb than pollen-deprived bees. Pollen foraging seems to be regulated by at least three mechanisms: young larvae, stored pollen, and empty space. The amount of brood is a positive stimulus; while the quantity of stored pollen acts as an inhibitory stimulus for pollen foraging activity. Brood pheromone affects pollen foragers but not nectar-foraging behaviour. Camazine (1991) argued that the pattern of comb contents could be generated by a self-organizing algorithm of three simple rules: (1) the queen lays eggs in the centre of the comb; (2) workers deposit pollen and nectar at random; and (3) bees preferentially remove pollen and nectar from the brood nest relative to the honey storage area. Subsequent theoretical work supports this view.

8.1 Pollen and Brood

Although the importance of pollen is generally well known to beekeepers of any honeybee species, it should be noted that all of the published literature on pollen and comb-building are solely derived from studies on *Apis mellifera*. The survival of honeybee colonies depends on the collection of nectar and pollen from flowers and is essential for colony development. The necessity of pollen as a foodstuff for brood-rearing has long been suspected (Hornbostel 1744; Hunter 1792). However,

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_8,

the surmise that pollen is of nutritional importance to honeybees was first given experimental support in the work of Peterka (1939), who showed that a diet including pollen increased the longevity of bees. But, it was still some time before it was established that the nutrients which workers derive from consuming pollen provide all the proteins, lipids, vitamins, and minerals required for brood-rearing (De Groot 1953; Haydak 1970; Herbert 1992; Manning 2001).

Perennial colonies of social insects like honeybees, *A. mellifera,* heavily depend on stored pollen and honey to survive the long, cold winters in the temperate zones (Seeley 1985, Southwick 1991). Several experiments demonstrated that pollen is important for developing brood. The efficiency of nurse bees in a colony increases when a supply of pollen is available near the time of brood development. Crailsheim et al. (1992) showed that the primary consumers of pollen are indeed the nurse bees which feed the brood, and noted that pollen storage near brood cells reduces the time and energy spent by nurse bees retrieving stored pollen to feed the larvae. Mattila and Otis (2006a) recently conducted studies in temperate Canada on the effects of changes in the spring pollen diet on the development of *A. mellifera*. In the spring of the 3 years they established a series of colonies, some of which were fed supplementary pollen, and others not. The developmental progress of the colonies and brood-rearing noted were recorded. They found for all 3 years that those colonies which were provided with pollen or a suitable substitute, began brood-rearing earlier than the other colonies, and produced the most brood by early May (Fig. 8.1).

The large increase in the number of workers produced by colonies that were supplemented with pollen in the spring of 2002, resulted in substantial long-term differences between treatments with double the amount of honey at year's end compared to the pollen-limited colonies (Fig. 8.2). However, in 2003 and 2004, all colonies had similar annual honey yields (Fig. 8.3). While Matilla and Otis (2006a, b) argued that pollen supplies enabled colonies to produce more workers earlier, the annual honey yields will ultimately depend not only on the onset of brood-rearing, but also the momentum of foraging opportunities from year to year. The experiments conducted by Matilla and Otis (2006a, b) were designed for application in a commercial beekeeping context. However, that aspect of importance from their results for this essay is that, under temperate zone conditions, it is the relative abundance of pollen-rich plants flowering in spring, or its artificial supplementation, that drives the rate of brood-rearing each year in a temperate zone.

Matilla and Otis (2006a, b) also gave pollen supplements to bees in spring and autumn. The results revealed that those colonies supplemented with pollen in the autumn tended to rear more workers over an extended length of time before normal brood-rearing ceased for winter compared to colonies with less pollen. Feeding the bees in autumn could have exacerbated brood-rearing 'burn-out' of workers reared in the autumn that needed to overwinter, perhaps causing them to be relatively less productive the following spring. They also examined the effect on improving colony productivity using commercially prepared pollen substitutes

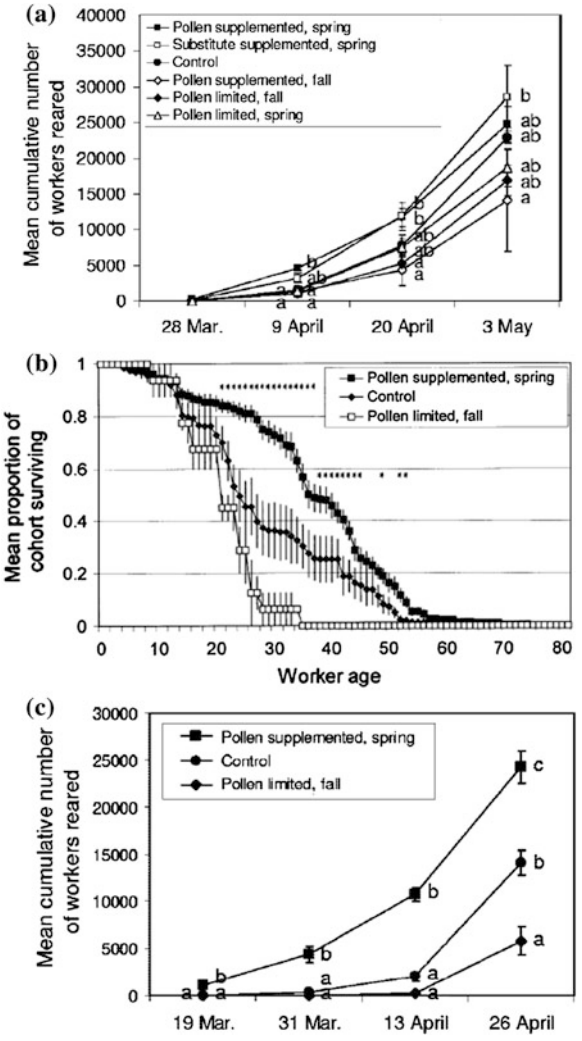

Fig. 8.1 Mean cumulative number of *A. mellifera* workers reared by colonies during 2004. Significant differences between means are indicated by different letters for each date (Mattila and Otis 2006b) **a** mean cumulative number of workers reared; **b** mean proportion of cohorts surviving; **c** mean cumulative number of workers reared

versus natural pollen. The results revealed that feeding pollen substitute in spring can also enhance colony population growth like natural pollen.

Bees, like many other kinds of animals can subsist for long periods in the absence of dietary protein. However De Groot (1953) performed an extensive series of experiments, the results of which established that the greatest net increase in the nitrogen content of bees was obtained when they were fed their normal pollen-based diet. The greatest growth rates of young workers occurred in their first week after eclosion, during which time the hypopharyngeal glands of the nurse bees reached their peak. Although Matilla and Otis (2006a, b) stated that the differences in the relationship between protein content and the longevity of bees between years was not significant (Figs. 8.4, 8.5, 8.6), these findings conflict with

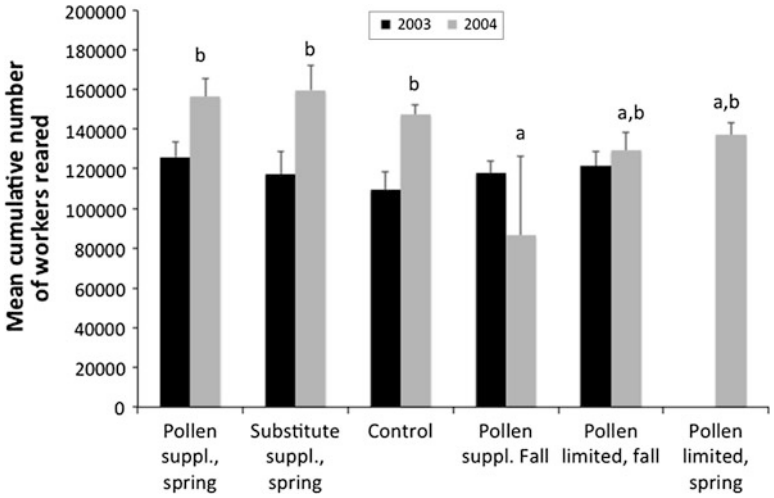

Fig. 8.2 Mean cumulative number of workers reared by *A. mellifera* colonies from June 2003 to June 2004 (Mattila and Otis 2006a, b)

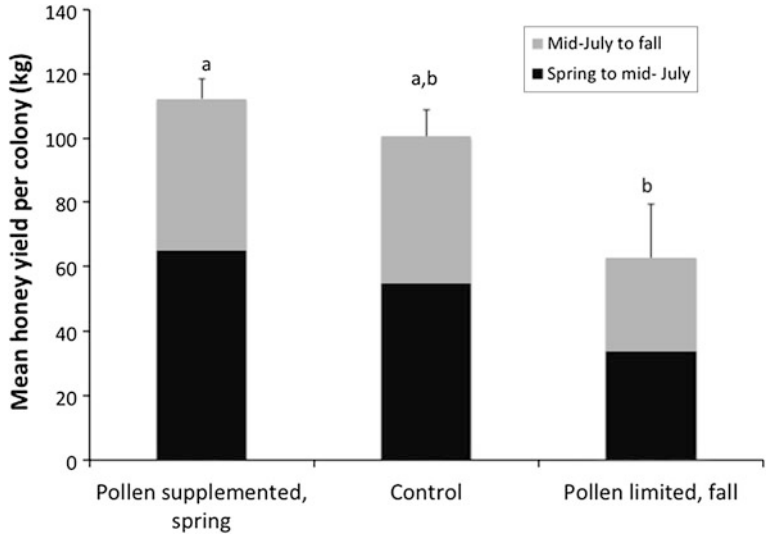

Fig. 8.3 Honey yields of *A. mellifera* under different pollen supplement conditions in 2002 (Mattila and Otis 2006b)

those of previous authors who observed that increased access to pollen or protein resources was positively correlated with worker longevity (De Groot 1953; Maurizio 1954, 1959; Crailsheim 1990).

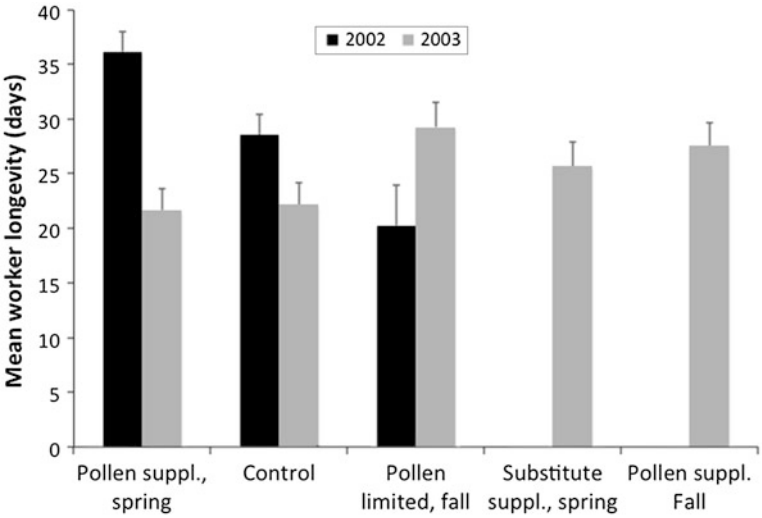

Fig. 8.4 The mean longevity of *A. mellifera* workers reared during spring 2002 and 2003 in pollen-supplemented and pollen-limited colonies. Differences within each year are indicated by different letters (Mattila and Otis 2006b)

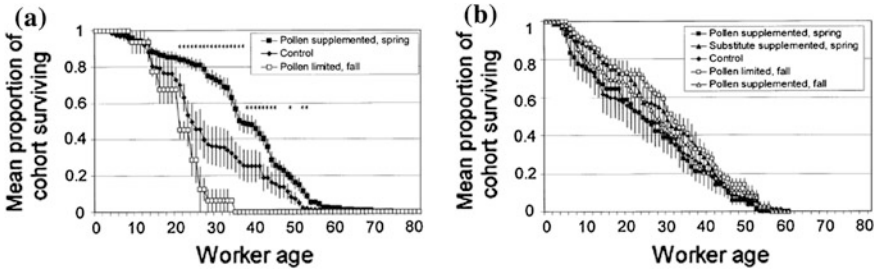

Fig. 8.5 The mean proportion of *A. mellifera* workers that survived over time during spring 2002, **a** pollen-supplemented workers, pollen-limited or control colonies; **b** during spring 2003 (Mattila and Otis 2006a)

8.2 Pollen and Wax Production

Having demonstrated that bees with no access to pollen could construct some comb (Huber 1814), a possible relationship between pollen and wax production was mooted on theoretical grounds only in the mid-19th century (Dzierzon 1861; Schmid and Kleine 1865). But, in retrospect, a stark insight into the importance of pollen in the development of the wax glands is evident in the 'failed' experiments of Dumas and Edwards (1843). Three out of four of their pollen-deprived experimental colonies produced no wax at all. This pointed the way for more rigorous

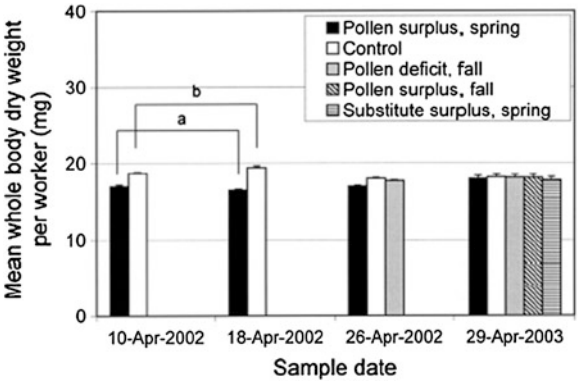

Fig. 8.6 The mean body dry weight of *A. mellifera* (workers reared in pollen supplemented or pollen-limited colonies) during spring 2002 and 2003 (Mattila and Otis 2006a)

investigations into the relationship between pollen as a dietary requirement and wax production, as illustrated by the work of Goetze and Bessling (1959). Goetze and Bessling (1959) prepared two queenright colonies each of only 100 newly emerged workers, and confined them to a cupboard for 3 weeks. One group was given only sugar, in excess, and the other group both sugar and pollen. After 20 days, the pollen-fed group had produced over 900 mg of comb, while those deprived of pollen had built less than 100 mg. Similarly, wax scales collected from equal samples of bees were fourfold greater in the pollen-fed group than in the pollen-deprived group. Histological analyses of the wax gland epithelium of both groups showed that the height of the glands of those fed pollen rose and fell, as had been shown by Rösch (1930), while the epithelial glands of the pollen-deprived bees began to degenerate after a week.

To determine when pollen must be included in the diet for normal wax production, Goetze and Bessling (1959) established additional small colonies that were fed pollen ad libitum after individual colonies had been deprived of pollen for 1, 2, 3, or 5 days; a control colony was only fed sugar. Those bees without pollen for 1 day commenced comb-building on the 4th day; those deprived of pollen for 2 and 3 days began building on the 5th day; those deprived of pollen 5 days did not build comb; the group receiving only sugar died, combless, after 8 days. Goetze and Bessling concluded that the earlier pollen feeding begins, the more rapid and extensive the development of the wax gland epithelium. To quantify how much pollen is necessary for wax gland development and comb production (the nutritional quality of protein was unknown), Goetze and Bessling (1959) established five more small colonies and serially gave them pollen at the rate of 5, 10, 20 and 40 mg/bee with no pollen fed to the control group. All groups received sugar. The control colony and that given 5 mg of pollen/bee built less than 50 mg of comb; that colony given 10 mg pollen produced about 200 mg of comb; and those with 20 and 40 mg of pollen/bee both built over 700 mg of comb.

The general conclusions that can be reached from the experiments of Goetze and Bessling (1959) on honeybees, and from similar observations on bumble bees by Röseler (1967), are: (1) that protein must be available immediately after

eclosion for the normal development of the wax glands and for comb construction; and (2) that wax gland development is just as dependent upon protein nutrition as is the development of the hypopharyngeal glands as shown by De Groot (1953). These results were confirmed and extended by Freudenstein (1960) using small, queenright colonies (with the queen caged) and an initial population of about 850 newly emerged bees. One colony was only fed sugar, the other an unlimited supply of both sugar and pollen. After 2 weeks, the colony that had access to pollen had built 30 cm^2 of comb, while the colony fed sugar had constructed nothing. Normal attrition reduced numbers by some 30 % in the sugar/pollen and 70 % in the sugar only colony; however, the reduced numbers of bees still exceeded the number of bees (100) necessary for comb-building (Darchen 1956, 1957; Goetze and Bessling 1959).

To compensate for total attrition, Freudenstein (1960) repeated the same basic experiment, but doubled the number of workers in the experimental, pollen-free colony. After two-and-a-half weeks, the bees fed on pollen had produced 75 cm^2 of combs, and the pollen-free colony only 34 cm^2 of comb; both colonies being of equal strength at termination of the experiment. The histological picture of the wax gland epithelium showed the same trend: the glands in the pollen-fed colony were significantly more developed than those in bees from the pollen-free colony. In two separate experiments, Freudenstein (1960) extended the pollen deprivation experiments of Goetze and Bessling (1959) using recently emerged bees and observing them over the first 11 days of adult worker life. In the first experiment, the bees were deprived of pollen for 3–6 days and then fed pollen. The control colonies which were continuously fed pollen, produced comb at the rate of 150 mm^2/bee for 3 weeks, while the experimental bees produced only 95 mm^2/bee; allowances were made for the differences in size of the colonies used in these experiments. Extending this experiment with new colonies deprived of pollen for 11 days, Freudenstein's pollen-fed colonies produced 100 mm^2 of comb per bee over 3 weeks, while the pollen-deprived bees only built 30 mm^2 of comb per bee. In both cases the height of the wax gland epithelium was greater in the pollen-fed colonies than in the sugar-fed experimental colonies.

Freudenstein (1960) then reversed his approach in a time-wise reciprocal experiment. He set up colonies, all of which were initially fed both sugar and pollen. Then the experimental group was deprived of pollen. In one experiment, pollen deprivation after only 5–7 days affected comb-building. At the end of the 6-week experiment, the pollen-fed bees had produced 677 cm^2 of comb, and the experimental group only 373 cm^2 of comb. Extending the feeding regimen to 2 weeks before pollen deprivation and artificially maintaining population density to compensate for attrition, Freudenstein (1960) found that both control and experimental groups produced combs at the rate of 70 mm^2/bee over the 6-week period. That a fortnight of pollen alimentation is adequate for comb-building was also confirmed in a slightly different way. Freudenstein (1960) formed two queenright colonies, each consisting of about 2500 old field bees, and deprived one colony of pollen. Two-and-a-half weeks later, the colony deprived of pollen had constructed only 13 % less comb (304 cm^2) than that given pollen (346 cm^2/colony).

From a histological analysis of his bees, Freudenstein (1960) found that all the factors that affected the height of the wax gland epithelium also affected the size of the oenocytes. He observed a linear correlation between increasing cell height and increasing oenocyte diameter, both in the natural progression of growth in young bees, and in foragers 'forced' to become wax bees again. Interestingly, he noted that the consumption of pollen decreased with the increasing age of the bees. To have equated pollen with protein as effectively as had been done in the experiments of Goetze and Bessling (1959) and Freudenstein (1960), recalls the admonition of De Groot (1953): more is known of the foodstuffs than of the nutritional requirements of bees. The fact that not a single pollen substitute (chosen on the basis of its apparent protein value) gave the same result as bee-collected pollen makes one wonder whether there is something more to pollen that still eludes us.

Apart from the question of growth and general physical maintenance, the actual definable usage of proteins from pollen in the development and activity of the wax gland complex has only been touched upon. Parallel studies of bacteria (Kaneda 1967) and plants (Kolattukudy 1968) have shown that some amino acids give rise to fatty acids, which are ultimately incorporated into wax. The discovery that beeswax contains proteinaceous material (Kurstjens et a1. 1985) also supports a fundamental role of protein in beeswax synthesis, that extending well beyond what is essential for the development of the cytoplasmic vacuolar system of the wax gland cells.

Hamdorf and Boehm (cf. Boehm 1965) showed that the metabolic rate of tissue, isolated from the fat body of bees fed on pollen, was higher than that of bees fed only sugar water. Because the oenocytes were much larger than the fat cells in old foragers induced to secrete wax again, Boehm (1965) argued that the increased oxygen consumption was due to the activity of the oenocytes during the preparations made by Hamdorf and Boehm. She coupled increased respiration with the larger volume of the oenocytes, and suggested that volume alone indicates activity. Finally, she concluded that there is a direct relationship between pollen feeding and the growth and size of the oenocytes.

Boehm (1965) went on to suggest two routes by which oenocytes could be reactivated in foragers (and hence explain initial activation in young bees): (1) either pheromonally; or (2) more directly through regulation in the central nervous system leading to an increased consumption of pollen, and consequently development of the oenocytes. She tested these ideas by establishing a colony of 600 foragers with regenerated wax glands and well-developed oenocytes. The bees had neither food stores in their combs nor access to any in the flight cage. Having starved the bees for 10 days, she gave them a mixture of pollen and sugar. She assayed the status of the oenocytes over the first 10 days, and again after having fed the bees. Bees from the starved period possessed thick wax scales, and their fat bodies were as well-developed as those of normal foragers with regenerated wax glands. The oenocytes of these bees remained large, even after their guts were completely empty. Following the administration of the pollen-sugar mixture on the 11th day (which the bees took very readily), Boehm assayed bees collected at

10-min intervals, over the next 10 h, and found no observable differences in the oenocytes of these animals. Finally, bees that had been starved for 2 weeks were analysed and, again, their oenocytes were no different from those of the starved bees or bees that were fed pollen on the 11th day. Therefore, once the wax gland system is maximally developed, even in foragers with regenerated wax glands, the bees can withstand starvation for a period of 2 weeks, the oenocytes remaining large.

Boehm (1965) tried to explain the apparent independence of pollen nutrition and the regulation of oenocyte and wax gland activity in another experiment, also with foragers with reactivated glands. She sampled bees from the building cluster on a daily basis over 10 days and plotted the changes in oenocyte diameter. On the 4th day of the experiment, the majority of bees that she examined bore wax scales, which became thicker over time. However, there were no scales on some bees, nor had there been any increase in oenocyte diameter, as would have been expected had the development of the wax gland system solely depended on pollen alimentation. Boehm (1965) concluded that the reactivation of the oenocytes and wax glands arise from a sensible 'need' to build a nest, possibly one that is pheromonally communicated.

8.3 Physical Presence and Regulation of Pollen in the Colony

Besides the physiological needs bees have for pollen for the development and maintenance of the wax gland system, there is an additional role of pollen: as a potent stimulus in a colony lacking such stores. Taranov (1959) alone considered the relationship between the flow of newly foraged pollen into the nest and wax production. In two separate experiments, he established 14 queenright colonies, each of about 5000 bees, on pollen-free combs from which pieces had been cut away. Each colony was fed 200 g of a 50 % sugar solution daily. The combined results obtained from all 14 colonies are shown in Fig. 8.7, from which it can be seen that there is a linear correlation between the rate of wax production and the influx of pollen, measured as pollen loads, entering the nest.

This interesting result must, however, be interpreted with extreme care. The reasons for this are evident in an argument by Butler (1974), as an example, if the field force of a colony is preferentially visiting the florets of white clover to secure nectar, as an example, the bees will inadvertently gather pollen as well. When the flow of nectar is strong, the bees will return laden with nectar but carrying only small amounts of pollen. If nectar secretion decreases in the florets, perhaps through lack of rain, the bees will be forced to visit many more blossoms than previously in order to obtain a large load of nectar. Then, if the bees still collect the same relative amount of pollen per floret as previously, they will return to the nest laden with pollen as well. Against this unpredictable flow of pollen, the need for pollen as protein will increase with the amount of brood which, in turn, can

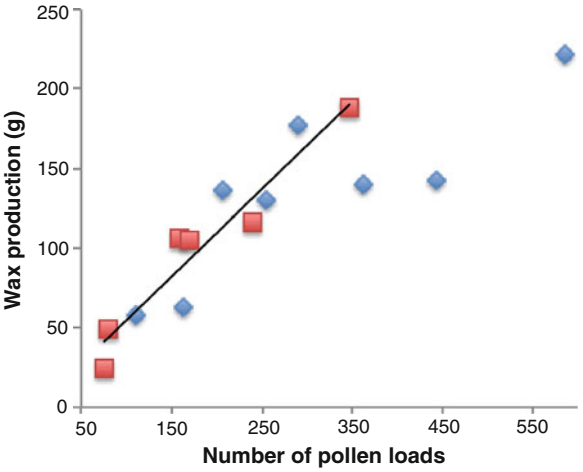

Fig. 8.7 Relationship between the influx of fresh, field pollen into colonies and the corresponding wax production obtained in two experiments on 14 *A. mellifera* colonies. The correlation coefficient for a linear relationship between the two variables is $r^2 = 0.91$ (Replotted from Taranov 1959)

only increase in proportion with the availability of pollen. This possible sequence of events is made more complicated by the discovery that strains of bees can be bred for either high or low pollen-hoarding behaviour (Nye and Mackensen 1970; Hellmich et al. 1985).

Colonies show a negative feedback associated with quantities of stored pollen such that excess stored pollen is as controlled as is comb-building. The quantity of pollen stored in nests affects the nest activities. When pollen was added to a colony, pollen-foraging activity decreased until the excess pollen had been reduced by the nurse bees and the quantity of stored pollen returned to near previous levels (Barker 1971; Free and Williams 1971; Moeller 1972; Fewell and Winston 1992). Conversely, when stored pollen was removed from colonies, there was a concomitant increase in the number of pollen foragers and the size of the loads collected, until the preexisting quantities were restored (Lindauer 1952; van Laere and Martens 1971; Fewell and Winston 1992; Eckert et al. 1994).

Pollen foraging activity is also directly affected by the relative quantities of brood in the combs. Several studies have demonstrated that pollen foraging behaviour increases in colonies that have large amounts of brood (Filmer 1932; Free 1967; Cale 1968; Todd and Reed 1970; Al-Tikrity et al. 1972; Calderone 1993). Thus two factors might be associated with the regulation of pollen foraging activity: (1) the amount of brood serves as a positive stimulus; and (2) the quantity of stored pollen acts as an inhibitory stimulus (Dreller et al. 1999). These two factors must eventually be integrated in to single inhibitory signal on a sliding scale, such as the mechanism of a slide rule used in engineering which is a mechanical analogue computer (Camazine 1993; Seeley 1995). Camazine (1993) separated pollen foragers from nurse bees by placing them on a comb located at the bottom in two observation hives. In one hive the bottom comb was separated from the rest of the hive with a single screen, which allowed trophallactic exchanges; in the second hive a double-screen blocked trophallaxis. The results

showed a decrease in pollen foraging in the colony with a single screen, compared to the double-screen treatment. Therefore, he interpreted these results to suggest that nurse bees conveyed information to pollen foragers, which inhibited pollen foraging activity. These results suggest a mechanism of negative feedback inhibition associated with quantities of stored pollen. Nonetheless, trophallaxis does influence the nectar foraging behaviour of honeybees.

Dreller et al. (1999) further tested this hypothesis on 20 colonies containing equal amounts of pollen, honey, sealed and unsealed brood, in which the foragers and nurse bees were separated by a single or double-screen. Using double-screens prevented any interaction between nestmates in both compartments, whereas the single screen still allowed trophallactic interactions. There were no differences in the number of pollen foragers between hives with a double-screen and those with a single screen. The authors concluded that there was no inhibitory signal or information transmitted by the nurse bees. Dreller et al. (1999) argued that even though it was possible that some nurse bees were indeed below the screens, there was an equal expectation for this condition to have occurred in both the single and double-screen treatments (Fig. 8.8).

In other experiments Dreller et al. (1999) tested the direct effects of excess pollen on foragers, by confining them in the lower hive body using a single screen, and then added pollen to this compartment, allowing pollen foragers direct access to pollen. The total number of pollen foragers in these experiments was significantly lower in colonies which were provided with supplementary pollen compared to the control colonies (Fig. 8.8). Unsealed brood acted as a positive factor in increasing pollen-foraging activity; but only if foragers had direct access to the brood nest. Under natural conditions, empty pollen cells in the nest might also provide information on pollen forage as an indirect stimulus.

Dreller et al. (1999) tested the role of empty cells on the pollen-foraging activity in 20 colonies by adding an empty frame to two treatment groups: (1) a frame was placed next to an unsealed brood comb (direct interaction); and (2) a frame was placed far away from the brood, as an outside comb at the outer edge of the hive body (indirect interaction). The results revealed that only the empty comb placed at the edge of the brood nest, where foragers normally unload their pollen, acted as a positive stimulus to increase pollen foraging activity (Table 8.1). Therefore, Dreller et al. (1999) suggested that there is little to support the hypothesis of indirect protein inhibitors of nurse bees; rather, their results support the hypothesis that pollen foragers directly assess pollen storage areas and are stimulated by empty space. In addition, it is unlikely that protein inhibition by nurse bees occurred, because trophallactic exchanges between nurse bees and other adult bees (including non-foraging adult bees) are rare, and occur on average about once per hour (Crailsheim et al. 1996).

Pollen foraging seems to be regulated by at least three mechanisms: young larvae, stored pollen, and empty space; factors for areas in which brood and pollen are stored are also negatively correlated. The effects of brood have been demonstrated to be direct, independent, and as a stimulus for pollen foraging (Pankiw et al. 1998). Pollen foragers can be directly and quantitatively modulated by

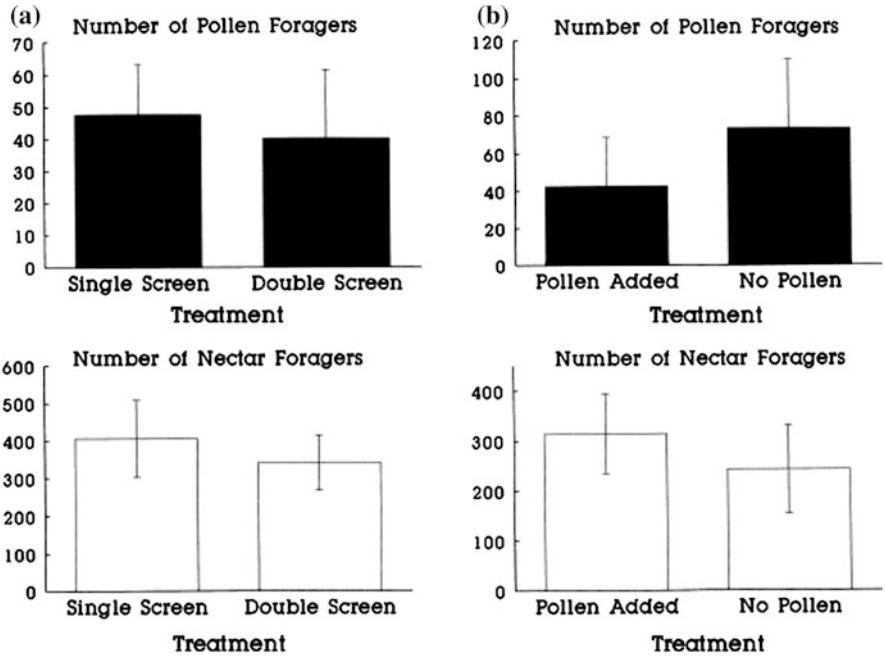

Fig. 8.8 **a** Mean number of pollen and nectar foragers when interactions between foragers and nurse bees were prevented by using a double-screen to separate them, or when interaction was allowed through a single screen; **b** Mean number of pollen and nectar foragers with additional pollen comb (pollen added), compared to colonies which were provided with an empty comb covered with aluminium foil (no pollen). In both groups, only foragers had access to the added comb (Dreller et al. 1999)

Table 8.1 The effect of empty space on pollen and nectar foraging activity

	Pollen forager		Nectar foragers	
	Day 1	Day 2	Day 1	Day 2
Comb inside ($n = 10$)	223 ± 69	278 ± 80 $P < 0.01$	263 ± 64	333 ± 81 $P < 0.05$
Comb outside ($n = 10$)	250 ± 75	274 ± 79 $P = 0.35$	287 ± 74	328 ± 67 $P = 0.14$

The empty frame was placed either next to uncapped brood (*comb inside*), or at the outer end of the hive body (*comb outside*) Dreller et al. (1999)

varying the amount of brood pheromone presented in a colony. Pankiw et al. (1998) used brood washed in hexane as a 'no-brood' condition. However, although much evidence exists for inhibitory effects of pollen, the actual mechanisms of inhibition remain to be demonstrated.

8.4 Pollen Pheromones

There is no doubt as to the efficacy of an influx of new pollen on comb construction, whether any effects are proximate, or a more removed stimulus, is not at all clear. A partial explanation of the way in which pollen stimulates wax working may well lie in the pheromonal (kairomonal) properties of its volatile constituents. In the case of emergency queen cell construction in a queenless colony, Fell and Morse (1984) noted that 6 of the 13 colonies tested actually constructed queen cells over pollen cells during the first 2 days of queenlessness. They speculated that the bees themselves might have added some substances to the pollen stores that incited queen cell construction, even though the queen larvae had not been fed with pollen at all.

The existence of a pheromone that induces construction of worker cell comb was proposed by Chauvin et al. (1961); but there are possibly many other chemical signals that affect wax production. During the summer of 1974, Chauvin (1976) placed various numbers of bees in small cages with a piece of beeswax foundation as a clustering/building site. The bees were fed candy and were also given sugar syrup to which various components were added. Every 5 days he measured the combs built, to obtain a baseline for wax production, and found that the average production, per 5 days, was as follows: 100 bees averaged 2.1 mg/bee; 200 bees 2.2 mg/bee; 300 bees 0.7 mg/bee; and, 400 bees 0.6 mg/bee.

Chauvin then reported that he learned, by accident, that extracts of pollen stimulate comb- drawing. He tested this by preparing an alcohol distillate of pollen trapped mainly from fruit trees. The solution was then added to the syrup fed to the bees (the control group were given only alcohol). He also tried a simple aqueous extract, fed in like manner, as well as alcohol extracts of boiled old combs. The results of several trials of these various extracts showed that the pollen fraction obtained from the alcohol and the water extracts of old combs, both resulted in more drawn comb than the corresponding control.

What we already know from these various experiments and observations is that a dietary intake of pollen, equated with protein, is essential for the normal development of the wax glands. Once developed, the glands may well function without additional pollen. Of the other two possible roles for pollen as wax-inducing stimuli, we have the single report (Pankiw et al. 1998) of an alcoholic extract of pollen, about which we would clearly like to know more. The findings of Taranov (1959) that the influx of fresh, field pollen into a colony stimulates wax production, hence comb construction, and are extremely difficult to interpret because the variables are conflated and inevitably coupled to fine weather, suitable ambient temperatures, and presumably the greater activities of the foragers. The task of unraveling the properties of these obviously complex stimuli remains as yet to be done.

Comb wax consists primarily of hydrocarbons and ester components (Tulloch 1973; Aichholz and Lorbeer 1999; cf. Phiancharoen et al. 2011). Honeybee comb used for food storage takes on a yellowish hue over time, due to the accumulation of pollen (Free and Williams 1974). Comb which is used for brood-rearing will

become darker with age and almost black, and more brittle (Hepburn 1998) because of the accumulation of faecal material (Jay 1964), propolis and pollen (Free and Williams 1974). The darker colour wax may contain a collection of undefined contaminants accumulated over time. Pheromones are also absorbed and transferred in wax combs and, depending on their volatility, may remain for a considerable length of time (Naumann et al. 1992).

Bees could express heightened sensitivity pheromonally when workers are exposed to secretions from the Nasonov gland; or, when they perceive new sources of honey, pollen, propolis, water, live queens or even 9-HDA (Ferguson and Free 1981). Highly sensitive reactions by bees arise not only in response to over-stimulation by real, potent stimuli, but also possibly due to what Lipiński (2006) termed 'psychogenic stress' caused by the lack of being able to express behaviour in response to real or expected stimuli. The release of attractant pheromones by bees increases after entering the nest entrance (Fergusson and Free 1981), or when bees are 'frustrated', such as when they do not find food at a feeding station to which they have been trained (Bittermann 1988, 1996). Thus one could speculate that the primary form of bee consciousness may reflect different primordial effects if, for example, the pollen pellets are gently removed from the hind legs of a forager as she is entering the hive, she will nonetheless go through the stereotypical behavioural motions of unloading the non-existent pollen pellets into the comb cell (McDonald 1968).

Pankiw et al. (1998) described how brood pheromone (whole hexane extracts of larvae) influences pollen foraging, whether it is an indirect or brood-food mechanism. The total number of pollen foragers was statistically similar in brood-pheromone- and brood-treated colonies, while there were significantly fewer pollen foragers in broodless colonies (Fig. 8.9), but the treatments had no effect on the number of sucrose foragers. The total number of foragers was significantly lower in broodless colonies compared to brood-pheromone-treated and broodright colonies (Fig. 8.9). The bees also responded to different levels of pheromone, so that the number of pollen foragers increased more than 2.5-fold when colonies were provided with extracts of 2000 larvae as a supplement to the 1000 larvae they already had. There was a significant treatment by time interaction for the number of pollen foragers entering colonies. This response appeared within 1 h of introducing brood pheromone to the colonies. Pollen foragers responded to the stimulus effects by showing significant differences between brood- and brood-pheromone treatments at 1, 2 and 6 h. Pollen foraging in the broodless treatment was significantly lower at all times.

Pankiw et al. (1998) further compared two treatments, (1) brood, and (2) brood-pheromone- supplement, so that an additional 2000 larval equivalents of brood pheromone were tested. The results clearly demonstrated that the total number of pollen foragers was significantly greater with the brood-pheromone-supplement treatment compared to the brood treatment (Fig. 8.10). Likewise brood-pheromone-supplemented colonies stored significantly more pollen and filled more empty cells than brood-treated colonies over the 6 h period. Changes in the areas

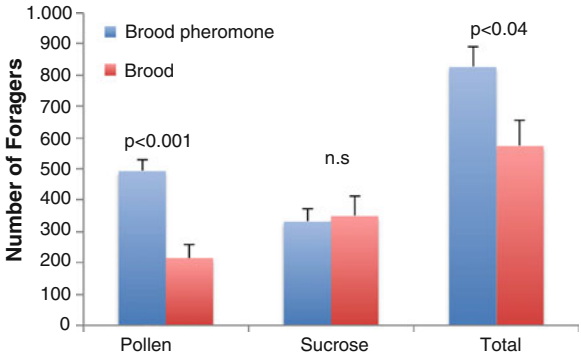

Fig. 8.9 The mean of *A. mellifera* paint-marked pollen and sucrose foragers visiting pollen and sucrose feeding stations with hexane extracts of larvae, brood and broodless treatments. The total number of foragers is the mean sum of pollen and sucrose foragers. No significant differences between treatments were noted (Pankiw et al. 1998)

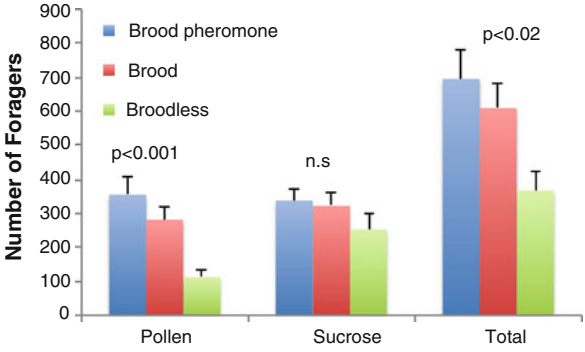

Fig. 8.10 The mean number of *A. mellifera*, paint-marked pollen and sucrose foragers visiting the pollen and sucrose feeding stations in brood- and brood-pheromone-supplement experiments (Pankiw et al. 1998)

occupied by honey, eggs, larvae and pupae did not differ significantly between treatments.

Pankiw et al. (1998) clearly demonstrated that hexane-soluble compounds associated with brood have strong effects on pollen-foraging behaviour. These results support the direct stimulus hypothesis for pollen foraging, and do not support the indirect inhibitor, brood-food hypothesis for pollen-foraging regulation by Camazine (1993). Although the data of Pankiw et al. (1998) support a direct stimulus effect, they argue that one cannot rule out the activities of nurse bees because it is not known how the pheromone is distributed. The pheromone had an immediate effect on foraging, rather than acting indirectly through physiological pathways of the nurse bees, as suggested by the inhibitor hypothesis.

The brood pheromone seems singular in its effect on pollen foragers, but not on sucrose-foraging behaviour. The dramatic increase observed for pollen foraging with supplemental brood pheromone suggests that the colony contains a pool of potential pollen foragers that are not actively foraging. These results support the stimulus response threshold hypothesis of division of labor (Robinson and Page 1988; Page and Robinson 1991; Pankiw et al. 1998); but, they are not clearly integrated into an inhibitory signal as proposed by Camazine (1993) and Seeley (1995).

8.5 Pattern and Function of Pollen Cells

The partitioning of comb into discrete areas contributes to maintaining temperature and humidity levels within narrow limits in the nest and, in particular, maintaining temperatures in the brood nest within the range 33–36 °C (Kleinhenz et al. 2003; Seeley 1985). Tautz et al. (2003) showed that the temperature at which pupae are incubated has a significant impact on their ability to perform foraging functions as adults. Several experiments have demonstrated the importance of pollen on developing brood. Maintaining a ready supply of pollen near the developing brood increases the work efficiency of nurse bees in a colony. Crailsheim et al. (1992) showed that the primary consumers of pollen are nurse bees which feed the brood; while Camazine et al. (1998) noted that pollen storage near brood cells would reduce the time and energy spent by nurse bees in retrieving stored pollen to feed larvae. The temperatures inside honeybee nests are determined by several complex, non-linear energy transfer processes involving: (1) radiation and convection of heat to and from the nest surfaces; (2) convection of heat and gaseous water in the air spaces between combs inside the nest; (3) heat conduction through the nest combs as affected by cells filled with air, honey, pollen or pupae and worker bees meandering over the comb surfaces; (4) the generation of energy in the nest through metabolic processes associated with passive and active bees; (5) the movement of bees in and out of the nest; (6) evaporation and water loss from the nest; and (7) bee fanning (Seeley 1989; Bujok et al. 2002; Jones et al. 2004; Humphrey and Dykes 2008).

Bujok et al. (2002) showed that there are specialized adult worker 'heating' bees that maintain temperatures within a suitable range in the vicinity of the brood by pressing their warm thoraxes (38.1–42.4 °C) onto capped brood cells for several minutes at a time. These heating bees also enter vacant cells among the sealed brood cells generate heat (Kleinhenz et al. 2003). These bees may have thoracic temperatures as high as 42.5 °C prior to entering a vacant cell, and can maintain temperatures ranging from 32.7–40.6 °C in the cell for several minutes (Kleinhenz et al. 2003). In this way, it has been determined that a cell-heating bee can establish a thermal radius-of-influence of about three brood cells, the energy for which is generated by non-shivering thermogenesis, the isometrical contraction of the bee flight muscles decoupled from the flight mechanism (Seeley 1985; Southwick and Heldmaier 1987; Moritz and Southwick 1992).

Fehler et al. (2007) simulated the efficiency of brood nest incubation using a multi-agent based computer mode l, SeSAm (Fehler et al. 2007). They investigated the efficiency of the bee cell-heating strategy for a range of biologically appropriate conditions. They approached the problem by solving the unsteady form of the heat conduction equation for 20 × 20 hexagonal cells, representing the brood section of a comb. The simulation allows us to understand the function of randomly wandering bees or cell gaps on the comb. Following prescribed temperature-related behaviour rules, some bees press their warm thoraxes to the caps of

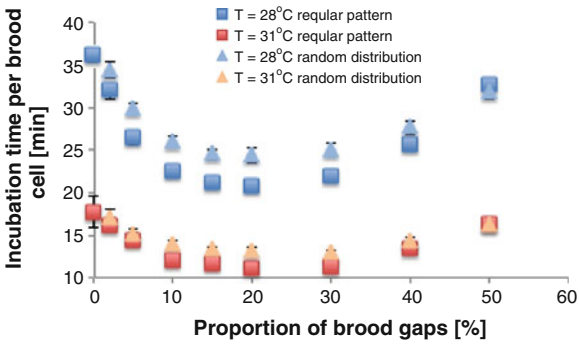

Fig. 8.11 Efficiency of brood incubation in *A. mellifera* colonies under different ambient conditions and with different proportions of brood gaps (Fehler et al. 2007)

cool brood cells to heat them; and others enter vacant cells (called 'gaps' by Fehler et al. 2007), adjacent to cool brood cells for the same purpose.

Fehler et al. (2007) stated that at any given set of simulation parameters (Fig. 8.11), a rise in the number of gaps from 0 % up to a certain optimum point, increases the efficiency of brood incubation, both in terms of incubation time and energy expenditure per brood cell. Therefore regularity in gap distribution is not essential, although that might lead to increases in comparison with the random distribution of gaps (Fig. 8.11) that are more likely to occur in natural colonies. The results of the energetic efficiency study are similar to those of the time measurements (Fig. 8.11).

Honeybee colonies also benefit from the presence and usage of a small proportion of gaps in the sealed brood area (Fehler et al. 2007). Although heat production inside gaps is not essential for the maintenance of optimum brood temperature, it clearly reduces the colony's costs (energy and time) per larva. This is a reasonable assumption because, for gap values ranging from 4 to 10 %, typical of healthy colonies, the Fehler et al. (2007) model predicts a significant reduction in the incubation time per brood cell to maintain the desired temperature. For gap values larger than 20 %, which would be unnatural in normal combs, the model predicts less efficient brood nest thermoregulation, so that the incubation time per brood cell would increase to maintain the required development temperature. Although not essential for maintaining optimal brood temperature conditions, the model shows that a small number of gaps improve heating efficiency whilst reducing the time required for heating.

Humphrey and Dykes (2008) performed a theoretical analysis to characterize the unsteady two-dimensional conduction of thermal energy in an idealized honeybee comb. They investigated the effects of cell-heating in combs by checking the heat fluxes to, and temperatures of, adjoining cells containing pupae, and nearby cells containing pollen, honey and air (Figs. 8.12, 8.13, 8.14).

The calculations of Humphrey and Dykes (2008) are based on different scenarios as follows:

To briefly summarize, the calculated results of Humphrey and Dykes (2008) are in accord with other experimental observations. The results provide an in-depth

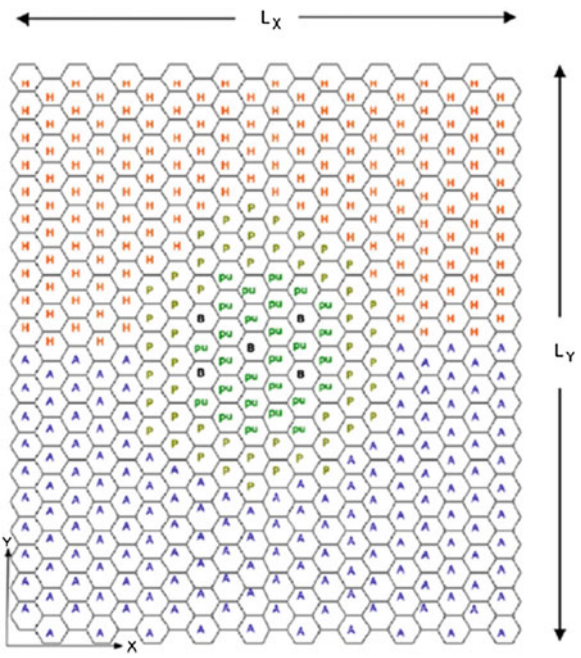

Fig. 8.12 Layout of *A. mellifera* comb cells identified by symbols (*H* honey, *A* air, *P* pollen, *pu* pupae, *B* cell-heating bee (Humphrey and Dykes 2008)

understanding heat transfer in a comb, which was not previously known. For the conditions explored, the calculated maximum temperatures due to cell-heating bees at the end of a 10 min heating phase, ranged from 37.4 °C for one bee, to 41 °C for five bees. Kleinhenz et al. (2003) found that bees raise their thoracic temperatures as high as 42.5 °C prior to entering a vacant cell in the brood region, and can maintain temperatures ranging from 32.7 to 40.6 °C in the cell for a few to several minutes. Similarly, the calculations revealed that the time rate of temperature increase, immediately around the cell of a heating bee ranges from $\sim$0.1 °C/min^{-1} for one heating bee, to $\sim$0.5 °C/min^{-1} for five heating bees. These values are in close agreement with the 0.1–0.2 °C/min^{-1} range measured by Kleinhenz et al. (2003). However, there do not appear to be any corresponding experimental values for the rates of temperature decrease during the cooling phase of a cell-heating/cooling cycle.

8.6 Cell Allocation

The cell allocation pattern is extremely important for the function of a comb. Seeley (1985) described a general cell allocation pattern in wild nests of honeybees: a dense brood clump surrounded by cells storing pollen, with honey stored in peripheral cells mostly in the upper region of the comb. In a ground-breaking study, Camazine (1991) further developed those observations and proposed a

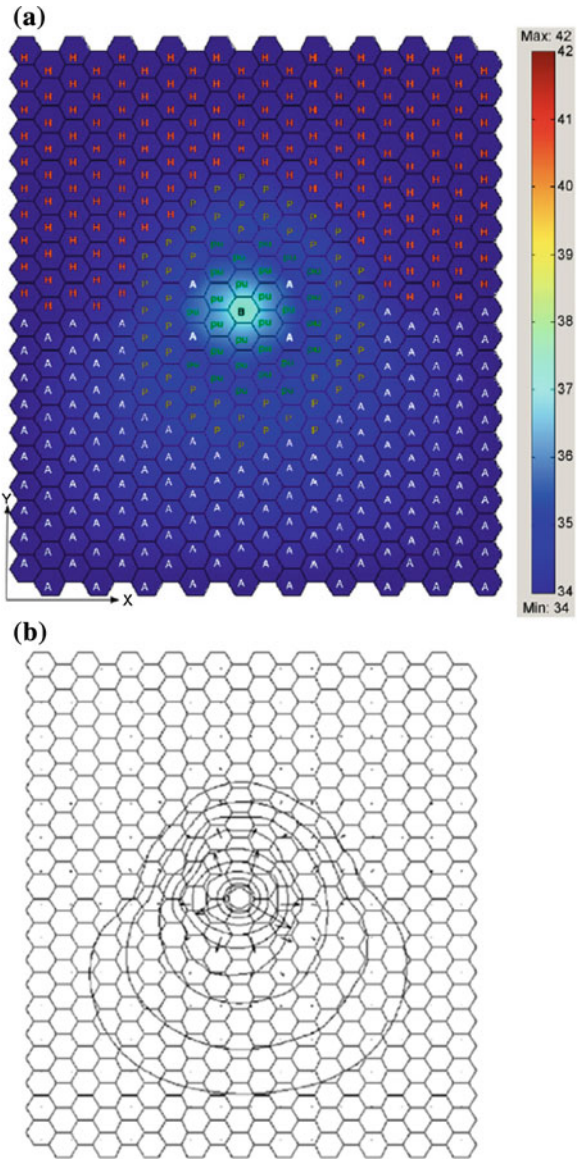

Fig. 8.13 One *A. mellifera* heating bee, in a cell at the comb center at t = 10 min: **a** colored temperature contours with cells labeled; **b** temperature line isotherms with heat flux vectors: outermost isotherm has a value of 34.41 °C, and innermost 37.05 °C (ΔT = 0.304 °C) (Humphrey and Dykes 2008)

self-organizing algorithm to explain the pattern of comb usage in *A. mellifera* honeybees, and showed that honeybees rear brood at the bottom of their nests, with pollen next to it and honey at the top and along the edges.

Camazine argued that the pattern could be generated by a self-organizing algorithm of three simple rules: (1) the queen lays eggs in the centre of the comb; (2) workers deposit pollen and nectar at random; and (3) bees preferentially

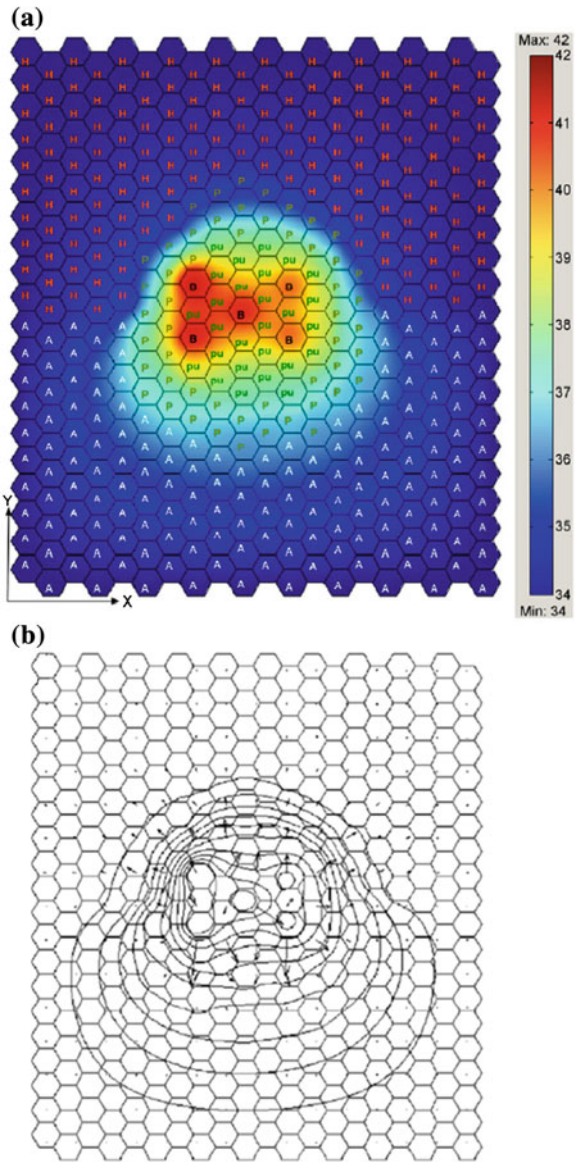

Fig. 8.14 Five *A. mellifera* heating bees in cells near the comb center at t = 10 min: **a** colored temperature contours with cells labeled; **b** temperature line isotherms with heat flux vectors: outermost isotherm has a value of 34.66 °C, and innermost 40.63 °C ($\Delta T = 0.663$ °C) (Humphrey and Dykes 2008)

removed pollen and nectar from the brood nest to the honey storage area. Subsequent studies supported this view (Camazine et al. 1990; Jenkins et al. 1992), and several reviews (Bonabeau et al. 1997; Camazine et al. 2001; Theraulaz et al. 2003) have since heralded this as a classic 'bottom-up' demonstration of self-organization in social insects. However, Camazine's model focuses on the pattern of the pollen band which can be explained with a simple self-organization algorithm. There is some inconsistency with what happens in reality because the model

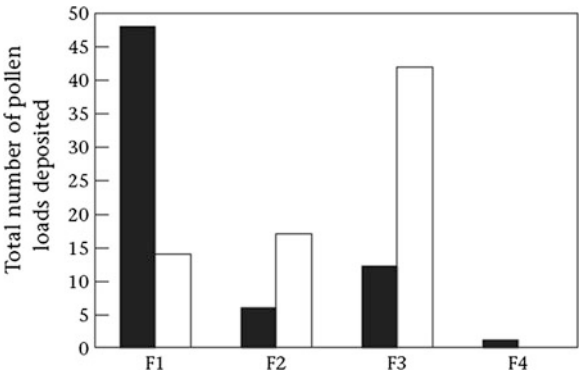

Fig. 8.15 Number of foragers which unloaded pollen on frames *F1–F4* while the frame with the unsealed brood was either in the first (*closed bar*) or third (*opened bar*) position (Dreller and Tarpy 2000)

explains that the pollen pattern is distributed as concentric (not requiring a directional component), whereas the pattern is actually strongly vertical, with the honey always being above and never below the brood (Seeley and Morse 1976). Camazine (1991) also concluded that workers unload pollen and nectar at random, while Dreller and Tarpy (2000) showed that pollen foragers prefer to unload and deposit their loads on open brood (Fig. 8.15).

Johnson (2009) re-examined pattern formation in honeybee combs by constructing an agent-based model of a honeybee colony that produces the characteristic pattern and elucidates the roles played by nectar receivers, pollen foragers and nurse bees in its construction. Running under conditions of a period of high nectar intake and no rain, the model showed that there is initially a disorganized phase, when pollen is unloaded throughout the nest and honey is present both above and below the brood. By day 14, most of the pollen, however, is at the bottom of the nest and a pollen band has not yet formed between the brood and honey. The brood zone occupies most of the nest (common in small colonies), and is below the honey zone, with a small empty zone in the middle. This space between the brood and honey contains pollen, but the pattern is not as strong as that described by Seeley and Morse (1976) which describes the characteristic patterns found in natural colonies (Fig. 8.16).

However, some simulations explored pattern formation during periods of rainy weather, which hold important consequences for honeybee foraging (Seeley 1985). Johnson (2009) therefore implemented ‘rain’ as a new parameter in the model with different combinations of mechanisms. Rain is reported to have two effects on the behaviour of the bees: (1) foragers do not forage on rainy days (Seeley 1985); and (2) on those days when rain led to the loss of pollen stores, the brood is cannibalized (Schmickl and Crailsheim 2001). Johnson hypothesized that during rainy spells, the bees eat through most of their pollen stores and the queen lays in many of the recently emptied cells. This leads to the only empty cells being between the brood and honey, where pollen foragers unload when foraging recommences. Thus, a larger pollen band forms in rainy weather. The electronic supplementary material shows the results of simulations for which rain occurred stochastically throughout the first 2 weeks of pattern formation. Both random and fixed patterns

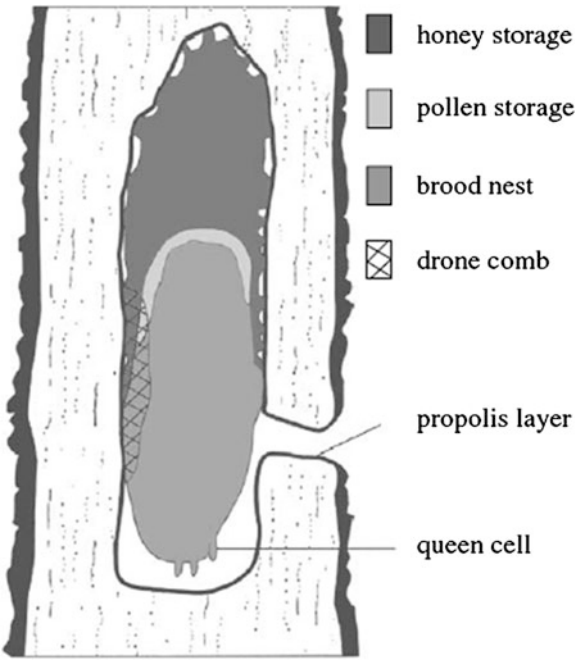

Fig. 8.16 Idealized drawing of the characteristic pattern on the surface of wild *A. mellifera* honeybee colonies (Seeley and Morse 1976)

of rain led to the formation of a thicker pollen band, relative to simulations without rain (Fig. 8.17).

Johnson (2009) concluded that pattern formation on honeybee combs is dependent on self-organization and at least two templates (e.g. the gravity-based template and the queen-based template, Fig. 8.18). This study also presented the role of each template without the 'queen based template' to allow the random unloading by pollen foragers as opposed to the template which resulted in pollen being scattered throughout the honey storage area (Fig. 8.18). Without the self-organizing mechanism to allow random removal of honey and pollen, this resulted in the absence of a pollen band, and the brood and honey zones became indistinct (Fig. 8.18). There are three possibilities to consider: (1) a gravity-based template; (2) the vertical random unloading of honey; and (3) the upward movement by nectar-receivers. Actually, the random unloading of pollen and nectar resulted in the pattern remaining concentric (Fig. 8.18d). However, only the original self-organization model of Camazine with pollen stores allows the differential removal of pollen and nectar from the brood nest; but this would still leave the pattern incomplete and not as well-developed as the natural pattern observed by Seeley and Morse (1976).

These processes are the result of the behaviour of four different groups of bees: the queen, the nectar receivers, the pollen foragers, and the nurse bees. The vertical pattern of honey on top and brood on the bottom arises from a gravity-based template effect, whereas the pollen band is the result of the combined effects of a

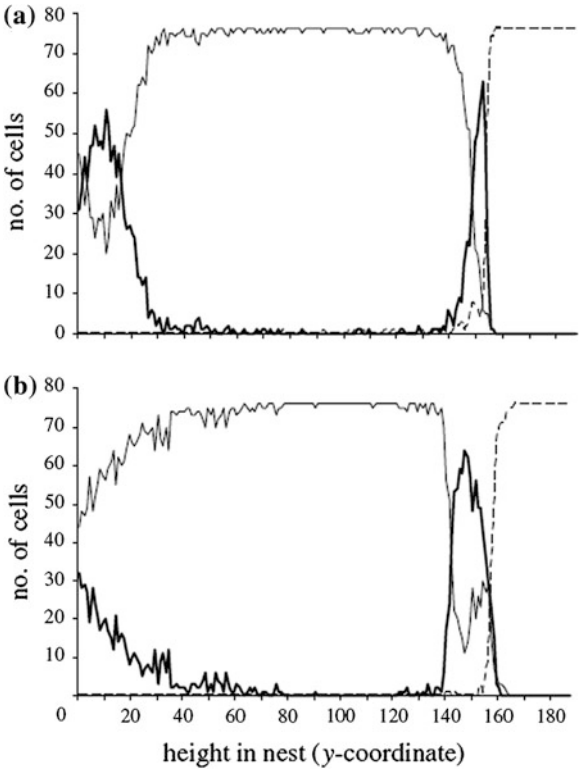

Fig. 8.17 Role played by rainy days in the formation of the pollen band between the brood and honey areas. The results of two simulations (with and without 3 days of rain), are shown; brood (*thin solid line*), honey (*dashed line*) and pollen (*thick solid line*) at different levels within the nest. The two representative simulations were chosen because their pollen bands were equal to the average of 30 simulations: **a** in the absence of rain, a relatively weak pollen band forms between the brood and honey, but most of the pollen is at the bottom of the nest; **b** during rainy spells, the bees consume most of their pollen stores and the queen fills in many of the recently emptied cells with eggs (Johnson 2009)

queen-based template and a self-organization process. Colonies using this complex pattern formation mechanism had higher growth rates in terms of egg-laying than colonies using self organization alone (Fig. 8.19). Without a bias in the direction of nectar unloading, honey quickly filled the whole nest and prevented the queen from laying at her optimal rate. Johnson (2009) combined the idea of self-organization with gravity-based templates (i.e. blueprint-like rules), which caused a bias in that the movement of nectar handlers was towards the top of the comb, and this produced a more natural pattern with the honey being stored near the top of the comb. This model includes two kinds of global information, templates for nectar storage and brood cells; but it only considers the pattern formation before young bees start to vacate their cells (the first 20 days). This model is suitable for the start-up of a colony, but could not maintain it in the long run.

A more recent model of the storage pattern developed by Montovan et al. (2013), presented a cellular automaton model after that of Johnson to maintain storage patterns over multiple brood cycles. Their model, together with that developed by Camazine (1991), can create a self-organizational pattern on an almost empty comb (now referred to as model 1), and change some of the rules in biologically reasonable ways to create models that both initially create, and then steadily maintain the comb allocation patterns once young bees begin to vacate their cells.

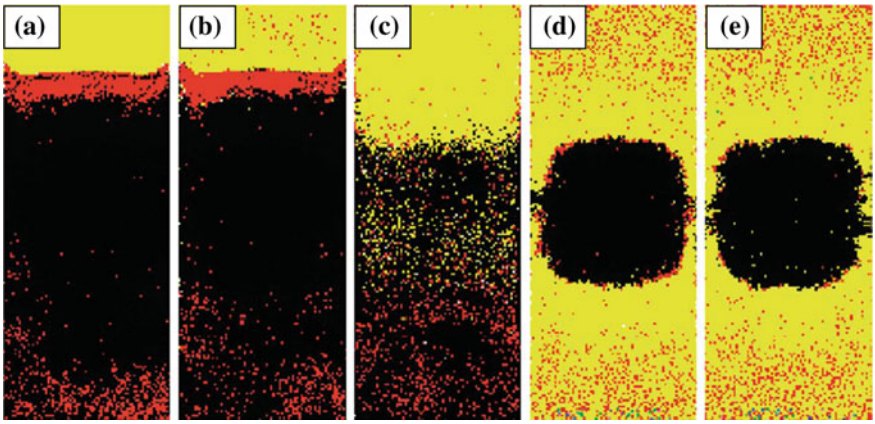

Fig. 8.18 Role played by self-organization, gravity-based template and queen-based template for *A. mellifera*. Each picture shows the pattern at 14 days: **a** full model (SO + T1 + T2) (with rain); **b** without the queen-based template (SO + T1); **c** without the self-organizing mechanism (T1 + T2); **d** without the gravity-based template (SO + T2); **e** the original self-organization model of Camazine + 3 days' worth of pollen stores. Honey cells are *yellow*, pollen cells are *red* and brood cells *black* (Johnson 2009)

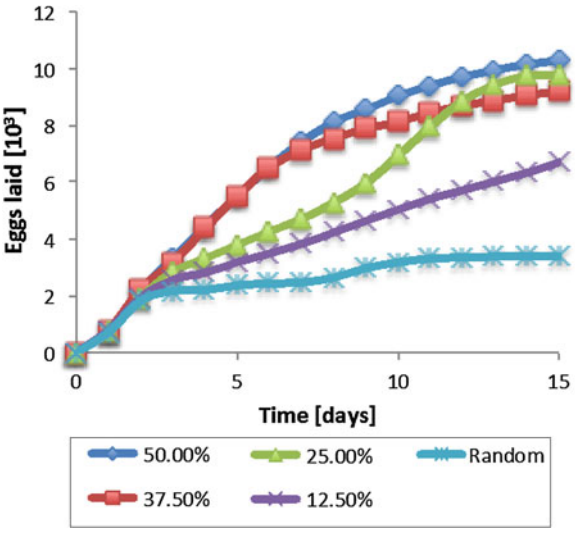

Fig. 8.19 Growth rate (in eggs laid) of colonies when *A. mellifera* workers varied in their bias towards unloading at the top of the nest, no upward bias or random (*solid line*), 12.50 % bias (*triangles*), 25 % bias (*diamonds*), 37.50 % bias (*filled squares*) and 50 % bias (*open squares*) (Johnson 2009)

Three different models have been developed with different conditions based on the egg-laying behaviour of the queen, deposition of honey and pollen, and honey and pollen consumption by worker bees. Two hundred unique parameter sets were used to analyze all three models, with ranges for the key parameters (see Table 8.2) chosen based on the relevant literature, and ranges extended to accommodate uncertainty in parameter estimates.

Table 8.2 Parameters used in simulations of models 1–3 and the sensitivity analysis (Montovan et al. 2013)

Parameter	Description	Estimate	Range
n	Queen's cell visitation rate (cells per hour)	60 Camazine (1991)	60–120
r_b	Brood requirement radius (cells)	4 Camazine (1991)	1–4
r_n	Preferential nectar consumption radius (cells)	4 Camazine (1991)	1–4
ω	Average honey collection(loads per day)	833 Montovan et al. (2013)	1000–4000
P_{ph}	Ratio of pollen collection to honey collection (dimensionless)	0.21 Camazine (1991)	0.2–1.0
P_p	Ratio of pollen consumption to pollen collection (dimensionless)	0.99 Camazine (1991)	0.9–1.1
P_h	Ratio of honey consumption to honey collection (dimensionless)	0.59 Camazine (1991)	0.9–1.1
X	Temporal distribution of daily nectar and pollen collection: uniform constant ($X = 0$), uniform random ($X = 1$) and Markov clumped random ($X = 2$)	NA	0–2
k	Model 1: Ratio of honey/pollen taken from cells fully surrounded by brood cells to honey/pollen taken from cells with no brood neighbours (dimensionless)	10 Camazine (1991)	5–20
k	Model 2 and 3: Ratio of probability that a cell fully surrounded by brood cells chosen for nectar consumption to the probability that a cell with no brood neighbours are chosen (dimensionless)	10	5–20

In the first model, the queen performs a random walk across the comb and attempts to oviposit in suitable cells, while workers attempt to randomly deposit honey and pollen in cells. But at the same time, the workers attempt to consume honey and pollen randomly from all cells. As it turns out, the actual number of loads taken is proportional to the number of neighboring brood cells. This model is not capable of maintaining the pattern beyond 60 day periods. The second model was developed by changing the honey/pollen consumption rules as the queen performs a random walk across the comb and attempts to oviposit in suitable cells. Workers still attempt to deposit honey and pollen randomly in all cells but, the probability that a cell will be selected is proportional to the number of neighboring brood cells. This second model showed nine parameter sets able to maintain the pattern over 60 days.

Later, a third model was adjusted by the preferential consumption rule of the second model, and incorporated workers attempting to consume one load of honey or pollen at a time, with the probability that a cell will be selected being

proportional to the number of neighboring brood cells and a bias for the queen's random walk towards the centre of the comb was added. Sixteen of the 200 simulations of the third model exhibited a well formed pattern. Pattern retention is more robust in the third than the second model. This work extends discussion to consider additional requirements for maintaining order after a honeybee colony has been established. The authors concluded that, maintenance could reasonably be expected from any process which can create order in some system, but in honeybees, the rules of initial pattern formation could not sustain the pattern of colony in later cycles (Montovan et al. 2013).

References

Aichholz R, Lorbeer E (1999) Investigation of comb wax of honeybees with high-temperature gas chromatography and high-temperature gas chromatography-chemical ionization mass spectrometry I. High-temperature gas chromatography. J Chromatogr A 855:601–615

Al-Tikrity WS, Benton AW, Hillman RC, Clarke WW (1972) The relationship between the amount of unsealed brood in honeybee colonies and their pollen collection. J Apic Res 11:9–12

Barker RL (1971) The influence of food inside the hive on pollen collection by a honeybee colony. J Apic Res 10:23–26

Bittermann ME (1988) Vertebrate invertebrate comparison NATO. ASI Ser Vol (G) 17:251–275

Bittermann ME (1996) Comparative analysis of learning in honeybees. Anim Learn Behav 24:123–124

Boehm B (1965) Beziehungen zwischen Fettkörper, Oenocyten und Wachsdrussenentwicklung bei *Apis mellifica* L. Z Zellforsch Mikrosk Anat 65:74–115

Bonabeau E, Theraulaz G, Deneuborg JL, Aron S, Camazine S (1997) Self-organization in social insects. TREE 12:188–193

Bujok B, Kleinhenz M, Fuchs S, Tautz J (2002) Hot spots in the hive. Naturwissenschaften 89:299–301

Butler CG (1974) The world of the honeybee. Collins, London

Calderone NW (1993) Genotypic effects on the response of worker honeybees, *Apis mellifera*, to the colony environment. Anim Behav 46:403–404

Cale GH (1968) Pollen gathering relationship to honey collection and egg laying in honeybees. Am Bee J 108:8–9

Camazine S (1991) Self-organizing pattern formation on the combs of honey bee colonies. Behav Ecol Sociobiol 28:265–272

Camazine S (1993) The regulation of pollen foraging by honeybees: how foragers assess the colony's need for pollen. Behav Ecol Sociobiol 32:265–272

Camazine S, Sneyd J, Jenkins MJ, Murray JD (1990) A mathematical model of self-organized pattern formation on the combs of honeybee colonies. J Theor Biol 147:553–571

Camazine S, Crailsheim K, Hrassnigg N, Robinson GE, Leonhard B, Kropiunigg H (1998) Protein trophallaxis and the regulation of pollen foraging by honey bees (*Apis mellifera* L.). Apidologie 29:113–126

Camazine S, Deneubourg JL, Franks N, Sneyd J, Theraulaz S, Bonabeau E (2001) Self-organized biological systems. Princeton University Press, Princeton, Oxford

Chauvin R (1976) Sur des substances qui provoquent l'étirage de la cire. Apidologie 7:237–242

Chauvin R, Darchen R, Pain J (1961) Sur l'existence d'une hormone de construction chez les abeilles. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 253:1135–1136

Cheshire FR (1888) Bees and beekeeping; scientific and practical, vol 1. L Upcott, London

Crailsheim K (1990) The protein balance of the honeybee worker. Apidologie 21:417–429
Crailsheim K, Schneider LHW, Hrassnigg N, Bühlman G, Brosch U, Gmeinbauer R, Schöffmann B (1992) Pollen consumption and utilization in worker honeybees (*Apis mellifera*). J Insect Physiol 38:409–419
Crailsheim R, Crailsheim K, Hrassnigg N, Stabentheiner A (1996) Diurnal behavioural differences in forager and nurse honeybees (*Apis mellifera carnica* Pollm.). Apidologie 27:235–244
Darchen R (1956) La reine d'*Apis mellifica* et les constructions cirières. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 243:439–441
Darchen R (1957) La reine d'*Apis mellifica*, les ouvrières pondeuses et les constructions cirières. Insectes Soc 4:321–325
De Groot AP (1953) Protein and amino acid requirements of the honeybee (*Apis mellifica* L). Physiol Comp Oecol 3:197–285
Dreller C, Page RE, Fondrk MK (1999) Regulation of pollen foraging in honeybee colonies: effects of young brood, stored pollen, and empty space. Behav Ecol Sociobiol 45:227–233
Dreller C, Tarpy DR (2000) Perception of the pollen need by foragers in a honeybee colony. Anim Behav 59:91–96. doi: http://dx.doi.org/10.1006/anbe.199.1301
Dumas JB, Edwards HM (1843) Note sur la production de la cire des abeilles. Ann Sci Nat Paris 20:l–8
Dżierzon J (1861) Rationelle Bienenzucht oder Theorie und Praxis. Falch'sche Buchdrukererei, Brieg
Eckert CD, Winston ML, Ydenberg RC (1994) The relationship between population size, amount of brood, and individual foraging behaviour in the honeybee, *Apis mellifera* L. Oecologia 97:248–255
Fehler M, Kleinhenz M, Klugl F, Puppe F, Tautz J (2007) Caps and gaps: a computer model for studies on brood incubation strategies in honeybees (*Apis mellifera carnica*). Naturwissenschaften 94:675–680
Fell RD, Morse RA (1984) Emergency queen cell production in the honeybee colony. Insectes Soc 31:221–237
Fergusson AW, Free JB (1981) Factors determining the release of Nasonov pheromones by honeybees at the hive entrance. Physiol Entomol 6:15–19
Fewell JH, Winston ML (1992) Colony state and regulation of pollen foraging in the honeybee, *Apis mellifera* L. Behav Ecol Sociobiol 30:387–393
Filmer RS (1932) Brood area and colony size as factors in activity of pollination units. J Econ Entomol 25:336–343
Free JB (1967) Factors determining the collection of pollen by honeybee foragers. Anim Behav 15:134–144
Free JB, Williams IH (1971) The effect of giving pollen and pollen supplement to honeybee colonies on the amount of pollen collected. J Apic Res 10:87–90
Free JB, Wiliams IH (1974) Factors determining food storage and brood rearing in honeybee (*Apis mellifera* L.) comb. J Entomol Ser A49:47–63
Free JB, Williams IH (1972) Hoarding by honeybees (*Apis mellifera* L). Anim Behav 203:321–334
Freudenstein H (1960) Einfluss der Pollennahrung auf der Bauvermögen, die Wachsdrüsen und den Fettkörper der Honigbiene (*Apis mellifera* L). Zoo1 Jahrb Allg Zoo1 Physiol Tiere 69:95–124
Goetze G, Bessling BK (1959) Die Wirkung verschiedener Fütterung der Honigbiene auf Wachserzeugung und Bautätigkeit. Z Bienenforsch 4:202–209
Haydak MH (1970) Honey bee nutrition. Ann Rev Entomol 15:143–156
Hellmich RL, Kulincevic JM, Rothenbuhler WC (1985) Selection for high and low pollen-hoarding honeybees. J Hered 76:155–158
Hepburn HR (1998) Reciprocal interactions between honeybees and combs in the integration of some colony functions in *Apis mellifera*. Apidologie 29:47–66

Herbert EW (1992) Honey bee nutrition. In: Graham JM (ed) The hive and the honey bee. Dadant & Sons, Hamilton, pp 197–233
Hornborstel HC (orig Mellitophilus Theosebastus) (1744) Neue Entdeckung, wie dar Wachs won den Bienen Komt. Hamburg Vermis Bibliothek 2:45–62
Huber F (1814) Nouvelles observations sur les abeilles. English trans: (1926). Dadant, Hamilton
Humphrey JAC, Dykes ES (2008) Thermal energy conduction in a honeybee comb due to cell-heating bees. J Theor Biol 250:194–208
Hunter J (1792) Observations on bees. Phil Trans R Soc 82:128–196
Jay SC (1964) The cocoon of the honey bee, *Apis mellifera* L. Can Entomol 96:784–792
Jenkins MJ, Sneyd J, Camazine S, Murray JD (1992) On a simplified model for pattern formation in honey bee colonies. J Math Biol 30:281–306
Johnson BR (2009) Organizing model for task allocation via frequent task quitting and random walks in the honeybee. Am Nat 174:537–547
Jones J, Myerscough M, Graham S, Oldroyd BP (2004) Honey bee nest theromregulation: diversity promotes stability. Science 305:402–404
Kaneda T (1967) Biosynthesis of long-chain hydrocarbons. 1. Incorporation of L-valine, L-threonine. L-isoleucine and L-leucine into specific branched chain hydrocarbons in tobacco. Biochem 6:2023–2032
Kleinhenz M, Bujok B, Fuchs S, Tautz J (2003) Hot bees in empty broodnest cells: heating from within. J Exp Biol 206:4217–4231
Kolattukudy PE (1968) Biosynthesis of surface lipids. Science 159:498–535
Kurstjens SP, Hepburn HR, Schoening FRL, Davidson BC (1985) The conversion of wax scales into comb wax by African honeybees. J Comp Physiol B156:95–102
Lindauer M (1952) Ein Beitrag zur Fräge der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 34:299–345
Lipiński Z (2006) The emotional nature of the worker honeybee (*Apis mellifera* L.). J Apic Sci 50:49–62
Manning R (2001) Fatty acids in pollen: a review of their importance for honey bees. Bee Wld 82:60–75
Mattila HR, Otis GW (2006a) Influence of pollen diet in spring on development of honeybee (Hymenoptera: Apidae) colonies. J Econ Entomol 99:604–613
Mattila HR, Otis GW (2006b) The effects of pollen availability during larval development on the behaviour and physiology of spring-reared honeybee workers. Apidologie 37:533–546
Maurizio A (1954) Pollenernährung und Lebensvorgänge bei der Honigbiene (*Apis mellifica* L.). Landwirt Jahrb Schweiz 62:115–182
Maurizio A (1959) Factors influencing the lifespan of bees. In: Wolstenholme GEW, O'Connor M (eds) Proceedings of the ciba foundation, symposium the lifespan of animals, Churchill, London, 231–243
McDonald JL (1968) The behaviour of pollen foragers which lose their load. J Apic Res 7:45–46
Montovan KJ, Karst N, Jones LE, Seeley TD (2013) Individual behavioral rules sustain the cell allocation pattern in the combs of honey bee colonies (*Apis mellifera*). J Theoret Biol 336:75–86. doi:10.1016/j.jtbi.2013.07.010, arXiv:1211.4298v2[q-bio.PE]
Moritz RFA, Southwick EE (1992) Bees as superorganisms-an evolutionary reality. Springer, Berlin
Moeller FE (1972) Honeybee collection of corn pollen reduced by feeding pollen in the hive. Am Bee J 112:210–212
Naumanna K, Winstona ML, Slessor KN, Prestwich GD, Latlia B (1992) Aromatic honey bee queen mandibular gland pheromone components: movement as a unit. Canad Entomol 124:917–934
Nye WP, Mackensen O (1970) Selective breeding of honeybees for alfalfa pollen collection: with tests in high and low alfalfa pollen collection regions. J Apic Res 9:61–64
Page RE, Robinson GE (1991) The genetics of division of labor in honeybee colonies. Adv Insect Physiol 23:117–169

Pankiw T, Page RE, Fondrk MK (1998) Brood pheromone stimulates pollen foraging in honeybees (*Apis mellifera*). Behav Ecol Sociobiol 44:193–198
Peterka V (1939) Sojamehl und die Fräge der Pollenersatzmittel. Schweiz Bienen-Ztg 62:143–153
Phiancharoen M, Duangphakdee O, Hepburn HR (2011) Biology of nesting. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 109–132
Robinson GE, Page RE (1988) Genetic determination of guarding and undertaking in honeybee colonies. Nature 333:356–358
Rösch GA (1930) Untersuchungen über die Arbeitsteilung im Bienenstaat. Die Tätigkeiten der Arbeistbienen unter experimentet veränderten Bedingungen. Z Vegel Physiol 12:1–71
Röseler PF (1967) Lage und Funktionsleistung der Wachsdrüsen bei einigen Hummerlarten im Vergleich zu anderen Apiden. Zool Anz 94:73–783
Schmickl T, Crailsheim K (2001) Cannibalism and early capping: strategy of honeybee colonies in times of experimental pollen shortages. J Comp Physiol A187:541–547
Schmid A, Kleine G (1865) Leitfaden für den Unterricht in Theorie und Praxis einer rationellen Bienenzucht. Bech, Nordlingen
Seeley TD (1985) Honeybee ecology. A study of adaptation in social life. Princeton University Press, Princeton
Seeley TD (1989) The honey bee colony as a superorganism. Am Sci 77:546–553
Seeley TD (1995) The wisdom of the hive: the social physiology of honey bee colonies. Harvard University Press, Cambridge
Seeley TD, Morse RA (1976) The nest of the honey bee (*Apis mellifera* L.). Insectes Soc 23:495–512
Southwick EE (1991) The colony as a thermoregulating superorganism. In: Goodmann LJ, Fischer RC (eds) The behaviour and physiology of bees. CAB International, Wallingford, pp 28–47
Southwick EE, Heldmaier G (1987) Temperature control in honeybee colonies. Bioscience 37:395–399
Taranov GF (1959) The production of wax in the honeybee colony. Bee Wld 40:113–121
Tautz J, Maier S, Groh C, Rossler W, Brockman A (2003) Behavioral performance in adult honey bees is influenced by the temperature experienced during their pupal development. Proc Nat Acad Sci 100:7343–7347
Theraulaz G, Gautrais J, Camazine S, Deneubourg JL (2003) The formation of spatial patterns in social insects: from simple behaviours to complex structures. Phil Trans R Soc Lond 361:1263–1282
Todd FE, Reed CB (1970) Brood measurement as a valid index to the value of honeybees as pollinators. J Econ Entomol 63:148–149
Tulloch AP (1973) Comparison of some commercial waxes by gas liquid chromatography. J Am Oil Chem Soc 50:269–272
van Laere O, Martens N (1971) Influence d'une diminution artificielle de la provision de proteines sur l'activité de collete de la colonie d'abeilles. Apidologie 2:197–204

Chapter 9
Nectar Flows and Comb-Building

Abstract In temperate zones, the onset of comb-building is associated with warm fronts, the more intense and closer together, the greater the colony response. European *A. mellifera* are commonly dormant during winter, but Asian bees are active during the tropical dry season. Comb-building occurs during the dry season and the rainy season is their dormant period. Some plants flower during the rainy season and provide sufficient forage for the dwarf honeybees to complete their comb within three weeks. Large *A. dorsata* colonies cannot subsist on such meager resources and seasonally migrate. Comb-building pulses require that comb fullness reach a threshold, with a balance of brood and stored food. Comb-building peaks are correlated with high comb fullness and with correlations between daily nectar intake and comb construction. Wax production is reduced in the absence of a nectar flow; likewise, the greater the supply of combs in the nest, the greater the increase in number of nectar foragers. Nectar forage, empty combs and free building space within the nest are correlated with engorgement of the honey stomach and wax secretion in workers. Once building has begun, the colony will monitor only nectar intake to control comb-building. They build when nectar can be collected in the field and the combs are filled above their thresholds for comb fullness and nectar intake. The amount of wax is constant among age cohorts and across the seasons. About half of the wax in a colony is borne by festoon bees, the remainder from non-festoon bees, except in winter when non-festoon wax production is higher than festoon wax production.

9.1 Introduction

Comb-building is conducted in different areas of the nest by many individuals, some clustered in festoons, others not, while other wax-workings are often the efforts of individual bees (Lindauer 1952). Yet, the basic stimulus for comb-building is 'flowering', which produces the nectar and pollen essential in providing the energy required for colony development. These two factors allow colonies to

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_9,

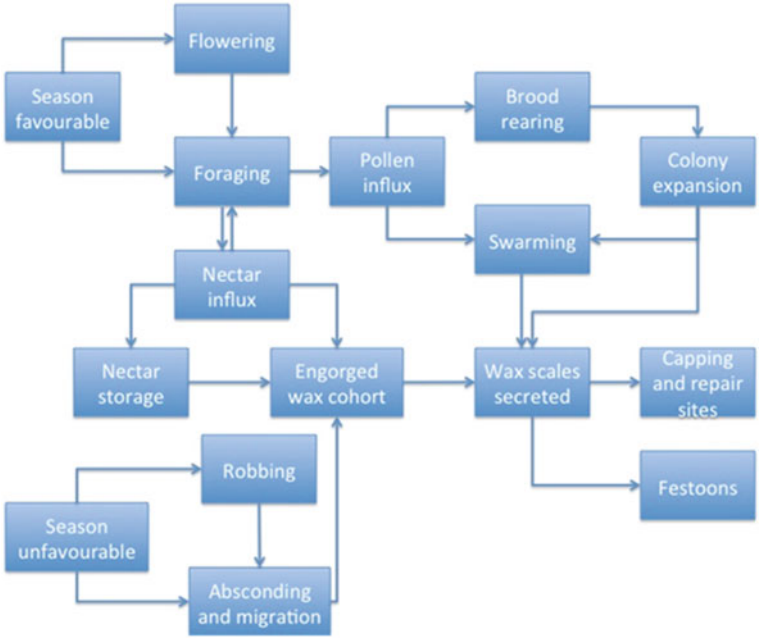

Fig. 9.1 Simplified flow diagram for the stimulation of wax secretion in *A. m. capensis*. The favourable season sequence should apply to all subspecies of *A. mellifera*, and probably also to *A. cerana*; the unfavourable sequence applies to African *A. mellifera* (Hepburn 1998)

grow and to complete annual cycles. If conditions are unfavorable, colonies will abscond. This is borne out in the readily observed differences in bee behavior between continents which have different climatic seasons (Hepburn 1998) (Fig. 9.1). This chapter explores the ways in which nectar affects comb-building. Here again, the preponderance of the relevant literature is based mainly on temperate zone *A. mellifera*, and to a lesser extent, tropical *A. mellifera.*

Forgetting fundamental differences between the species of *Apis*, there are major differences between the climatological regimes of the temperate zone regions and the way the 'spring' is the stimulus for kick-starting colony cycles (Koch 1957, 1959, 1961), whereas in the tropical and neotropical belts nearer the equator honeybees do not over-winter in the northern sense. It has long been known that the gathering of nectar and the construction of combs are related; when the flow of nectar ceases, comb construction falls into abeyance.

If we consider the additional problem of restricted space for storing incoming nectar then it is worth remembering the various observations, stretching back two centuries, to Huber (1814), Gundelach (1842) and Miner (1849) and, more recently Ribbands (1953) on the bloated appearance of wax-secreting bees pieces of the puzzle begin to fall into place. Taking the focal points of these observations, Butler (1974) formulated a very attractive hypothesis on how the influx of nectar results

in the secretion of wax. Butler argued that if there is insufficient space for house bees to store incoming nectar, the bees are compelled to retain nectar in their honey stomachs or crops for some time. This inevitably results in the assimilation of sugar by the bees retaining the nectar, followed by the activation of the wax glands. The greater the rate of nectar influx, the greater the additional comb space required to store it. The longer the house bees serve as reservoirs, the more sugar is assimilated, wax glands activated and wax secreted. If the nest cavity is so occluded with combs and there is no place in which more combs can be constructed, the bees still secrete wax, but the scales are simply dropped on the bottom of the nest cavity.

These intriguing ideas, as expressed by Butler (1974), remained untested. As a first approximation, Hepburn and Magnuson (1988) performed experiments using *A. m. scutellata* to assess nectar forage and the availability of empty combs as well as free building space within the nest cavity in relation to wax secretion. They found a positive correlation between engorgement of the honey stomach and wax secretion. While empty combs and free building space were positively associated with wax secretion, their intensities as stimuli are relative to context. However, a real impetus to resolving these relationships was recently provided in series of experimental works by Pratt (1998a, b, 1999, 2004) as shown in the text below.

9.2 Temperate Zone Spring as a Stimulus

In 1609, Charles Butler engagingly described honeybees as 'summer birds' because each year colonies begin afresh the founding of nests or greater expansions of combs in an old one. This knowledge is contained in mediaeval calendars and probably reaches back into prehistory. The convergence of the myriad factors and events by which we all recognise spring contains a seemingly endless number of possible stimuli to which, in various permutations, honeybees might be encoded to respond: blossoming, nectar flow, season, ambient temperature, the number of young bees available to produce wax and the gathering of nectar and pollen (Hepburn 1986). In the temperate zone, where the seasons have distinctly different temperature profiles from the tropics, they are clearly divisible into spring, summer, autumn and winter. Spring is when many flowering plants are in bloom, and the dissection of spring into testable hypotheses has become crucial to apiculture in particular, and many branches of insect biology in general (Tauber et al. 1986). Although honeybees do not hibernate, they remain in the nest during winter, poised for the first signals of spring.

There has been only one significant series of studies to date for which an attempt was made to define the elements of spring to which honeybees might respond; that of Koch (1957, 1959, 1961). The last chapter of this trio is devoted to an analysis of the relationship between the onset of comb construction and some meteorological aspects of the European spring. Koch shrewdly chose a range of sites in Germany where there are large temporal differences in the frequency and

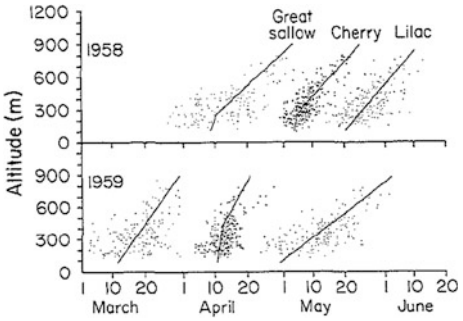

Fig. 9.2 The onset of blossoming in the great sallow (*Salix caprea*), cherry (*Prunus avium*) and lilac (*Syringa vulgaris*) in relation to altitude for 1958 and 1959 at eight different stations extending from the Baltic Sea to the central German mountains. In this altitude-time diagram, the restricted period of flowering in the cherries is particularly noteworthy for 1959. The curves indicate the arithmetic averages of the points at 50 m intervals; they are shifted to the right if the delay in flowering is greater at altitude than at sea level (Koch 1961)

origin of springtime warming phases and where there are striking thrusts or surges in the renaissance of trees and flowers. Koch (1961) studied six sites comprising three different weather patterns, extending from the Baltic Sea to the central German mountains. His method was one of survey, in which he was assisted by professional apiarists who noted in detail comb construction activities over three spring seasons (1958–1960).

Koch's first examinations were botanical ones; he found that plant species could be grouped, more or less, into three successive surges of leafing or flowering, irrespective of when the warming spring might have actually begun. As a further generalisation, surges in flowering were always in the second half of the primary spring. Comb construction was always found to begin in the second surge of renewal; when the sweet cherry (*Prunus avium*), early peaches (*P. persica*), plums (*P. domestica*) and dandelions (*Taraxacum officinale*) were in flower and the leaves of plane trees (*Platanus* sp.) and white birches (*Betula* sp.) unfurled. Koch (1961) found that the narrowest flowering period was that of the cherry (Fig. 9.2), and that the onset of comb-building went hand-in-hand with the appearance of these blossoms (Figs. 10.3–10.5).

It is implicit in Koch's writings that there need not be a direct link between any particular plants and comb construction, even though the plants might provide the stimuli for building activities. Put in question form, Koch (1961) asked: Under what circumstances, if any, is weather correlated with the onset of comb-building? He plotted the frequency distributions of comb-building and found temporal variations within and between sites over the seasons. The relationships between the onset of building and temperatures over three years at his sites, Gatersleben, Altenberga and Bergen are shown in Figs. 10.3–10.5.

The warmth of spring was gradual in 1958; there were no sharp peaks or heat surges and the daily mean temperature hovered around 10 °C. Comb-building

began at all sites when there were small peaks in the daily mean temperature above 10 °C. In contrast, 1959 was strikingly different because there were two early surges in spring temperature during which the fruit trees blossomed and the bees began comb-building. These two warm peaks were sufficient to get most of the colonies started. A third peak at Bergen roused the remaining few colonies into comb-building. In the following year, 1960, a short warm front aroused a few colonies to begin, but they subsequently subsided until the next warm front; sustained comb-building only began with the second and third warm spells. Despite enormous variation in weather over the three years, the onset of comb-building was always associated with warm fronts (Figs. 10.3–10.5); the more intense and close together the fronts, the greater the colony response.

Having noted how the flowering times of great sallows, cherries and lilacs varied with altitude, Koch (1961) then examined the relationship between the onset of comb construction and plant development in 1958 and 1959 (Fig. 9.2). Of these major plants, the wild cherries had the narrowest flowering period across Germany, regardless of whether spring came early or late. The flowering of the cherries also followed the narrow band of warm spells so closely that Koch was able to conclude that, given surges of warmth, the flowering of the cherries was condensed; but when spring extended over a longer period of time, then the blossoming of the cherries was also prolonged. The cherry blossoms proved to be an excellent bioindicator of comb-building in the German landscape.

The commencement of comb-building at all three stations was associated with a mean daily high of 10–11 °C (Figs. 9.3, 9.4, 9.5); if the temperature dropped, none of the colonies began construction in the ensuing trough. This is not to say that colonies which had previously begun to build comb ceased doing so in a trough of low temperature; cessations only came about if there had been a severe enough frost to stem the flow of nectar. This led Koch to suggest that a 10 or 11 °C set point is a sufficient, but not necessary, condition for comb construction. Because he felt that the daily maximum temperature expressed the 'warmth' of the day, Koch thought this value a good threshold index. A far greater number of colonies responded to daily maximum temperatures above 15 °C. When the onset of spring was prolonged and the daily mean temperature hovered between 10 and 12 °C, and the daily maximum temperature did not rise above 15 °C, colony response was poor.

9.3 Tropical Areas: Environmental-Based Construction

Closer to the equator colonies of bees do not over-winter in the northern sense. Hot regions have two or three seasons; the rainy (wet or monsoon) season, the dry season, and in some tropical areas, a cool or mild season. Flowering in tropical regions is highly variable and many regions between 10° and 25° latitude have seasonal wet and dry cycles. With an influx of humid air, the wet season stimulates growth in some perennial plants as a precursor to flowering, whereas the

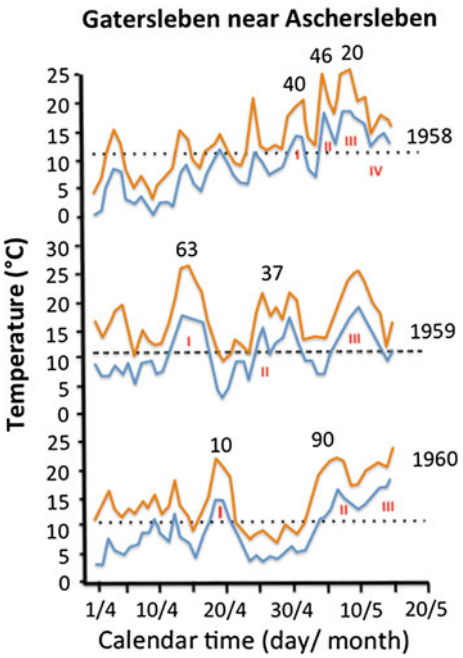

Fig. 9.3 The daily average (lower trace) and maximum (upper trace) temperatures of spring for three successive years at Gatersleben (51.49N, 11.28E, Alt 110 m) near Aschersleben. The Roman numerals indicate periods during which some colonies began comb construction, while an estimated percentage of the total is given above the peaks for each year and place (Koch 1961)

herbaceous plants follow normal cycles independently of the season. Some perennial plants become partially or fully dormant in the dry season when sunlight is less intense, temperatures are cool and rainfall scant; the foliage is shed to conserve water and prevent death from drought during prolonged dry periods; the plants then enter the monsoon season and follow a new cycle of growth and flowering (Hepburn and Radloff 1995).

However, in the evergreen forests of Southeast Asia, which are primarily dominated by trees, Dipterocarpaceae, general flowering events occur between 2 and 10 years (Sakai et al. 1999; Oldroyd and Wongsiri 2006; Rattanawanee et al. 2012a). In this type of forest, flowering occurs year round, but at low densities, and individual trees of each species tend to be spaced far apart. Another unique feature of aseasonal forests, which produce another unique and fascinating phenomenon, is mass flowering and mass fruiting. The majority of dipterocarp species, and members of several other families, explode into almost simultaneous flowering episodes over wide regions which last for about six months (Ridley 1901; Wood 1956; Corlett 2011). An entirely different flowering system occurs in the highlands of Ethiopia, where there is extensive swarming and migration by the ecotypes of *A. mellifera* in response to changing flowering seasons (Nuru et al. 2002; Shenkute et al. 2012). The availability of food is vital to tropical bees starting a new colony. The major period of comb construction is during the dry season while the dormant period is over the rainy season.

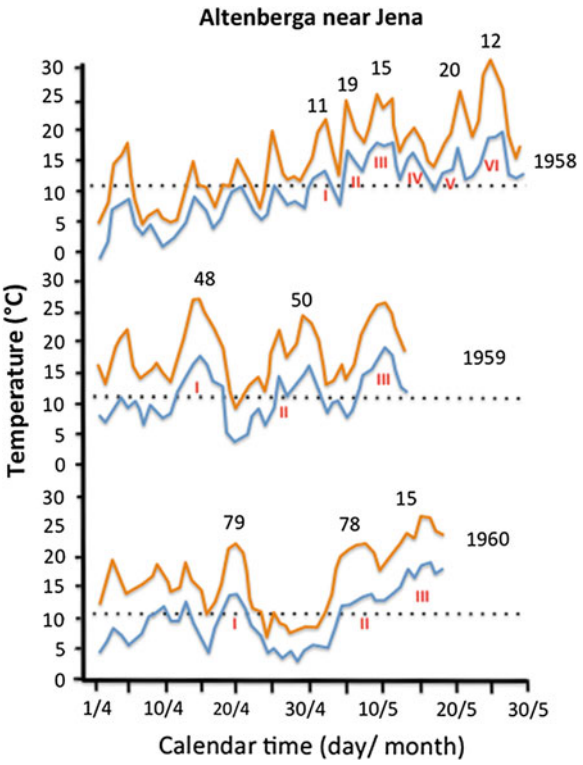

Fig. 9.4 As in Fig. 9.3, but data from Altenberga (50.49N, 11.31E, alt 265 m) near Jena (Koch 1961)

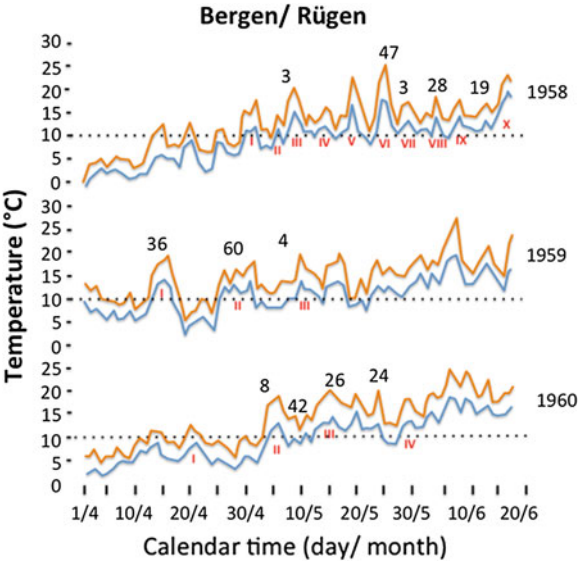

Fig. 9.5 As in Fig. 9.3 but data from Bergen/Rugen (54.25N, 13.25E, Alt 49 m) (Koch 1961)

Fig. 9.6 Comb development of *A. florea;* **a** day 6, **b** day 21

While the European honeybees, *A. mellifera* are commonly dormant during the dry season (winter), Asian bees are active during the tropical 'dry season' when ample food is available. In the northern tropical hemisphere of Southeast Asia above the equator (Myanmar, Thailand, Vietnam, Laos and Cambodia), two major peaks of precipitation occur in the rainy season. Normally, the rainy season begins in March and ends September/October. Given the normal monsoon pattern in these areas, there is massive precipitation at the beginning of the season (May to June), and another at the end of the season (September). Apart from the period of heavy monsoon rains at the beginning and the end of the season, some deciduous plants and crops flower throughout this time and honeybees can still effectively forage. Given the different requirements of Asian bees, the dwarf honeybees, *A. florea* and *A. andreniformis*, need only a small amount of resources available to establish a new colony at any time of the year. They are very fast comb-builders, constructing fully functional combs within 21 days (Fig. 9.6). Within such a short time frame, *A. florea* can found new colonies even during periods when resources are limited during the rainy season (July to August).

Duangphakdee et al. (2013a) studied seasonal migration of *A. florea* at Ratchaburi Campus, King Mongkut's University of Technology, Chombueng, Ratchaburi, Thailand (13.59N, 99.51E, A 86 m). *A. florea* founded new colonies throughout the year; and numerous colonies either immigrated into the study area, or, conversely emigrated therefrom. However, the flowering season affects the sedentary time of the colonies because the most stable phase of the seasonal cycle begins in the middle of the dry season and continues into the beginning of the wet season (February to July in this study), when major flowering occurs.

On the other hand, the giant honeybees, *A. dorsata*, are dependent on abundant available food resources due to their massive colony populations (up to 100,000 individuals). *A. dorsata* cannot subsist on the meager flowering that sustains *A. florea* colonies. They have therefore adapted to seasonal migration, between

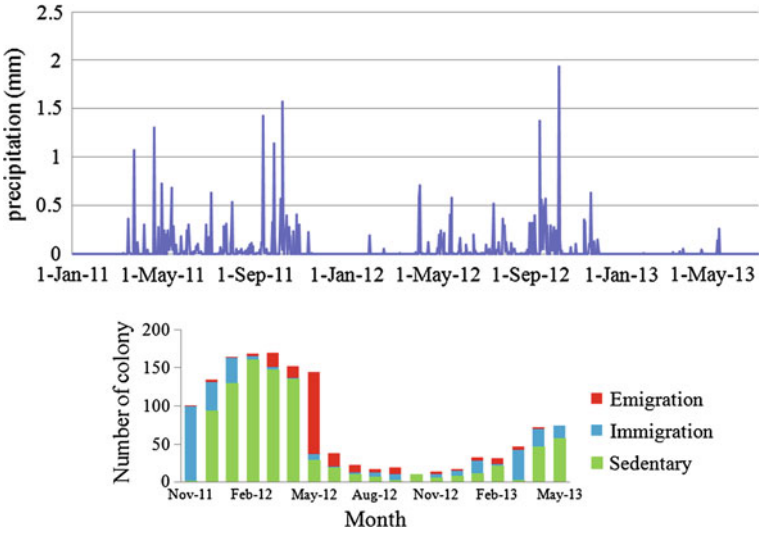

Fig. 9.7 Migration pattern of *A. dorsata* in Suan Phung District, Ratchaburi, Thailand showing a negative correlation to precipitation in the area (Duangphakdee et al. 2013b)

alternative nesting sites, following available forage resources (Paar et al. 2004). The construction of new *A. dorsata* nests is related to seasonal cycles; in lowland forests, *A. dorsata* migrations are negatively correlated with precipitation. They immigrate into areas during the dry season when flowering begins, and emigrate when the rainy season starts again (Duangphakdee et al. 2013b) (Fig. 9.7).

9.4 Nectar, the Unqualified Stimulus for Comb Construction

Comb construction by *A. florea* begins immediately after a colony settles and rapidly builds a new nest (Duangphakdee et al. 2013a). After the nest has been constructed, the building pulse varies greatly, depending on various factors such as flowering, nectar flow, season, ambient temperature and the number of young wax-producing bees. Just as we are thus far unable to separate the potential stimulative properties of flowers from the warmth of a season on the inception of comb-building, we are equally hard-pressed to define the effects of location, duration, intensity and qualities of different nectars on comb construction. Some diverse examples are given below.

Gontarski (1936) found that, even though *A. mellifera* were fed during the German winter, they produced very little wax. During the balmy summer months in Baton Rouge, USA, honeybees constructed a lot of comb when fed (Whitcomb 1946). In Japan, *A. cerana* did not construct as much comb during the summer

dearth, even when fed, as they had done in spring (Tokuda 1955). According to Taranov (1959) honeybees produced wax in direct proportion to the rate at which they were fed during the Moldavian autumn. In the Transvaal highveld, South Africa, during the warm month of April, *A. m. scutellata* were fed copious volumes of sugar syrup but produced no comb (Hepburn 1986). By the same token, during exceptionally heavy nectar flow, large colonies of bees (50,000) may collect over 10 kg of nectar over a fortnight (Hepburn 1986). In such cases foraging begins much earlier in the day than usual, and a considerable number of field bees carrying wax scales can be seen. The implication is that among the younger bees recruited to harvest nectar, there are many that would otherwise be engaged in comb-building activities (S Taber, pers. comm.).

The location, duration and intensity of 'nectar' flow has thus become of apparent, but undefined, importance. The quality of 'nectar' is even more difficult to judge. Fine details on the chemistry and biology of nectar in relation to honeybees and other animals is given by Nicolson et al. (2007), but the information is not directly related to comb-building. Turning to relevant apicultural examples, Huber (1814), for example, obtained twice as much wax from bees fed brown sugar or maple syrup than from those fed white sugar. Given the chemical and calorific differences in these syrups these results are bizarre. White sugar has an energy content of 1,619 kJ against 1,576 kJ for brown sugar and 1,093 kJ for maple syrup.

Viallon (1885) claimed that bees ought to produce more wax when they are fed nectar as opposed to honey, another untested idea. But against this, Zherebkin and Martinov (1977) found that bees fed sugar syrup had a more developed wax gland epithelium than bees having only honey as a sugar foodstuff. Pratt (1998a) noted that after a colony was deprived of a feeder and given a comb filled with honey, comb construction was much reduced compared to the period during active feeding. Several of these observations have been converted into testable hypotheses in recent years, particularly in the work of Pratt (below).

One important phenomenon which is poorly understood is how a honeybee colony controls the trigger for new comb construction. One of the pulses to stimulate comb construction coincides with periods of nectar intake (Hepburn 1986). However, Pratt (1998a, b) showed that the initiation of these building pulses depends on two conditions; a colony that is currently collecting nectar and the fullness of the comb is above a threshold level; and there is a balance of brood and food stores. Nectar flow and comb fullness are strongly correlated to comb-building (Pratt 1999). Pratt (2004) also observed a newly established swarm and tracked it through the spring and summer until the colony was fully developed. Peak comb-building was significantly correlated both with periods of high comb fullness and weight gain. Significant positive correlations were found between daily nectar intake and comb construction the following day; between comb fullness each day, and the amount of comb built that day; and between comb fullness each day and nectar intake that day (Fig. 9.8).

Knowledge of the basic biology and behaviour of honeybees has been accumulating over the past few centuries and can be codified by Huber (1814): (1) wax production is reduced in the absence of a nectar flow; (2) if bees are denied forage

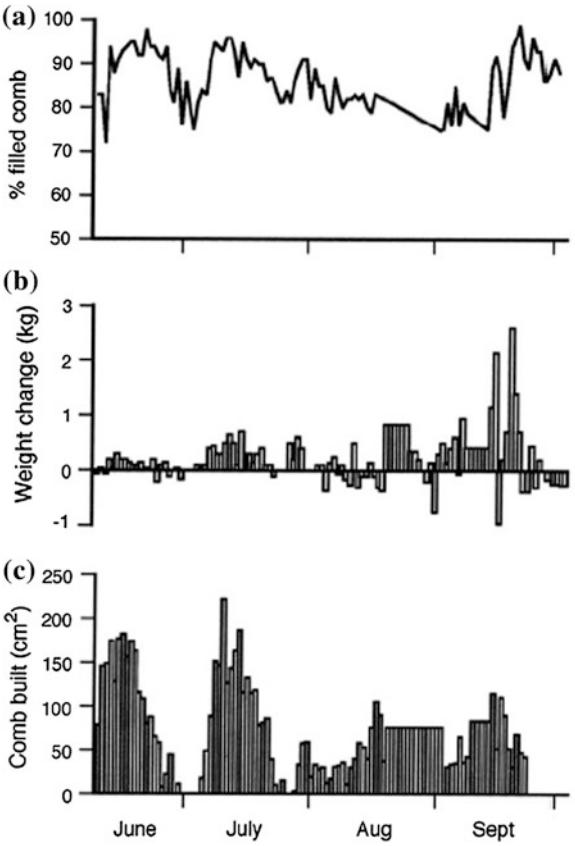

Fig. 9.8 Daily measurements of comb fullness, nectar intake and new comb construction in an *A. mellifera* colony in an observation hive colony, over the course of a nectar collecting season. **a** Percentage of comb area comprised of cells containing food or brood each day. **b** The colony's daily weight change, an estimate of its nectar intake. **c** The area of new comb constructed each day (Pratt 2004)

through cold or rainy weather, comb-building is reduced; (3) comb production ceases during a nectar dearth even if pollen is available; and (4) gathering nectar and comb-building go hand-in-hand. There are just about as many endorsements for these statements as there are beekeeping texts! The statements are undoubtedly true, but how they have come to be true is another matter.

9.4.1 Hoarding Assays

The first experimental attempts to quantify the rate of ingress of sugar into the honeybee nest in relation to the qualities of comb produced were those of Free and Williams (1972). They devised a sugar-hoarding assay based on small units of caged bees (n = 50), an approach widely used today. They discovered that old combs were more attractive storage depots than new ones. This effect was enhanced by temperature and inhibited by the presence of brood and light. The extrapolation of this technique for predicting hoarding behaviour of field colonies

Table 9.1 Hoarding of sugar by *A. mellifera* bees after changing the available storage areas of their combs

Transfer		No of cells used for storage
From	To	
1 comb	3 combs	61.4 ± 3.3
3 combs	3 combs	53.8 ± 2.8
3 combs	1 comb	34.3 ± 3.6
1 comb	1 comb	29.5 ± 1.9

Rinderer and Baxter (1979) n = 24 (12 hives of bees for each group)

was published the following year by Kulincevic and Rothenbuhler (1973). Then Rinderer and Baxter (1978) picked up the thread and they analysed the storage of a sucrose solution by small groups of caged bees (n = 50) and of field colonies in relation to the amount of empty comb available in the nest. Each of the colonies of one group was given about 4 m^2 of empty comb, and those of the other group slightly less than 2 m^2. The former group stored, on average, 50 % more nectar than the latter. In the reciprocal experiment, the colonies with the larger areas of comb stored 20 % more nectar. The same trend was observed in small colonies of caged bees. These authors concluded that empty comb itself somehow stimulates the collection of nectar.

Encouraged by their results, Rinderer and Baxter (1979) expanded their hoarding experiments. Each of a dozen cages, containing 50 young bees, was given about 47 cm^2 of comb and each of another dozen cages of bees received three times that area of comb (three combs). The bees were allowed to feed and hoard for 3 days. Significantly more sugar was stored by bees given the greater area of combs. The bees were then re-allocated to four new sets of cages. The bees that had been given only three combs were placed either in an identical cage with three combs or given only one comb; the same was done with those bees that had been given a single comb. The results (Table 9.1) are interesting indeed; those bees with three combs stored significantly more sugar than those given one, regardless of the size of their previous nests. That comb stimulates nectar-gathering as opposed to the collection of pollen or water was also shown in another experiment (Rinderer and Hagstad 1984).

These discoveries of the possible interactions that can occur between the extent of hoarding a surrogate nectar, such as a sugar syrup, and the size of the nest itself, soon led Rinderer (1981) to suggest that empty combs might have, among their volatile fractions, a constituent that could directly stimulate hoarding behaviour. By controlling the flow of air into experimental colonies, he tested normal air against that drawn over empty combs or over combs containing capped honey. He found significantly greater hoarding behaviour by bees stimulated with comb air. There was an effect of temperature as well; air pulled over combs held at 5 °C lacked the efficacy of that from combs at 35 °C. This experiment is certainly highly suggestive of an odoriferous, volatile principle in comb that stimulates hoarding. An odouriferous comb could also be derived from the modifications of comb by the presence of proteins and water (Hepburn and Kurstjens 1988), but

whether it is native to the comb or is placed there by the bees is unknown. Likewise, whatever the constituents may be, they are obviously not the same as those of old combs, as can be deduced from the experiments of Free and Williams (1972) and confirmed by Rinderer and Baxter (1979).

While the laboratory experiments are of interest, it was important to learn how bees behave with respect to comb stimuli and free foraging during a nectar flow. Rinderer and Baxter (1979) investigated the behaviour of queenright colonies of about 2,500 bees in vertical observation hives. The experimental manipulation was simple; all the hives contained a brood comb. Twelve hives were given one empty comb, and another twelve were each given three empty combs. The bees were allowed to forage during a major spring flow of white clover (*Trifolium repens*). The authors then counted the number of dancing foragers and the bees that they recruited, and measured the amount of honey that was stored by the one (control) versus the three (experimental) extra-comb colonies. The bees from the three-comb colonies had more dancing foragers and recruited more foragers that collectively stored eight times more nectar than did the control colonies.

During an autumnal dearth, Rinderer and Baxter (1979) trained bees from their one or three extra-comb colonies to forage at feeding dishes, where they changed the concentration of sugar. The results of the experiment showed that the bees from colonies with the greater amount of comb were less likely to forage on thin sugar syrup than the control colonies; but, having done so, were more likely to seek recruits on their return to the nest than the control bees. These results suggested that naturally occurring variation in the amount of available empty comb in the nest would, sensibly, be reflected in the seasonal way in which nectar is gathered. Reverting to colonies in the field in which half were given 4 m^2 and the other half 2 m^2 of empty comb, they kept this ratio of available comb throughout 5 months of summer by replacing filled combs on a monthly basis. Those colonies with the greater comb area stored, on average, 25 % more nectar than the colonies with smaller areas of comb (Table 9.1). The available storage space in both cases was always in excess of the volume of nectar collected by the bees. But it is interesting to see how the colonies differed on a monthly basis (Fig. 9.8). When the seasonal flow of nectar was great, the bees with more comb space outstripped those with less space, and vice versa as the flow declined (Fig. 9.9).

The seasonal study was restricted to a comparison of differing amounts of comb area coupled to natural seasonal changes and the availability of nectar, probably a very complex interaction. However, Rinderer and Baxter (1984) had the good fortune to examine records on the nectar-gathering traits of several colonies, used as honey production units, whose nectar yield had been measured monthly and continuously over a period of 25 years (Oertel et al. 1980). These colonies had been handled from a beekeeper's point of view for the production of honey. These startling results are shown in Fig. 9.9. Those colonies which stored, on average, the least amount of honey during major nectar flows over 25 years, consistently stored the most nectar during both early and late weaker flows, and conversely for the other colonies. It is probable that the differences were genetically based.

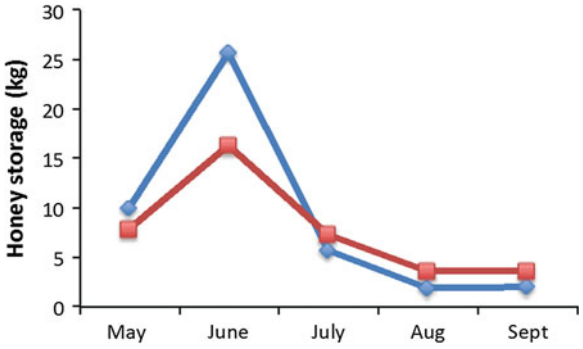

Fig. 9.9 The average monthly weight of honey produced by *A. mellifera* colonies with 4 m^2 (*closed circles*) or 2 m^2 (*open circles*) of empty comb (Rinderer and Baxter 1979)

Table 9.2 Rate of honey production and weight of honeybees, *A. m. meda*, in old and new combs over two years (Dizaji et al. 2008)

Items	2005			2006		
	Old comb	New comb	Sign	Old comb	New comb	Sign
Honey production (kg)	2.7 ± 0.35	3.6 ± 0.72	*	2.5 ± 0.3	2.9 ± 0.45	*
Weight of honey bee (mg)	99.03 ± 4.1	105.2 ± 1.2	*	93.19 ± 3.1	107.4 ± 2.0	**

While it is very fashionable to discourse upon 'foraging strategies', in this context we are more concerned with assessing the ways in which empty combs, of variable number, might explain the kinds of results that Rinderer and his colleagues obtained. Their argument hinges on the flow of volatiles in the nest; because the vapour pressure of these volatiles is related to temperature, it is presumed that during warm times more volatiles will be circulating in the nest and this is indicative of the availability of nectar in the field. Similarly, during winter the combs of the nest are most likely to be at a lower temperature than in summer, so reduced concentrations of volatiles are available to stimulate foraging. That foraging bees might be stimulated by the volatile scents of their nests in a dose-dependent way is an attractive idea. However, once a bee has taken flight and left its nest, it is subject to a host of stimuli, known and unknown, that will interact to modify its subsequent behaviour. Is the remembered scent of empty combs among them?

Dizaji et al. (2008) found that the age of honeycomb wax affects honey production. A two year dataset (2005–2006) showed significant differences in honey production, with higher yields produced in new combs and less in older ones (Table 9.2). Piccirillo and de Jong (2004) suggest that although honeybees are attracted by pheromones in old combs, given a choice they will use newer ones. Old combs can harbour pathogenic microbes, unhealthy antigens and other biological hazards which have detrimental effects on honeybees.

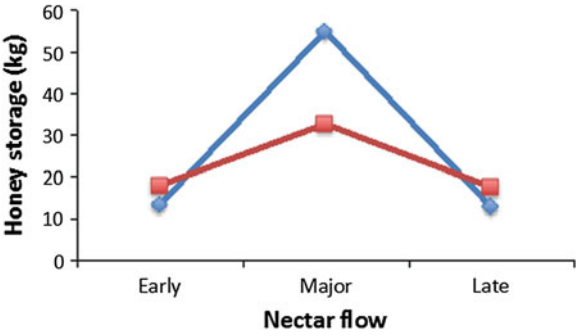

Fig. 9.10 The average amount of honey stored on a seasonal basis by *A. mellifera* with respect to the major flow at Baton Rouge (USA) over 25 years. Colonies that were most successful in the major flow (closed circles) and least successful (*open circles*) (Rinderer and Baxter 1979)

9.4.2 The Honey Stomach

It has long been known that the gathering of nectar and the construction of combs are related; when the flow of nectar ceases, comb construction falls into abeyance. Pratt (1998 et seq.) developed more direct tests for the additional condition of restricted space for the storage of incoming nectar, testing the hypotheses of Huber (1814), Gundelach (1842), Miner (1849) and Ribbands (1953). He tested the relationship of nectar crop size to the onset of comb construction. In his experiment, he divided the bees into three groups: (1) colonies with full combs and, (2) and (3) replicate colonies with empty frames. This experiment tracked crop distension of potential builders which were chosen from two groups: nectar receiver bees, and 10 days-old bees which are in the middle age range of wax secretion (Rösch 1927) and building behaviour. As expected, the experimental colonies collected nectar at a high rate and filled the storage combs and the number of empty cells gradually declined. The colonies began to build new comb when less than 5 % of cells were completely empty (Fig. 9.10). Both groups of bees showed significant effects on crop weight and nectar receivers had consistently larger crop sizes than 10-day-old bees (Figs. 9.11 and 9.12).

9.5 Decision-Making and Regulation of Comb-Building

Experimentally it has been well established that newly settled swarms are prodigious comb builders (Lee and Winston 1985; Hepburn 1986), but in a temporo-spatial framework, comb-building only reaches parity with other wax-working (capping and repairing) at the height of the colony growth cycle (Muller and Hepburn 1992). Comb-building is conducted in different areas of the nest by many individuals, some clustered in festoons others not, while other wax works are often the efforts of individual bees (Lindauer 1952). Changing ratios of what work is done and where it is carried out can be assessed by following the raw wax in a colony with the changing seasons. At the very beginning of nest founding, the swarm builds a full complement of combs as rapidly as possible to reach a fully

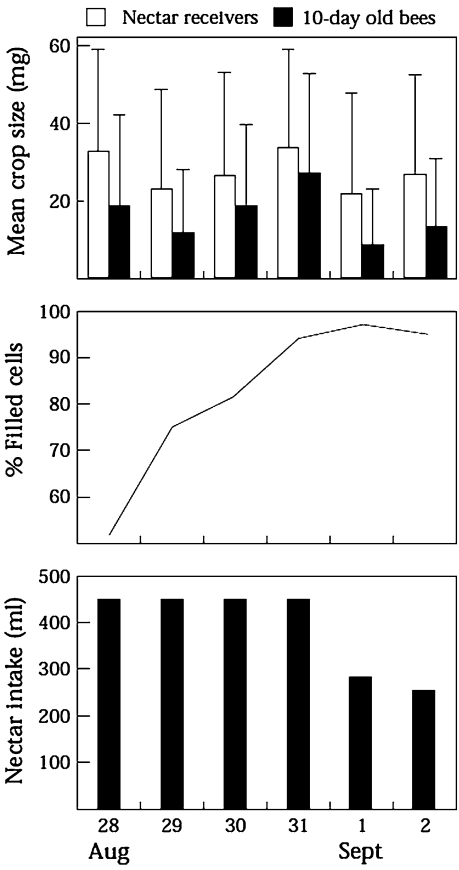

Fig. 9.11 Tracking changes in crop weight of *A. mellifera* nectar receiver bees and 10-day-old bees (Pratt 1998a, b)

developed nest consisting of stored energy (honeycomb) and rearing compartments (brood cells) for producing subsequent generations of brood. After the colony reaches maturity, instead of building in an intense way as in nest founding, they build new comb in pulses (Hepburn 1986). The available evidence suggests that the timing of these pulses depends on both colony state and environmental conditions (Pratt 1998a, b, 1999, 2004). In beekeeping all evidence indicates that bees will build only when they are collecting nectar (Hepburn 1986). Kelley (1991) confirmed this idea of building depending on the availability of comb to attain a threshold quantity of food and brood.

Because beeswax is the basis for both for housing and food storage, to build excessive combs would quickly deplete honey stores and increase the risk of starvation. Consequently, a balance between the energy costs of construction and the opportunity provided by nectar flows is of the utmost importance. The total cost of building 1 kg of comb has been conservatively estimated at 6.25 kg of honey (Weiss 1965). Thus, 1.2 kg of comb in a fully-developed colony consumed an impressive 7.5 kg of the 60 kg of honey consumed each year by a typical

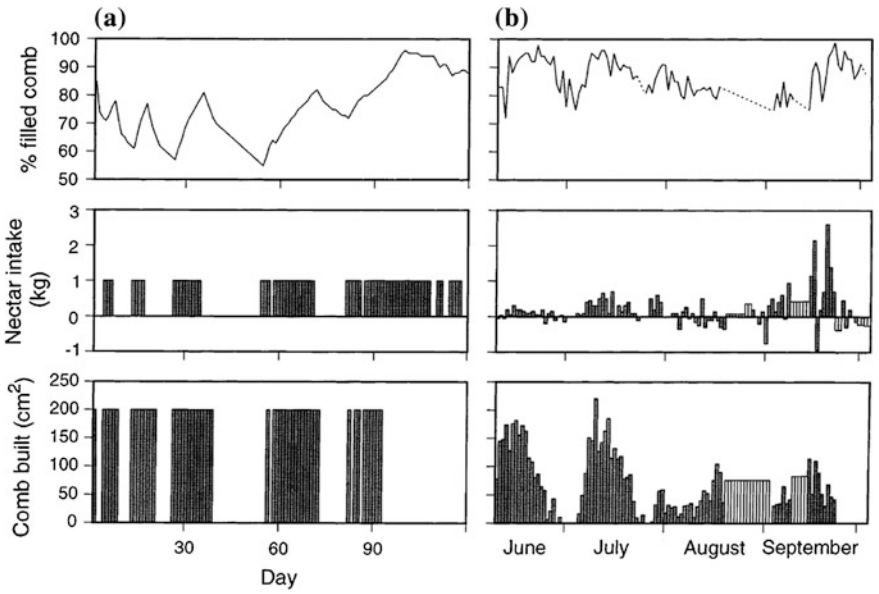

Fig. 9.12 Simulated trajectories of comb construction, honey stores and comb fullness for a colony following and optimal condition-dependent building policy. **a** Shows the percentage of comb area is comprised of cells containing food or brood for each day of a 120-day foraging season. The middle plot shows the nectar available in the field each day. The lower plot shows the area of new comb constructed each day. **b** Daily measurements of comb fullness, nectar intake and new comb construction in an *A. mellifera* observation hive over a nectar-collecting season. The upper and lower plots show the same information as corresponding plots in (**a**). The middle plot shows colony daily weight change, an estimate of nectar intake. *Dotted lines* in upper plot and paler bars in two lower plots indicate interpolated values for days on which data were not collected (Pratt 1999)

temperate-zone colony (Seeley 1985). Pratt (1999) tried to address the optimal timing of new comb construction in honeybee colonies. This work explores a resource allocation problem underlying the growth of two crucial functions of honeybee colonies: the honey hoard which serves as an energy reserve, and the comb in which this energy is stored. The building rules and comb growth trajectory predicted by his model were compared with data from actual honeybee colonies. This model primarily depends on a colony's decision to store nectar to avoid a highly probable death by starvation over winter during the first year (Lee and Winston 1985), and to avoid reproductive swarms.

The results between that predicted by the model and the observation colony, exhibited two major features. Comb-building is optimal only when the colony has stored greater than some threshold amount of honey, even though the available comb for storage is still half empty (Fig. 9.13). There was one slight difference in that the total amount of comb constructed was smaller in the observation colony than in the simulated model colony. Data from the observation colony also showed a significant difference between daily nectar intake and comb construction on the

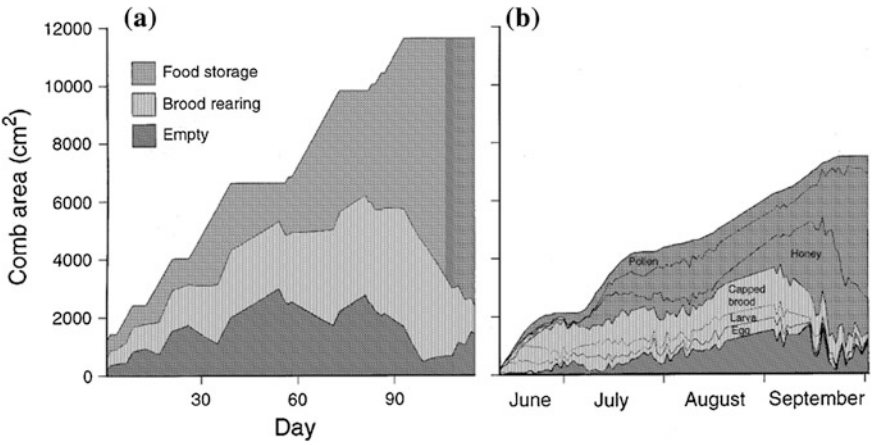

Fig. 9.13 **a** Growth and change in pattern of comb use for the same simulation (depicted in 9.12a). **b** Growth and change in pattern of comb use for the *A. mellifera* observation hive (described in Fig. 9.12b (Pratt 1999) Pattern

following day; between comb fullness on each day and the amount of comb built on that day and between comb fullness on each day and nectar intake on that day (Pratt 1999).

The model also indicates that the magnitude of the degree for a building threshold varies with the quality of foraging conditions and the strength of the constraints of nectar collecting efficiency implied by the empty comb area. This threshold is typically rather low, even when there are enough empty combs to store several kilograms of honey. The experimental colony confirmed the threshold of the model, even when it had enough empty combs to hold 1.5 kg more honey. These results again confirm that nectar is a critical stimulus for comb-building, both directly through nectar intake, and indirectly through the affect of nectar collection on internal colony conditions. Regulation of the timing to begin building is partly independent of the amount and duration of building. Once building has begun, the colony will only track nectar intake to control comb-building, and not the amount of comb already built. Interference in this natural building cycle by beekeepers removing large quantities of combs from colonies, an unlikely occurrence in nature, create differences in colony behaviour where a continual demand for comb to store nectar is experienced (Pratt 1999).

9.6 Who are the Comb Builders?

Following the discovery of a dramatic, age-related transition in honeybees from nest to field activities (Dönhoff 1855), more subtly differentiated tasks were found among house bees(Rösch 1925, 1927, 1930; King 1928), and this led to the

Table 9.3 Percentage bees with wax (%), sample sizes (n) and the mean amount of wax (mg/bee ± s.d.) produced by festoon and non-festoon *A. m. capensis* worker honeybees for each age group (Muller and Hepburn 1992)

Age (days)	Festoon			Non-Festoon		
	n	%	mg/bee	n	%	mg/bee
3	307	22.2	0.06 ± 0.14	1053	36.7	0.11 ± 0.18
6	682	60.3	0.21 ± 0.26	861	42.5	0.16 ± 0.25
9	676	71.0	0.32 ± 0.35	959	56.4	0.23 ± 0.3
12	727	62.5	0.28 ± 0.37	1286	40.5	0.15 ± 0.27
15	694	54.9	0.27 ± 0.45	1279	28.9	0.1 ± 0.23
18	515	39.2	0.17 ± 0.31	1037	23.9	0.08 ± 0.2
21	390	28.2	0.1 ± 0.24	840	13.7	0.04 ± 0.15
	3981	X = 53.3	X = 0.22 ± 0.34	7315	X = 35.5	X = 0.13 ± 0.24

concept of an age-related division of labour of tasks among honeybees (polyethism). These interpretations were essentially correct (Ribbands 1953), even if early interpretations were somewhat rigid. Knowledge of polytheism was refined through the experimental demonstration that worker bees form age-related cohorts and that have a high probability of performing only a limited set of tasks, each usually restricted to certain areas of the nest (Seeley 1982). The tasks change as the worker bee becomes older; there is also a shift away from the brood area until she eventually becomes a forager and leaves the nest.

Although research on the activities of house bees has out-paced that on the physiological basis of behaviour (Seeley 1982), the classic works have shown that the activities of some glands (e.g. wax glands) are age-related, and might be closely linked to task differentiation (Rösch 1925, 1927; King 1928; Ribbands 1953). For example, wax-working includes festooning behaviour (King 1928) and cell capping (Lineburg 1923a, b; Lindauer 1952), both of which are spatially separated (Hepburn 1986), and either or both of which may be driven by the secretory cycle of the wax gland complex (Hepburn et al. 1991). In view of the above, there is now sufficient information to ask how the probability of a cohort performing given tasks is constrained by underlying physiological characteristics (glandular secretions), and whether the tasks are susceptible to modulating stimuli (queen pheromones, nectar influx etc.). To this end, the temporal and spatial characteristics of wax secretion and wax-working behaviour and how they are integrated in colonies of honeybees, were investigated by Muller and Hepburn (1992) (Table 9.3).

They showed that wax secretion is significantly related to worker age and that bees between 3 and 21 days old form such a cohort. Comb-building festoons, previously thought to be the site of wax secretion, represent only a small fraction of newly secreted wax in the nest. Wax secretion remains constant relative to age in the cohort, but varies significantly with season, as does the participation of bees in festooning behaviour. Wax secretion and wax-working are both definable in

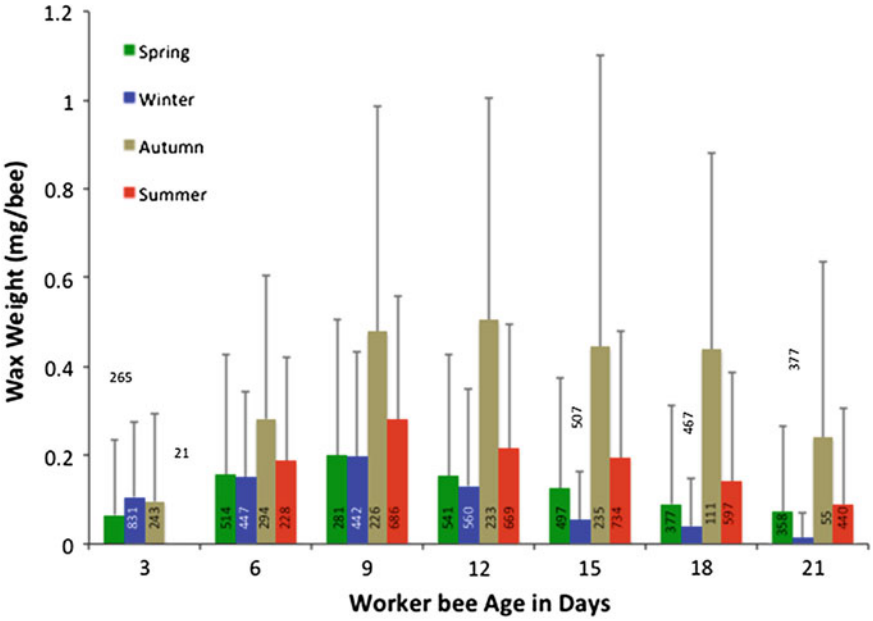

Fig. 9.14 Seasonal wax production in *A. m. capensis* honeybees (mean ± SD). Sample sizes are indicated in the bars (Muller and Hepburn 1992)

terms of time and space in the nest, the relative probability of activity changing with season. Wax secretion itself is constrained by the cyclical activity of the underlying wax gland complex.

Muller and Hepburn (1992) performed experiments in the field throughout the year using introduced marked *A. m. capensis* bees into queenright colonies and harvested festoons from building frames. These experiments revealed that bees producing wax do not differ significantly whether in festoons or elsewhere in the nest. There were, however, significant differences in bees of the same age cohorts between festoon bees and non-festoon bees, thus resolving a two hundred year old question. The mean amount of wax borne varied significantly with the season (Fig. 9.14), the least amount of wax was produced in winter, significantly more in summer and spring, and even more in autumn. However the amount of wax among each age cohort remained constant, and the amount of wax recoverable from any particular cohort relative to age remained constant among cohorts across the seasons (Fig. 9.14). About half the raw wax was recovered from festoon, bees and the other half from non-festoon bees (Fig. 9.15), except in winter, when non-festoon wax production was significantly higher than festoon wax.

The age composition of festoons is rather constant, but the percentages of bees that participated in festoons varied. Approximately 56 % were captured in summer, 37 % in spring and autumn and only 7 % during winter (Fig. 9.15). The ages of the bees in both festoon and non-festoon areas ranged from 3 to 21 days old

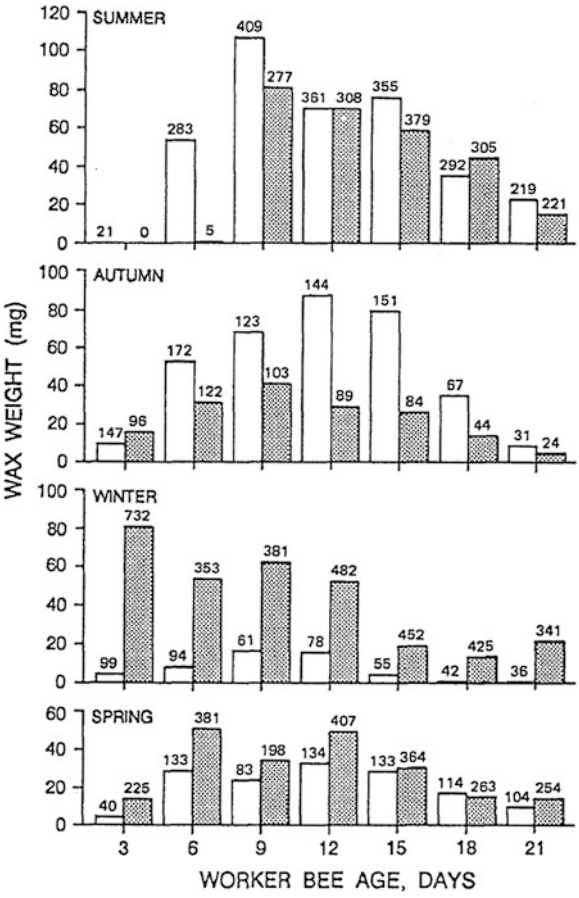

Fig. 9.15 Seasonal total wax production by festoon and non-festoon *A. m. capensis* worker honeybees. *Open bars*—festoon bees; *stippled bars*—non-festoon bees. Sample sizes are indicated above the *bars* (Muller and Hepburn 1992)

(Fig. 9.16). These results correspond with those of Seeley and Kolmes (1991) who reported on age polymorphism and comb construction. Calculations from the proportion of marked bees revealed that only half were caught in festoons, and of those, only half carried wax scales, which means that the workers producing wax in the festoons were only 25 % of the total number of workers producing wax in the colony. The amount of wax borne by bees varies with age and season, as does wax production in festoons (Figs. 9.15 and 9.16).

Festoon size also varied with time of the day and seasonally. During summer, festoons were larger at night, whilst during the day all field bees were out foraging. Day-time festoon size also varied with the prevailing weather, being larger on cool days than warmer ones, hinting that foraging bees also participate in festoons. Even so, the festoons were not stable clusters, but had a high turnover rate of bees coming and going with the length of time a bee spent in a festoon ranging from 30 min to 4 h. Pratt (1998a, b) tested the participation of nectar receivers in comb

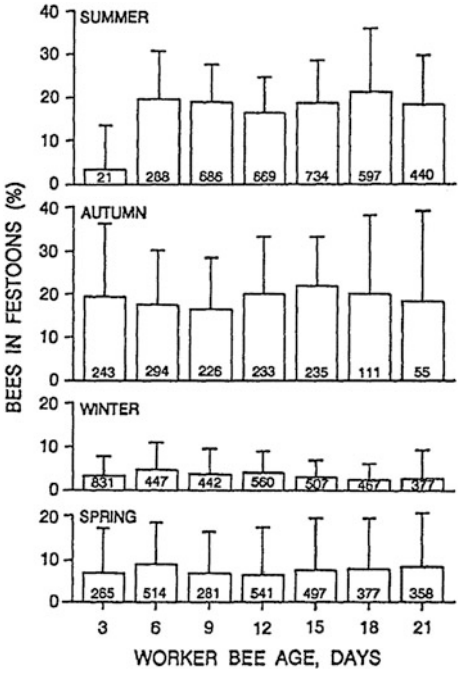

Fig. 9.16 Seasonal festoon participation by *A. m. capensis* honeybee workers (mean ± SD) Total sample sizes for each age category are indicated in the bars; bar represents the percentage of bees of that age recaptured in a festoon (Muller and Hepburn 1992)

construction. He marked the colony's nectar receivers to see what proportion is involved in comb-building tasks. Three replicates of this experiment were performed. Interestingly, marked bees appeared in the building festoons of all three replicates. This indicated that some nectar receivers switched to comb construction.

Pratt (1998a, b) marked the receiver bees and observed the comb builders and whether nectar receiver bees take part in comb-building. Three replicates of an experiment were performed. The results showed 43 nectar receivers of a total 1449 comb builders in replicate 1; 2 nectar receivers of a total 123 builders in replicate 2; and 13 nectar receivers of total 279 builders in replicate 3. These data revealed that comb-building is not actively undertaken by nectar receivers even though some builders were recruits from among the nectar receivers, and other recruits probably from another subpopulation of the colony (Table 9.4). These findings that only a very few nectar receivers are among the comb-builders, seems to contradict previous findings that these tasks are performed by bees of the same age caste (Rösch 1927; Seeley 1982, 1989; Hepburn et al. 1991; Muller and Hepburn 1992). Assuming that 75 % of the colony's workers were nectar receivers, 25 % of a colony's population may be engaged in foraging (Seeley 1995).

Table 9.4 Results of three replicates of an experiment on *A. mellifera* testing whether a colony's builders are recruited from amongst its nectar receivers (Pratt 1998a, b)

Age (days)	Festoon			Non-festoon		
	n	%	mg/bee	n	%	mg/bee
3	307	22.2	0.06 ± 0.14	1053	36.7	0.11 ± 0.18
6	682	60.3	0.21 ± 0.26	861	42.5	0.16 ± 0.25
9	676	71.0	0.32 ± 0.35	959	56.4	0.23 ± 0.3
12	727	62.5	0.28 ± 0.37	1286	40.5	0.15 ± 0.27
15	694	54.9	0.27 ± 0.45	1279	28.9	0.1 ± 0.23
18	515	39.2	0.17 ± 0.31	1037	23.9	0.08 ± 0.2
21	390	28.2	0.1 ± 0.24	840	13.7	0.04 ± 0.15
	3981	X = 53.3	X = 0.22 ± 0.34	7315	X = 35.5	X = 0.13 ± 0.24

Date	Colony population	Estimated number of nectar receivers (20 % of total population	Number (%) of marked receivers	Number of builders	Expected number (%) of marked builders	Observed number (%) of marked builders	G
16/6/94	7637	1527	532 (34.8)	1449	50.4 (34.8)	43 (3.0)	906
22/7/94	2416	483	380 (78.7)	123	97 (78.7)	2 (1.6)	357
7/7/95	3716	743	227 (30.6)	279	85 (30.6)	13 (4.7)	119

The data from this experiment supports the interpretation that the comb-building signal operates via nectar intake and is not derived from direct measurements of nectar receiver bees. The results also confirm that the trigger does not come from crop size even though it varied between days and may have been influenced by the amount of nectar intake. On the other hand, the low frequency of nectar receivers among builders does not conflict with the perspective of colony functional design. To avoid the interruption of the active food-collecting and storing activities, comb builders may be drawn from a pool of inactive or unemployed reserves bees within the colony.

9.7 Nectar Intake and Comb Fullness

Pratt (1998a, b) showed that bees build comb when two conditions are at: (1) adequate nectar collection in the field; and (2) the filling of the comb is above their threshold; which means that the start of building requires both comb fullness and nectar intake. In a colony with full combs, when they are regularly replaced with empty combs, the bees will not build new combs even if they are fed a

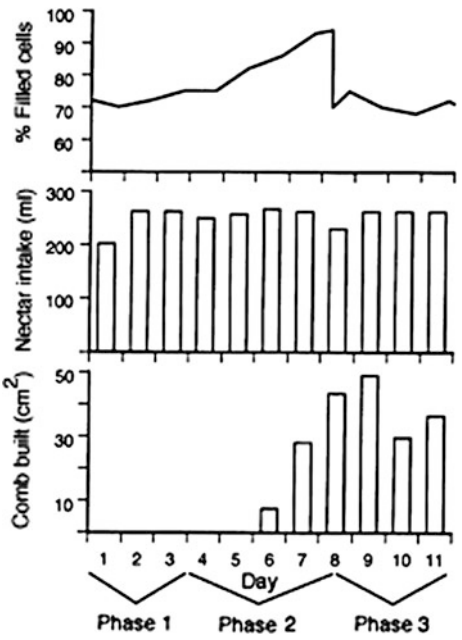

Fig. 9.17 Experiment on *A. mellifera* to test the role of comb fullness on whether a colony will begin new comb construction. In Phase 1 the colony had a heavy influx of sucrose but was maintained at a low level of comb fullness. In Phase 2, sucrose intake continued, but bees were allowed to fill their combs. In Phase 3, the colony was returned to the condition of phase 1. The bees built no new comb in Phase 1, beginning construction only in Phase 2 after the level of colony fullness had markedly increased. However, the bees did not stop building comb when the level of comb fullness was lowered in phase 3 (Pratt 2004)

concentrated sucrose solution. They begin to build only when the comb is almost full. A companion experiment showed that building is highly correlated to both comb fullness and nectar intake (Pratt 2004). Colonies fail to start building if deprived of nectar even if the comb is completely full (Figs. 9.17 and 9.18). While both nectar intake and comb fullness are necessary for building to start, nectar intake alone controls construction after that. Figure 9.17 shows that the bees started to build when the combs begun to fill but did not stop building when the fullness of combs was reduced, by replacing the full combs with empty ones. In contrast, Fig. 9.18 shows that when a colony is deprived of field nectar, they cease construction after 2 days even if the combs are full.

However, the opposite demand of turning off nectar intake causes the cessation of comb-building, a delayed effect which reflects that the bees collect data to enable them to make building decisions. The regulation of building therefore requires some time from within which information is collected and evaluated according to physiological and behavioral changes. It may take longer for comb-building to commence because reactivation of the wax glands is slow (Fig. 9.19, Pratt 1999).

Nectar foraging in honeybees is a complex process requiring the coordinated efforts of nectar foragers and receivers. Foragers collect nectar and unload it to receiver bees in the lower region of the nest near the entrance. Receiver bees then transport the nectar to the honey-storage area at the top of the nest. Previous work has shown that the process of unloading foragers and depositing the nectar in the honey-storage region of the nest is tightly linked (Seeley 1995). This is a

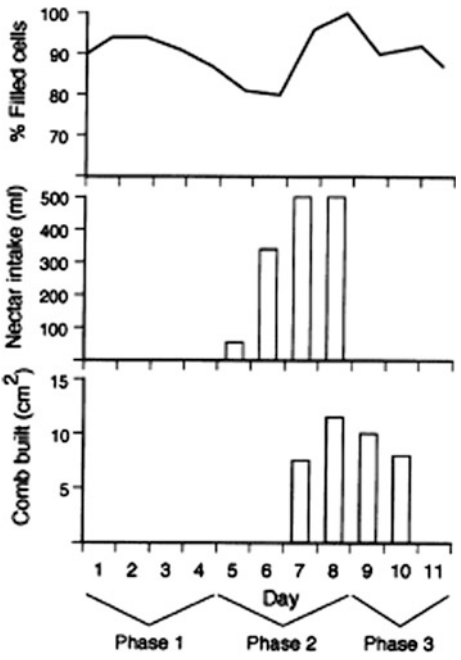

Fig. 9.18 Experiment on *A. mellifera* to test the role of nectar intake on whether a colony will begin constructing comb. In phase 1 the colony had a high level of comb fullness, but received no nectar at all. In phase 2 the bees were fed a sucrose solution and their level of comb fullness remained high. In phase 3 the colony was returned to the condition of phase 1, starting construction again only after experiencing 2 days of nectar intake. Bees ceased construction 2 days after nectar intake was cut off in phase 3 (Pratt 2004)

physiology-dependent task (cf. Fig. 9.1). Therefore, an increase in the demand for this task should be met solely by receiver bees, despite the fact that the labour demand for the task is that of house bees.

Figures 9.20 and 9.21 show the effects of an increased nectar flow on the task distributions of nurse and food-processing bees. During the first experimental trial the rate of nectar foragers entering the nest per minute increased from 1.1 to 5.9. During the second trial, the rate of foragers entering the hive showed a greater increase relative to the first trial, with the number of foragers entering rising from 2.8 to 17.6 per minute. A significant interaction between caste and environment was found, indicating that the two castes responded differently to the rise in nectar influx. Food processing bees had both a larger decrease in merely standing and a larger increase in walking than the nurse bees. In addition, there was a significant interaction between trial and time-period which was to be expected, since the level of increase in nectar influx was considerably greater in the second experimental trial (Johnson 2003).

9.8 Termination of the Stimulus

One must bear in mind that autumn and winter are peculiar to those regions away from the equator; lands closer to the equator have rainy and dry seasons (even if the odd mountain like Kilimanjaro is akin to high latitudes in temperate zones).

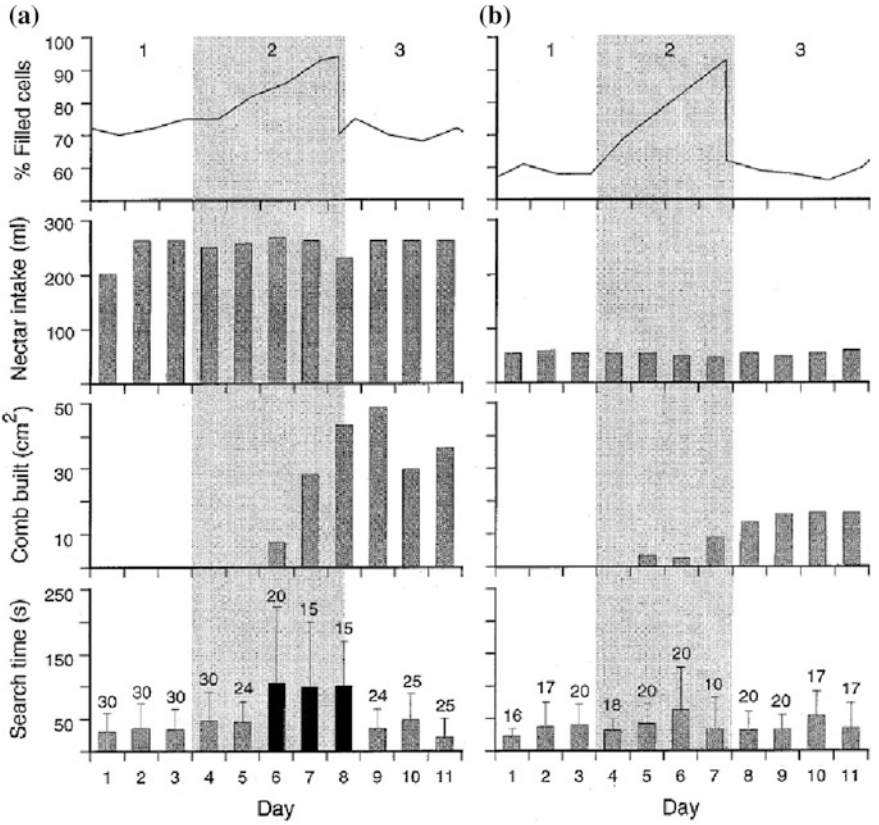

Fig. 9.19 Results of two replicates of an experiment on *A. mellifera* testing the role of comb fullness in a colony's decision to start building new comb. **a** In Phase 1, the colony experiences a heavy nectar influx but maintained a low level of comb fullness. In Phase 2, the nectar influx continued and the bees were allowed to fill their combs with honey. In Phase 3, the colony was returned to the condition of Phase 1A. **b** In Replicate 1 the bees built no new comb in Phase 1, beginning construction only in Phase 2, after the level of comb fullness had markedly increased. The bees did not, however, cease construction when the level of comb fullness was lowered in Phase 3. In the lowest plot, bar height (+SD) shows the mean search time of returning foragers looking for receiver bees to take their nectar. *Dark bars* are significantly greater than the others (Pratt 1999)

Following Rösch's extensive studies (1927, 1930) on the development of wax glands in *A. mellifera* bees of the far north in spring and summer, we are left to consider how the wax biology varies with the seasons, however defined. Dönhoff (1855) noted that bees in one of his observation hives, fed sugar in the autumn, bore heavy wax scales in winter; indeed scales have been recorded from bees even in the dead of the northern winter (Kustenmacher 1922; Farrar 1927).

Although none of these colonies were observed to build any combs, those of Szalök (1928) in Hungary certainly did. In Russia, Koschevnikov (1900) had also

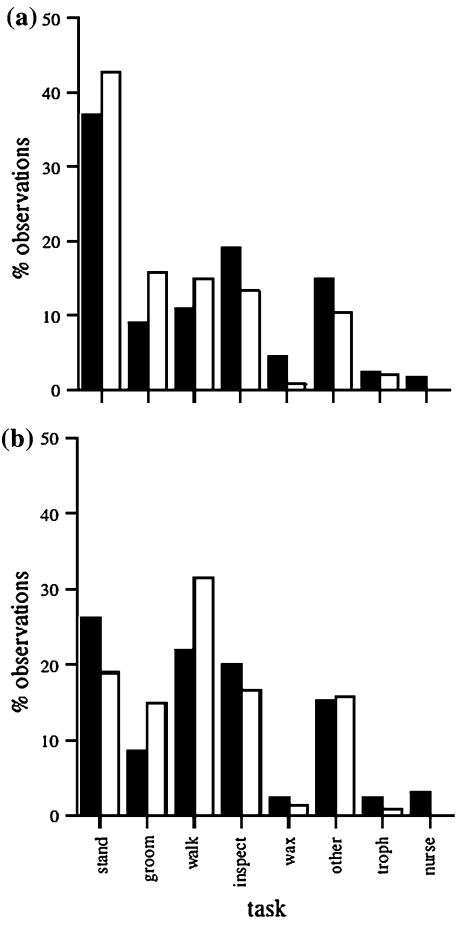

Fig. 9.20 Effect of increased nectar influx on task distribution of *A. mellifera* nurse bees (*black bars*) and receiver bees (*open bars*). Trial 1 **a** task distributions before nectar influx: nurse bees = 144/481; receiver bees = 83/248; **b** task distributions in response to increased nectar influx: nurse bees 141/492; receiver bees = 80/222 (Johnson 2003)

seen wax scales on winter bees and wondered whether such scales were carried over from the summer or were produced in winter. In a not entirely clear investigation into the matter, Tuenin (1928) studied bees from field colonies over a Russian winter and found that, while some bees bore scales, the epithelium of the wax gland remained undeveloped (as noted in passing by Dreyling 1903); and he concluded that the wax scales were a carry-over from summer. Nonetheless, when the warmth and blossoms of spring returned, these old, over-wintered bees, from whose colony Tuenin had removed all the young brood of spring, began the construction of combs. Örösi-Pál (1931) came to much the same conclusion.

In areas of about 30° latitude, some colonies of bees will certainly build combs if artificially fed during winter. In such places, one can encounter winter swarms which have absconded. On settling, they begin comb-building, but seldom survive the cold conditions. While efforts, such as those of Koch, are highly suggestive, we are still left in some doubt as to what mechanism precisely the bees respond; the

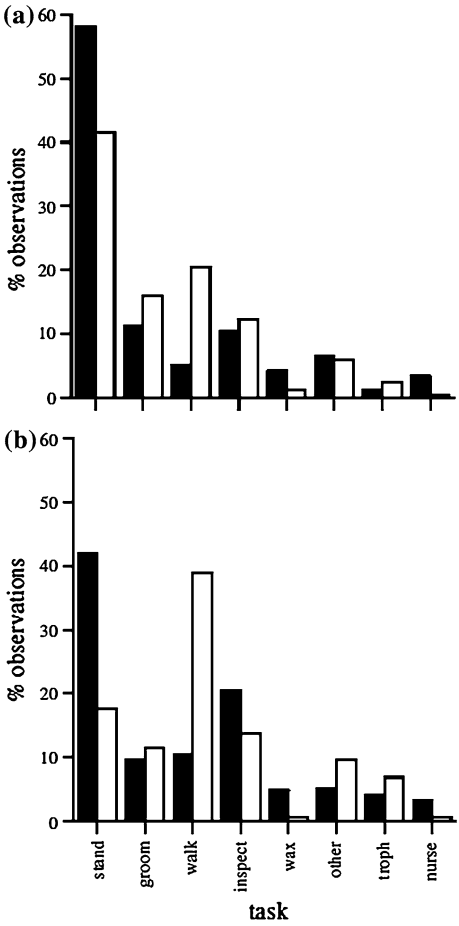

Fig. 9.21 Effect of increased nectar influx on task distribution of *A. mellifera* nurse bees (*black bars*) and food-processing bees (*open bars*). Trial 2 **a** task distributions before nectar influx: nurse bees = 63/241; food processing bees = 96/237; **b** task distributions in response to increased nectar influx: nurse bees 63/248; food processors = 85/277 (Johnson 2003)

flow of nectar, the temperature, both, or some other not as yet recognised factor(s). Natural experiments are difficult to interpret, and a solution may lie in the experimental techniques of Worswick (1987). For studies on honeybee metabolism and temperature regulation in *A. m. capensis* and *A. m. scutellata*, Worswick (1987) constructed what amounts to a refrigerator in which he placed entire Langstroth colonies. He could simultaneously control and monitor the ambient temperature of the refrigerator and measure the core temperature of the cluster of bees within the hive. A suitably programmed regime of temperatures to find the temperature to induce comb-building could easily be plugged into this kind of experimental design. An alternative and acceptable confirmatory approach might include the relative responsiveness to flowering vis-à-vis warming weather; in a cold temperate country, in cellared versus field colonies.

Not only does a limited amount of comb restrict the volume of honey stored, but a shortage of empty comb may also reduce foraging efficiency, as nest bees

become mired in lengthy searches for empty cells in which to place incoming nectar (Seeley 1995). Pratt (1999) tested the idea that the termination of comb-building was relative to nectar flow by running his simulation model and comparing the results with actual bees. The model showed that the building threshold rises during the last part of the foraging season, which means building never reaches an optimal level by the end of the season. Results from the experiment with actual bees corresponded with the model, as colonies built no comb in the face of oncoming winter, even though the nectar flow and comb use conditions were similar to those in the earlier part of spring. Brood-rearing decreased to near zero and food storage increased dramatically in the final week of the season (Fig. 9.13b). The physiology of the bees also corroborates this idea because the wax glands of the bees become inactive at the end of the season, but are reactivated again at the end of winter.

References

Akratanakul P (1987) The natural history of the dwarf honeybee, *Apis florea*. Thesis, Cornell University, Ithaca

Butler C (1609) The feminine monarchie or a treatise concerning bees, and the due ordering of them. Joseph Barnes, Oxford

Butler CG (1974) The world of the honeybee. Collins, London

Corlett RT (2011) Honeybees in natural ecosystems. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 215–225

Dizaji AA, Alishah HM, Shaddel AA, Sis NM (2008) Effects of comb wax age on the brood and honey product performance in honeybees. Asian J Anim Vet Adv 3:51–53

Dönhoff E (1855) Über das Herrschen verschiedener Triebe in verschiedenen Lebensatten bei den Bienen. Schweiz Bienen-Ztg (Cited from Ribbands 1953)

Dreyling L (1903) Über die wachsbereitenden Organe der Honigbiene. Zoo1 Anz 26:710-715

Duangphakdee O, Hepburn HR, Rod-im P (2013a) Seasonal movements of the red dwarf honeybee, *Apis florea* (In ms.)

Duangphakdee O, Hepburn HR, Rod-im P (2013b) Development of the nest of *Apis florea*. (In ms.)

Farrar CL (1927) Wax secretion in winter. Am Bee J 67:52

Free JB, Williams IH (1972) Hoarding by honeybees (*Apis mellifera* L). Anim Behav 20:321–334

Gontarski H (1936) Die Wachserzeugung der Arbeiterinnen von *Apis mellifica* während der Winterruhe. Z Wiss Zool 145:526–536

Gundelach FW (1842) Die Naturgeschichte der Honigbienen. Kassel, Bohne

Hepburn HR (1986) Honeybees and wax. An experimental natural history. Springer, Berlin

Hepburn HR (1998) Reciprocal interactions between honeybees and combs in the integration of some colony functions in *Apis mellifera* L. Apidologie 29:47–66

Hepburn HR, Magnuson P (1988) Nectar storage in relation to wax secretion by honeybees. J Apic Res 27:90–92

Hepburn HR, Kurstjens S (1988) The combs of honeybees as composite materials. Apidologie 19:25–36

Hepburn HR, Radloff SE (1995) First approximation to a phenology of the honeybees (*Apis mellifera*) and flora of Africa. Oecologia 101:265–273

Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax in honeybees. Apidologie 22:21–36

Huber F (1814) Nouvelles observations sur les abeilles. [English translation 1926], Dadant, Hamilton
Johnson BR (2003) Organization of work in the honeybee: a compromise between division of labour and behavioural flexibility. Proc R Soc Lond B22(270):147–152
Kelley S (1991) The regulation of comb-building in honeybee colonies. Cornell University, Ithica (unpubl. cited from Pratt, 1998a)
King GE (1928) The larger glands in the worker honey-bee. A correlation of activity with age and physiological functioning. Thesis, University of Illinois
Koch HG (1957) Zur Phänologie der Bienenvölker. Z Angew Met 3:33–43
Koch HG (1959) Witterung und Nektartracht der Bienenvölker. Z Angew Met 3:278–292
Koch HG (1961) Der Baubeginn der Bienenvölker als phänologische Ereignis. Z Angew Met 4:69–82
Koschevnikov GA (1900) Über den Fettkörper und die Oenocyten der Honigbiene (*Apis mellifera* L.). Zool Anz 23:337–353
Kulincevic JM, Rothenbuhler WC (1973) Laboratory and field measurements of hoarding behaviour in the honeybee. J Apic Res 12:179–182
Kustenmacher M (1922) Der Aufbau des Bienenkörpers und seine Tätigkeiten. (Cited from Örösi-Pål 1931)
Lee PC, Winston ML (1985) The influence of swarm size on brood production and emergent worker weight in newly founded honeybee colonies (*Apis mellifera* L.). Insectes Soc 32:96–103
Lineburg B (1923a) Conservation of wax by the bees. Am Bee J 63:615–616
Lineburg B (1923b) What do bees do with brood cappings? Am Bee J 64:235–236
Lindauer M (1952) Ein Beitrag zur Frage der Arbeitsteilung in Bienenstaat. Z Vergl Physiol 34:299–345
Miner B (1849) The American bee keeper's manual. Wiley, London
Muller WJ, Hepburn HR (1992) Temporal and spatial patterns of wax secretion and related behavior in the division of labour of the honeybee (*Apis mellifera capensis*). J Comp Physiol A171:111–115
Nicoloson SW, Nepi M, Pacini E (2007) Nectaries and nectar. Springer, Berlin
Nuru A, Amssalu B, Hepburn HR, Radloff SE (2002) Swarming and migration in the honeybees (*Apis mellifera*) of Ethiopia. J Apic Res 41:35–41
Nuru A, Awad AM, Al-Ghamdi AA, Alqarni AS, Radloff SE (2012) Nectar of *Ziziphus spina-christi* (L.) Willd (Rhamnaceae): dynamics of secretion and potential for honey production. J Apic Sci 56:49–59
Oertel E, Rinderer TE, Harville BG (1980) Changing trends in southeastern Louisiana honey production through 25 years. Am Bee J 120:763–765
Oldroyd BP, Wongsiri S (2006) Asian honeybees: biology, conservation, and human interactions. Harvard University Press, Cambridge
Örösi-Pål (1931) Die Wachrdrüsen der Winterbienen. Arb Ung Biol Forsch lnst 4:108–121
Paar J, Oldroyd BP, Huettinger E, Kastberger G (2004) Genetic structure of an *Apis dorsata* population: the significance of migration and colony aggregation. J Hered 95:119–126
Piccirillo GA, de Jong DD (2004) Old honey bee brood combs are more infested by the mite *Varroa destructor* than areas of new combs brood. Apidologie 35:359–364
Pratt SC (1998a) Condition-dependent timing of comb construction by honeybee colonies: how do workers know when to start building? Anim Behav 56:603–610
Pratt SC (1998b) Decentralized control of drone comb construction in honeybee colonies. Behav Ecol Sociobiol 42:193–205
Pratt SC (1999) Optimal timing of comb construction by honeybee (*Apis mellifera*) colonies: a dynamic programming model and experimental tests. Behav Ecol Sociobiol 46:20–42
Pratt SC (2004) Collective control of the timing and type of comb construction by honeybees (*Apis mellifera*). Apidologie 35:193–205

Rattanawannee A, Chanchao C, Lim J, Wongsiri S, Oldroyd B (2012) Genetic structure of a giant honey bee (*Apis dorsata*) population in northern Thailand: implications for conservation. Insect Cons Divers. doi:10.1111/j.1752-4598.2012.00193.x
Ribbands CR (1953) The behaviour and social life of honeybees. Bee Research Association, London
Ridley HN (1901) Garu and chandan. J Straits Roy Asiatic Soc, Malaysian Branch 35:1–107
Rinderer TE (1981) Volatiles from empty comb increase hoarding by the honeybee. Anim Behav 29:1275–1276
Rinderer TE (1982) Regulated nectar harvesting by the honeybee. J Apic Res 21:74–87
Rinderer TE, Baxter JR (1978) Effect of empty comb on hoarding behaviour and honey production of the honeybee. J Econ Entomol 71:757–759
Rinderer TE, Baxter JR (1979) Honeybee hoarding behaviour: effects of previous stimulation by empty comb. Anim Behav 27:426–428
Rinderer TE, Baxter JR (1980) Hoarding behaviour of the honeybee: effects of empty comb, comb color and genotype. Environ Entomol 9:l04–105
Rinderer TE, Baxter JR (1984) Hoarding behaviour of the honey bee: effects of empty comb, comb color and genotype. Env Ent 9:104–105
Rinderer TE, Hagstad WA (1984) The effect of empty comb on the production of foraging honeybees collecting nectar. J Apic Res 23:80–81
Rinderer TE, Hagstad WA (1963) The effect of empty comb on the production of foraging honeybees collecting nectar. J Apic Res 23:80–81
Rösch GA (1925) Untersuchungen über die Arbeitsteilung im Bienenstaat. I. Die Tätigkeiten im normalen Bienenstaate und ihre Beziehungen zum Alter der Arbeitsbienen. Z Vergl Phyriol 2:571–631
Rösch GA (1927) Über die Bautätigkeit im Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 6:265–298
Rösch GA (1930) Untersuchungen über die Arbeitsteilung im Bienenstaat. 2. Die Tätigkeiten der Arbeitsbienen unter experimentell veränderten Bedingungen. Z Vergl Physiol 12:l–71
Sakai S, Momose K, Yumoto T, Nagamitsu T, Nagamasu H, Hamid AA, Nakashizuka T (1999) Plant reproductive phenology over four years including an episode of general flowering in lowland mixed dipterocarp forests of South-East Asia. Biol J Linn Soc 75:233–248
Seeley TD (1982) Adaptive significance of the age polyethism schedule in honeybee colonies. Behav Ecol Sociobiol 11:287–293
Seeley TD (1985) The social physiology of honeybee colonies. Harvard University Press, Cambridge
Seeley TD (1989) The honey bee colony as a superorganism. Am Nat 77:546–553
Seeley TD, Kolmes SA (1991) Age polyethism for hive duties in honeybees—illusion or reality? Ethology 87:284–297
Seeley TD (1995) The wisdom of the hive. Harvard University Press, Cambridge
Shenkute AG, Getachew Y, Assefal D, Nuru A, Ganga G, Abebe W (2012) Honey production systems (*Apis mellifera* L.) in Kaffa, Sheka and Bench-Maji zones of Ethiopia. J Agric Ext Rural Develop 4:528–542
Silberrad REM (1976) Bee-keeping in Zambia. Apimondia, Bucharest
Szalök J (1928) Viasztermeles, epitkezes teli idoben. Meheszet 25:275. (Cited from Örösi-Pál 1931) [in Hungarian]
Tan K, Guo YH, Nicolson SW, Radloff SE, Song QS, Hepburn HR (2007) Honeybee (*Apis cerana*) foraging responses to the toxic honey of *Tripterygium hypoglaucum* (Celastraceae): changing threshold of nectar acceptability. J Chem Ecol 33:2209–2217
Taranov GF (1959) The production of wax in the honeybee colony. Bee Wld 40:113–121
Tauber M, Tauber C, Masaki S (1986) Seasonal adaptations of insects. Oxford University Press, Oxford
Tokuda Y (1955) Experimental studies on beeswax production by the honeybee colonies. Beeswax yield by the honeybee colonies under sugar syrup feeding. Bull Nat Inst Agric Sci Chiba Ser G [Anim Husb] 11:111–133 (in Japanese)

Tuenin F (1928) The wax glands of bees. Opyt Pas 11:502–508 (in Russian)
Viallon PL (1885) Amount of honey consumed by bees to make one pound of beeswax. Am Bee J 21:153–158
Weiss K (1965) Über den Zuckerverbrauch und die Beanspruchung der Bienen bei der Wachserzeugung. Z Bienenforrch 8:106–124
Whitcomb W (1946) Feeding bees for comb production. Glean Bee Cult 74:198–202
Wood GH (1956) Dipterocarp flowering season in Borneo. Malasian For 19:193–201
Worswick PV (1987) Comparative study of colony thermoregulation in the African honeybee, *Apis mellifera adansonii* (=*scutellata*) Latreille, and the Cape bee, *Apis mellifera capensis* Escholtz. Comp Biochem Physiol A86:95–102
Zherebkin MV, Martinov AG (1977) Effect of supplementary feeding with sugar on bees' condition. Proceedings of 26 international apiculture congress, Adelaide, pp 377–379

Chapter 10
Construction of Combs

Abstract The construction of cells and regulation of the space between combs are separate but related problems. The space between combs, affected by the bees themselves, is the very basis of contemporary practical beekeeping. Within a honeybee multiple comb nest, there are several independent comb starts within the building clusters. Then the "rule of parallelism" comes into play because the building bees modify their constructions to keep equable and parallel spaces between combs. Comb construction is the result of interplay of vertical and lateral forces which lead to many imperfections that are eventually hidden by retouching. A building cluster can exert torsional and tensile loading on a piece of comb. When twisting combs, cell walls become broken; however, the bees rapidly repair them. To achieve parallel combs bees must maintain a tolerance distance between combs which may be due to the detection of gravity. Building bees appear to exploit a sense of gravity which was shown by disrupting the function of sense organs and then observing the effects on comb construction. Bees detect gravity by an unfettered sense organ of the neck and orient themselves during comb construction, based on magnetic material in a band across the abdomen. Different magnetic oxide nanoparticles have been observed in all body parts of honeybees, but greater concentrations occur in their abdomens and antennae.

10.1 Introduction

The construction of cells and the regulation of the space between combs are separate but related problems. The space between combs, affected by the bees themselves, is the very basis of contemporary practical beekeeping. Within a honeybee multiple comb nest there are several independent comb starts within the building cluster and at different attachment sites. Then Darchen's "rule of parallelism" comes into play because the building bees modify their constructions so as to keep a reasonably equable and parallel space between combs. Parallelism overrides other considerations, such as the length of cells.

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Comb construction is the result of interplay of vertical and lateral forces acting on the combs which, over time, lead to many imperfections that are eventually hidden by retouching. A building cluster can independently exert torsional and tensile loading of a piece of comb. In the process of twisting comb, cell walls will inevitably be broken; however, the bees rapidly mend such tears and fractures. Honeybees achieve reasonably parallel sets of combs, but in the end, they have some means both of achieving this and of maintaining the distance between combs within limits that we can recognise as tolerances. This may be due to the detection of the vertical axis of gravity.

Building bees might be able to exploit a sense of gravity that would allow them to build vertical combs. This was shown by disrupting the function of a sense organ and then observing the effects on comb construction. It was shown that an unfettered sense organ of the neck is the instrument by which bees detect gravity and so orient themselves during comb construction. The basis for this ability is supported by the discovery of magnetic material in a transverse band across the abdomen. Indeed, different magnetic oxide nanoparticles, ranging from super-paramagnetic to multi-domain particles, are found in all body parts of a honeybee, but greater concentrations occur in their abdomens and antennae.

10.2 Parallelism Between Combs

The building of a honeybee nest involves both the construction of cells and the regulation of the space between combs; separate but related problems. The space between combs, affected by the bees themselves, is the very fundament of practical beekeeping. The realisation of the importance of this space is contained in the correspondence of Langstroth (Naile 1942), but is not explicit in his laborious account of its management (Langstroth 1853). In any event, although Langstroth is usually cited as the 'discoverer' of bee space, the first practical application of the principle was that by Dżierzon (1852). The way in which the space between the combs might be regulated by bees occupied Darchen through many years of research on *A. mellifera.* Summarising and expanding on three of his earlier research letters (Darchen 1952a, b, 1954) presages his experimental work with the observation that a straw skep is a more 'natural' nest container than a beekeeper's hive. In the former, the combs curve below and are not constrained by the rectilinear design of the latter. In a skep, or feral nest, the combs are also parallel to one another, even when they curve about a horizontal axis (Fig. 10.1).

Viewed as a crystal, the combs from a skep may contain a dislocation of the lattice (Fig. 10.1). This indicates that there are several independent comb starts within the building cluster and at different attachment sites. Darchen's "rule of parallelism" then comes into play; the building bees modify their constructions so as to keep a reasonably equable and parallel space between the combs. The finished comb is only the final result of how the bees have reacted to the many stimuli for construction. Interference with the forming nest gives some insight into what

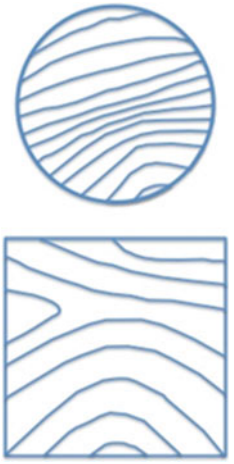

Fig. 10.1 *Top* Disposition of combs naturally built by *A. mellifera* in a skep or a hive without frames; *bottom* bees may interpose an additional comb (on *left*) depending upon constraints of the nest cavity (after Darchen 1954)

stimuli may have influenced the bees. It also provides examples of how bees retouch their constructions to achieve parallelism. In the early stages of construction, a comb is often twisted (Fig. 10.2), but the torsion is obscured by retouching. Similarly, breaches may also be inflicted on combs and these too are quickly repaired with retouching. That parallelism overrides other considerations, such as the length of cells, was shown by juxtaposing two pieces of comb and obtaining the building solution shown in Fig. 10.3.

In another series of tests, Darchen fixed a sheet of wax between two existing combs, but the sheet was abnormally close to one of the combs (Fig. 10.4a, top), with the result that new wax added to the bottom of the given sheet was gradually re-contoured to obtain a parallel result (Fig. 10.4a, bottom). If, however, the comb closest to the inserted sheet of wax was covered with a piece of cardboard, the bees then built so as to connect the sheet of wax (Fig. 10.4b). If the cardboard was placed on the opposite comb, then the new comb built was contoured to lie equidistant between the apparent faces of the two combs (Fig. 10.4c). Darchen (1954) concluded that parallelism operates within a perceptible range of distances, deviations only occurring when a space between two combs is unacceptably small. That the distance between the cell walls themselves is the likely element that bees could measure is shown in Fig. 10.4.

10.3 Festoons and Torsion

The forming combs are generally extended in the vertical plane, but they may well lean to one side and thus grow obliquely. Darchen (1956) suggested that some force might act on the combs during construction, such as a mass of building bees working on only one side of the comb. He concluded that comb construction is the result of interplay of vertical and lateral forces acting on the combs which, over

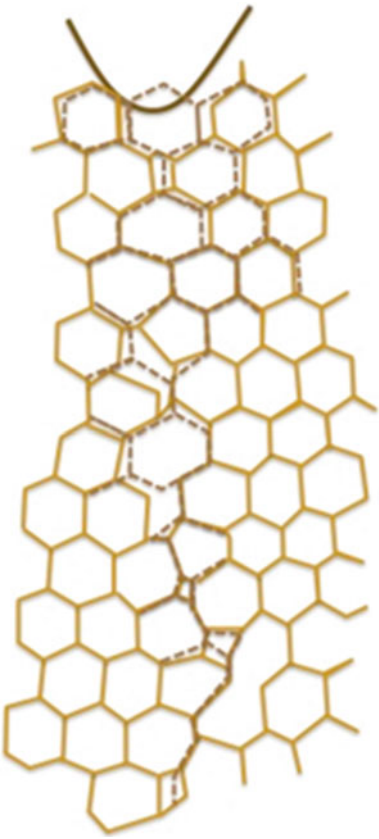

Fig. 10.2 The retouching of cells in the second phase of the construction by *A. mellifera* indicated by the *dark brown* broken line (after Darchen 1954)

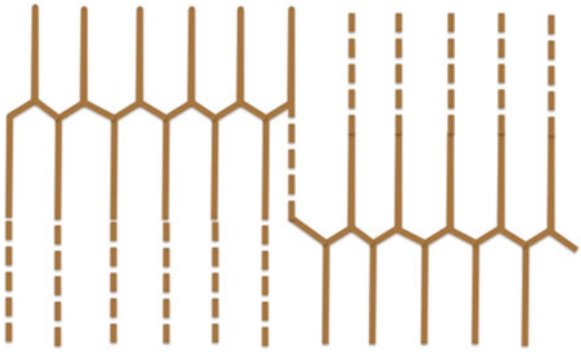

Fig. 10.3 Juxtaposition of two pieces of constructed comb (*solid lines*) results in reconstruction so that parallelism is maintained in *A. mellifera* combs (*dashed line*) (after Darchen 1954)

time, lead to many imperfections that are eventually hidden by retouching (cf. Fig. 10.2). As we shall see, evidence for forces acting on combs during construction comes from several experimental studies on comb-building.

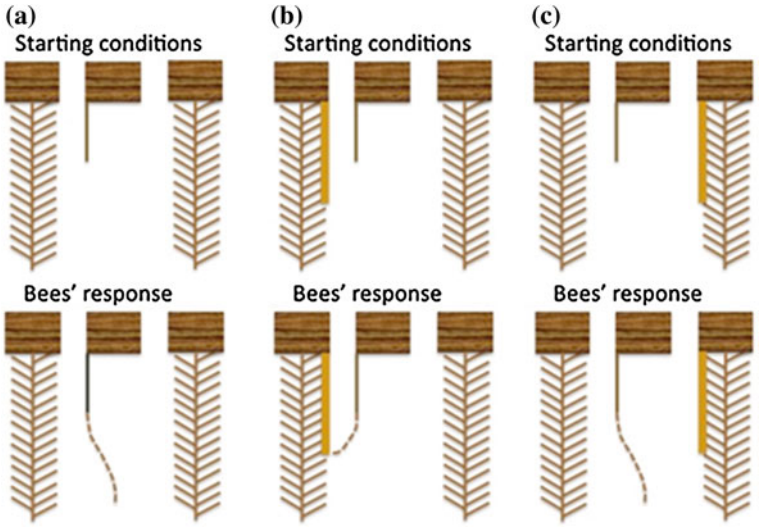

Fig. 10.4 **a** Experimental insertion of a piece of beeswax foundation is placed unacceptably close to an *A. mellifera* comb as the starting condition (*top*) which leads to the bees' response (*bottom*). **b** When the space between the beeswax sheet and an adjacent comb is further reduced by adding a piece of cardboard to the comb face the starting condition (*top*) leads to reconstruction as shown (*below*). **c** In the third sequence, a combination of the interferences shown in (**a**) and (**b**) (*top*) leads to the new construction re-establishing the parallelism between combs (*bottom*) (after Darchen 1954)

In his numerous observations on comb-building, Darchen (1959b) began an analysis of how building festoons congregate on combs and the loading effects the bees may exert on them. Before discussing Darchen's work in any detail, it is important to note that in wild nests, bees make their combs parallel in two ways. They either lengthen the cells of one side of the comb, or they tear down what they have built and reconstruct the comb, the latter tack is imperceptible in the completed combs. In his ingenious experiments, Darchen (1958, 1959b) placed a piece of beeswax foundation normal to and in between two parallel combs (Fig. 10.5). Soon after the bees had settled, this new sheet of wax was gradually twisted about the vertical axis so that the bottom-most portion of the wax sheet was properly aligned to both the adjacent combs as shown in Fig. 10.5. However, the embossed pattern of the middle section of the wax sheet showed that the cells were elongated as well.

In order to separate the torsional effects of the bees from the stretching of the wax, Darchen then introduced a piece of foundation coated with an alcohol extract of propolis (said to inhibit construction). Several hours later, this new piece of wax had been twisted into alignment with the adjacent combs, but the cell embossment showed no stretching at all.

It appears, then, that a building cluster can independently exert torsional and tensile loading of a piece of comb. In the process of twisting comb, cell walls will

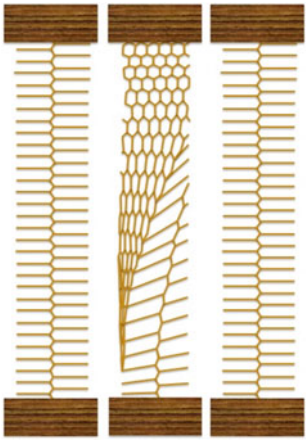

Fig. 10.5 Embossed beeswax foundation inserted in the opposite direction to two adjacent combs, is twisted by the bees into alignment with the pre-existing *A. mellifera* combs (after Darchen 1959a)

inevitably be broken; however, the bees rapidly repair such tears and fractures. These kinds of repairs obscure the fact that bees may well have twisted combs and retouched whatever rents may have appeared. Darchen went on to provide an experimental mechanical model to simulate the torsional deformation of combs, and was able to conclude that simple, horizontal traction, applied to opposite ends of a strip of wax or of a comb, produces sufficient torsion to twist the forming wax of their nests. Since these sheets of wax were twisted, Darchen investigated the chirality of 49 such specimens. He found that 22 of them had a left-handed sense and the other 27 a right-handed sense, results that imply randomness. Similarly, the amplitude or angle of torsion appeared to be related to the distance between the sheet of foundation wax and an adjacent piece of comb. The amplitude of torsion increased with increasing distance between the two combs, in which the experimental sheet of wax was placed.

These simple little experiments of Darchen (1958, 1959b), and his earlier observations on the inter-conversions of worker and drone cells (Darchen et al. 1957), contain a wealth of information and suggestions. They demonstrate considerable plasticity in the building behaviour of bees and show how they effectively 'hide' their extensive retouching of nest combs to produce a final product of parallel constructs. In another series of experiments Darchen (1962a) developed further generalisations about nest construction. In essence, his work is really a test of stereotypy, a mechanistic perspective of animal behaviour that dominated ethology over three decades.

By presenting bees with a wide range of different kinds of triangular and several other irregular shapes, Darchen (1955, 1962a) was able to observe how, in such cases, a comb would be constructed. While he regarded the bees' initial modes of construction as 'incoherent', he was able to establish a more orderly second phase of construction in which the wax is gradually drawn and rounded into an ellipsoid body, followed by a rapid vertical increase in comb length, and finally the

development of cell walls. This second phase, in fact, reflects exactly what bees do when initiating the building of a nest, as shown by the confirmatory experiments of Naulleau and Montagner (1961).

10.4 Festoons and Comb Growth

Even more comb handling can be directly attributed to the behaviour of festoons of building bees, as Darchen (1962b) learned when he established an observation hive within an incubator held at a temperature of 30 °C. It was under these same conditions that Huber's (1814) thick curtain of bees admitted some light, as the workers began to spread out, and clearly defined chains of bees become visible (Fig. 10.6). (As an aside one must be instantly alerted to the possibility that the extremely dense clustering of bees in an unheated nest is in fact for the production and conservation of heat). Darchen (1962b) found that he could predict the points of growth on the combs from the positions of the festoons. He drew the positions of festoons, or chains of bees, on the glass of his observation hive and, the following day found that the newly constructed comb closely matched the outlines of where the bees had previously hung. Thus the position of the chains of wax-secreting bees could serve as a daily blue-print for comb construction, an idea first suggested by Hubbe (1957) and finally confirmed by Darchen (1962b).

Towards the end of his study, Darchen (1962b) made 12-hourly recordings of the chains and subsequent growth of the combs; the correspondence between the two is evident (Fig. 10.7). Additional information on the chain bees also emerged. Temporarily, the most stable chains were those closest to sites where the comb was actually being extended. Once a chain is formed, other bees rarely join it. Marked bees were observed to remain in a chain for several days. Oddly enough, Darchen could not see wax scales on the bees in a chain, yet when individual bees left the chain there was always a vigorous rubbing of their abdomens, perhaps to loosen scales? We can add confirmation of Darchen's (1962b) observations from very similar observations of our own, on African *A. m. scutellata* and *A. m. capensis,* as well as *A. cerana* in Asia (Hepburn and Duangphakdee, pers. obs.).

Both at the inception of a honeybee nest, or during extensions within an existing nest, groups of wax- bearing worker bees gather in vertical, elongated chains in which individual bees may remain there for some time. These chains of bees, also termed festoons, are easily seen in the frame hives used for *A. cerana* and *A. mellifera*, especially if there are empty frames from which they can be suspended. Often several chains may be seen at different sited and on different frames (cf. Fig. 10.9—Hepburn 1986). Indeed, photographs have been published showing *A. cerana* x *A. mellifera* mixed-species chains of building bees (Yang et al. 2010a, b). To observe chains of building bees in nests of the single comb species is more difficult. The inception of a nest and of a chain of comb-building bees of *A. florea* was recently photographed (Fig. 10.6).

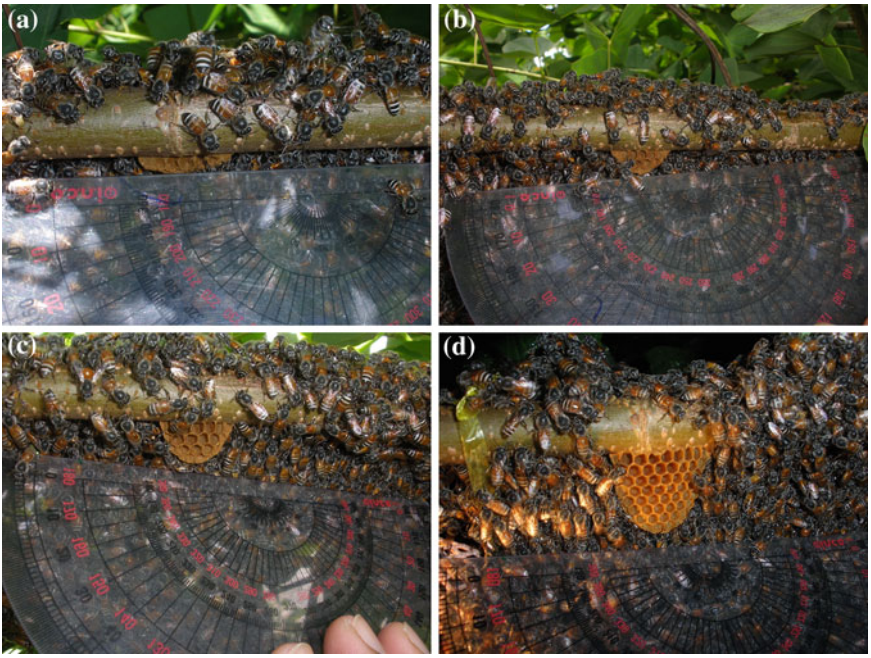

Fig. 10.6 Inception of an *A. florea* nest. **a** shows the worker bees gathering both *above* and *below* the nest twig at 11.31 h after settling on twig; **b** even more bees are present at the site by 12.39 h; **c** distinct chains of workers have constructed a few cells below the twig at 13.54 h; **d** construction is in full swing at 19.56 h and at the same time other bees have begun constructing the crown cob above the twig. Plastic piece with numbering is a protractor

Fig. 10.7 Correspondence between the positions of chains of wax building bees and the construction of new comb by *A. mellifera*. Festoons are represented by *thickened lines*, the thickness of which indicates the density of bees present. *Broken lines* represent additional new comb (after Darchen 1959b)

10.5 Evidence of a Sense of Equilibrium

The thrust of Darchen's many experiments and observations, which he summarised in 1968, is that, through retouching their constructions, honeybees achieve reasonably parallel sets of combs. Bees must, in the end, have some means both of achieving this and of maintaining the distance between combs within limits that we recognise as tolerances. That this may be due to the detection of the vertical axis of gravity was shown by Gontarski (1949), the mechanism investigated by Martin and Lindauer (1966), or rather by a self-organising process related to the substrate (Pratt 2000), and similar to the self-organisation of the hexagonal pattern (Pirk et al. 2004; cf. Chap. 12).

The combined cell bases constitute a mid-wall from which the cells extend perpendicularly. Gontarski (1949) investigated the means by which bees almost invariably achieve a vertical relationship between the vertical axis of the mid-wall and the pull of gravity. In his experiments, Gontarski (1949) placed small queenright colonies (1000 bees) into single-frame hives, which were thermostatically warmed and also kept covered for darkness. Each hive in turn was placed on a rotating stage, with the flight hole in the axis of rotation. By use of a synchronous motor he was able to maintain a constant loading on the combs in a desired axis.

Because the posture of the bees changes depending on their position in relation to the combs, the centre of gravity may act either through the median plane of the animal (dividing a bee into mirror halves when the bee itself is vertical), or through a frontal plane (between top and bottom halves of the bee if it stands on the horizontal). In Gontarksi's first experiment, the bees hung vertically on the combs so that the frontal axis of the bees remained constantly vertical, but there was a continual change about the median axis (Fig. 10.8). Surprisingly, after 10 days or so of continuous rotation, the bees had constructed 'normal' combs. This experiment argues for the mid-wall being constructed in the vertical axis if the frontal plane of the bees building is vertically orientated. It should be noted that the median plane would have been random in this experiment. These results are entirely consistent with natural constructions where the bees build vertically upwards, downwards or even sideways, the mid-wall always being vertical in such cases. A disruption of the median plane does not hinder a bee's ability to build with respect to gravity.

In a second experiment, Gontarski (1949) placed the comb and bees such that they were loaded tangentially on a rotating horizontal plate (Fig. 10.8). In this way centrifugal forces act normal to the broad comb face. In this situation the frontal plane, important for a vertical orientation (see above), is taken out of the vertical mode; likewise, the gravitational and centrifugal forces were not aligned and a resultant was obtained. The median plane of the bees remained vertical. In the configuration of this experiment the mid-wall of the combs would be expected in the direction of the resultant, and this is precisely what Gontarski (1949) obtained. This implies that the bees posturally reorient themselves to obtain a resultant vertical orientation of their frontal plane.

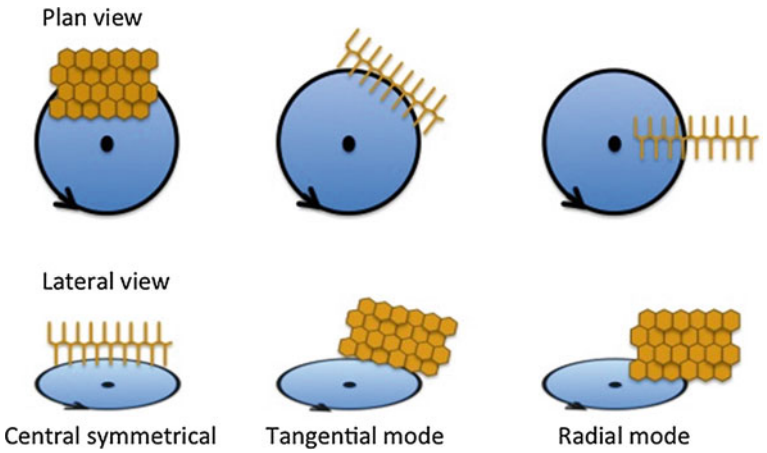

Fig. 10.8 The plane and nearly lateral view of the experimental design of Gontarski (1949) to assess the gravitational sense of bees. The position of the bees in his first experiment was as on the *left*, the tangential mode is shown diagrammatically in the *middle*, and the radial arrangement on the *right*

The role of gravitational forces acting on the median plane was studied in a third experiment. Here the hive was placed radial to the axis of rotation on the horizontal plate (Fig. 10.8). In this case the frontal axis of the bee could remain vertical and its median plane thrown in the direction of the result and of both centrifugal and gravitational forces. Again, the mid-walls of the combs were in the vertical plane.

Thus the bees followed the vertical axis, which must have been perceived through the frontal plane. The orientation of the hexagons themselves appears not to be mediated through a perception of the vertical. The skewed orientations which Gontarski (1949) observed in all his rotating experiments varied with the speed of rotation. He concluded that the degree of skewness results from the vertical orientation of the bees with respect to their median axis. This may be, but this interpretation does not explain the natural occurrences of horizontal, vertical or tilted cells in normal combs.

As a finale to Gontarski's experiments, it is extremely interesting to note that one of his colonies had been rotated continuously for 6 weeks in the radial mode. When removed from the experimental platform, the bees continued building comb. In this new comb the mid-wall was acute to the vertical and opposite in direction from the resultant that had prevailed during 6 weeks of rotation. This obviously implies either an overcompensation on cessation of the stimulus (Hepburn 1986), or an overcompensation during the 6 weeks of constant exposure to the abnormal influence of the hyper-gravitational forces (up to 1.2 g) during the experiment (Pratt 2000).

10.6 Application of the Sense of Equilibrium

In a continuation of their heroic experimental efforts, Martin and Lindauer (1966) further investigated how building bees might be able to exploit a sense of gravity that would allow them to build vertical combsvertical comb. Their experimental approach was to establish small colonies of bees, to disrupt the function of an organ, and then to observe the effects of their various interventions on comb construction. By trial and error, they eliminated surgical ablation as too time-consuming a procedure, and in the end they set about plastering over different sense organs with a wax-resin mixture (how they came about the right proportions is a story in itself). Their procedure was to take 500 to 1000 bees from the building cluster of a strong colony, to anaesthetise every bee and to gum over a sense organ of interest. The bees were then given a queen, put on empty building frames and kept at 25 to 30 °C during the experiments. Since it had previously been shown that bees have sense organs which detect the direction of gravity (Lindauer and Nedel 1959), Martin and Lindauer (1966) performed a series of five experiments to assess the possible role of gravity detection in comb construction.

In their first experiment, Martin and Lindauer (1966) anaesthetised 490 bees and immobilized their heads by gluing them to their thoraces using the wax-resin mixture. These bees were hived and formed a cluster on the building frames. After 8 days there was not a speck of wax on the frames, but wax scales had accumulated on the bottom of the hive. On repeating their experiment using 600 bees there were a few spots of wax here and there on the frames but no combs. The authors noted that the head-thorax join of 121 bees had become loose. Although the setae of the neck hair plates were still gummed over, this may account for the spots of wax. From this we can only conclude that 1090 bees, with their heads glued fast to their thoraces, did construct any comb. The implication is that mobility of the head is somehow necessary for comb construction, but not for wax secretion.

In two more refined, and technically more difficult procedures, Martin and Lindauer (1966) plastered only the sensory plates on the necks of the bees (Fig. 10.9). About 1000 bees in each trial failed to produce proper combs over a two-week period. However, after about two weeks (having checked daily for any loosening of the glue), the first bees were detected in which the glue had become loose. From that time onwards the bees constructed only few erratic triangles. These results are considered sufficient evidence to show that an unfettered neck organ is required for comb construction.

Since bees hold their abdomens in an obliquely downward position when lengthening cell walls (and often when they fly), Martin and Lindauer (1966) decided to assess the possibility that the sense cells of the abdominal petiole (Fig. 10.9) might contribute to comb construction. They performed two trials, with 660 bees in each group. In one group the sensory setae were gummed over, and in the second group the thorax was immobilized and glued to the abdomen to prevent any movement at that joint. Both groups of bees constructed normal combs. The immobility of the abdomen in Martin and Lindauer's (1966) experiment is supportive of a decisive role of

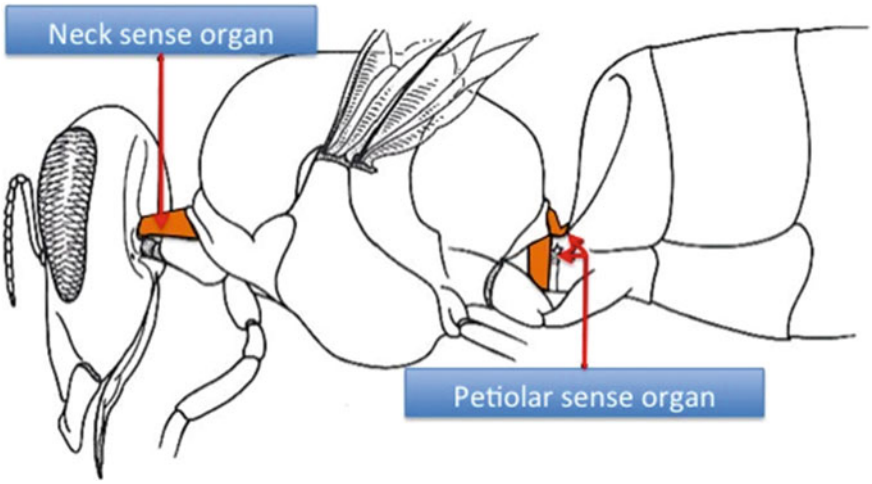

Fig. 10.9 Location of the sense organs of an *A. mellifera* honeybee worker thought capable of perceiving the direction of the force of gravity. Those of the neck (stretched here) are usually covered by the head (after Martin and Lindauer 1966 and modified from Hepburn 1986)

the sensory setae on the neck for comb-building, however they may work. Their results are also consistent with those of Gontarski (1949).

Martin and Lindauer (1966) concluded that an unfettered sense organ of the neck is the instrument by which bees detect gravity and so orient themselves during comb construction. This interpretation is made all the more plausible by the discovery by Gould et al. (1978), that worker bees have magnetic material in a transverse band across the abdomen. This material has been described during the intervening years and was recently reviewed by Wajnberg et al. (2010). To paraphrase these authors, honeybees show sensitivity to small changes in magnetic fields. Different magnetic oxide nanoparticles, ranging from super-paramagnetic to multi-domain particles, were observed in all body parts of honeybees, but relatively greater concentrations occurred in their abdomens and antennae. It is not yet known how magnetic information could be processed by the honeybee nervous system. Nonetheless, results from recent studies on honeybee magnetism published by Hsu et al. (2007) certainly support the original thinking that underlies the experimental work of Martin and Lindauer (1966).

An interesting experiment that relates to their work was the dispatch of a small colony of bees for 6 days on a space-shuttle flight beyond the earth's gravitational pull. It is said that the bees built perfectly normal combs under conditions approximating zero gravity (Vanderberg et al. 1985). This experiment very simply indicates that bees can build normal combs in the absence of gravitational cues. This supports an alternative idea; that not gravity but a substrate-dependent mechanism, because the cell walls are always perpendicular to the substrate (Wedmore 1929; Lau 1959). In comb-building the subsequent rows use the previous row as templates resulting in a cascade of propagating orderliness over the

whole comb (Pratt 2000). However, the ultimate test would be to measure the orientation in relation to the substrate and gravity in natural nests of *A. mellifera*, and furthermore in other species to include an evolutionary perspective.

10.7 The Orientation of Combs

The detailed observations of Darchen (1968) clearly show that a newly settled swarm may well begin the construction of combs at several different and apparently independent sites. However, parallel sets of comb are the end result of a building operation that is heavily dependent on retouching. Superimposed upon this parallelism is a planar orientation of combs with respect to compass directions. In one of the very few studies of comb orientation by feral bees, Seeley and Morse (1976) concluded that the arrangement of combs was independent of both the position of the nest entrance (previously noted by Owens and Taber 1973), and the magnetic field of the earth.

When swarms of honeybees are allowed to build combs freely, without the constraints of beekeeping, they build their combs parallel to the same plane and compass direction as were the combs of their mother colonies. Lindauer and Martin (1972, 1973) showed that by taking swarms from hives and placing them in cylindrical containers, these bees built combs of essentially the same orientation that had prevailed in their former nests. The removal of these bees to yet other fresh cartons gave the same results. In some cases, Lindauer and Martin (1972, 1973) placed Helmholtz coils around the second cartons in such a way as to deflect the apparent magnetic field by some 40°. The combs built under these conditions were likewise deflected by 40°. However, several other researchers, including Gould et al. (1978), who established that bees have magnetic remanance in the first place, failed to obtain the same results in similar experiments.

Whether or not bees retain memory of comb orientation in the construction of a new nest, or use the earth's magnetic field for orientation was reinvestigated by de Jong (1982). In his first experiment, he placed 25 swarms, which he had caught in trap boxes (containers with no beekeeping furniture), into specially designed building boxes. He measured the orientation of the combs as they had been constructed in the trap boxes and subsequently in the special building boxes. These bees showed a significant and positive tendency to maintain comb direction. de Jong then proceeded to place five colonies in his special comb-building boxes which were situated within a series of coils designed to generate a magnetic field. When he engaged the coils, the horizontal component of the magnetic field was shifted clockwise through 90°. Every few days the bees were transferred to fresh boxes and the coils engaged or not in alternate trials. He found that the bees had maintained, to a significant degree, their comb construction relative to a shifted magnetic reference. He concluded that the magnetic field of the earth is an important cue utilised by bees in the orientation of their combs during building. Thus, de Jong (1982) was able to confirm the original work of Lindauer and Martin (1972, 1973).

10.8 Behavioural Aspects of Comb Construction

Exposing mixed colonies of the two sister-species of the Western, *A. mellifera*, and the Eastern honeybee, *A. cerana*, to foundations made of pure wax from either species resulted in normal building behaviour, only the number of irregular cells was noticeable. In both pure controls, no worker brood was reared in the cells built on the foundation made of the wax of the opposite species. In the pure *A. mellifera* colonies the cell size was modified, whereas *A. cerana* constructed comb without modification but used the cells based on *A. mellifera* wax only to rear drones or for storage (Yang et al. 2010a, b; cf. Chap. 4).

References

Darchen R (1952a) Régulation sociale et écartement des rayons chez les abeilles. C R Hebd Seances Acad Sci Ser D Sci Nar Paris 235:820–822

Darchen R (1952b) Sue quelques régulations sociales dans la construction chez les abeilles. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 234:671–673

Darchen R (1954) Quelques régulations sociales dans la construction chez les abeilles. Insectes Soc 1:219–228. doi:10.1007/bf02222947

Darchen R (1955) Nouvelles régulations sociales dans les constructions d'*Apis mellifica*. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 241:1081–1083

Darchen R (1956) La construction sociale chez *Apis mellifica*. Insectes Soc 3:293–301. doi:10.1007/bf02224312

Darchen R (1958) Les abeilles peuvent tordre une lame de cire pour la rendr parallèle aux rayons. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 245:2208–2210

Darchen R (1959a) Observation et expirimentation sur un essaim nidifiant artificiellement l'air libre. Ann Abeilles 1:5–11

Darchen R (1959b) Un des roles der chaines d'abeilles: la torsion des rayons pour les rendre parallèles entre eux. Ann Abeille 2:193–209

Darchen R (1962a) Le comportement des cirieres d'*Apis mellifica* devant un "theme" de construction. Le role des destructions et des reconstructions des rayons. Insectes Soc 9:23–38

Darchen R (1962b) Observation directe du developement d'un rayon de cire. Le role des chaines d'abeilles. Insectes Soc 9:103–120

Darchen R (1968) Le travail de la cire et la construction dans a ruche. In: Chauvin R (ed) Traité de biologie de l'abeille. Masson, Paris, pp 252–331

Darchen R, Vizier C, Vuillaume M (1957) Sur le determinisme de la construction des cellules de males chez les abeilles. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 244:391–394

de Jong D (1982) Orientation of comb building by honeybees. J Comp Physiol A 147:495–501

Dżierzon J (1852) Nachtrag zur Theorie und Praxis des neuen Bienenfreundes. CH Beck'schen Buchhandlung, Brieg

Gontarski H (1949) Über die Vertikalorientierung der Bienen beim Bau der Waben und bei der Anlage des Brutnestes. J Comp Physiol A 31:652–670. doi:10.1007/bf00348364

Gould JL, Kirschvink JL, Deffeyes KS (1978) Bees have magnetic remanence. Science 201:1026–1028

Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin

Hsu CY, Ko FY, Li CW, Fann K, Lue JT (2007) Magnetoreception system in honeybees (*Apis mellifera*). PLoS ONE 2(4):e395. doi:10.1371/journal.pone.0000395

Hubbe W (1957) Beobachtungen zum Wabenbau der Honigbiene *Apis mellifica* L. Arch Geflügel Kleintier Kd 6:343–358
Huber F (1814) Nouvelles observations sur les abeilles. [English translation 1926] Dadant, Hamilton
Langstroth LL (1853) Langstroth and the hive and the honeybee. Root, Medina
Lau D (1959) Beobachtungen und Experimente über die Entstehung der Bienenwabe (*Apis mellifica* L.). Zool Beitr 4:233–306
Lindauer M, Martin H (1972) Magnetic effect on dancing bees, animal orientation and navigation. NASA US Government Printing Office, Washington
Lindauer M, Nedel JO (1959) Ein Schweresinnesorgan der Honigbiene. Zeit Vergl Physiol 42:334–364. doi:10.1007/bf00298125
Martin H, Lindauer M (1966) Sinnesphysiologische Leistungen beim Wabenbau der Honigbiene. Z Vergl Physiol 53:372–404
Martin H, Lindauer M (1973) Orientierung im Erdmagnetfeld. Forts Zool 21:211–228
Naile F (1942) America's master of bee culture: the life of LL Langstroth. Cornell University Press, Ithaca
Naulleau G, Montagner H (1961) Construction de cellules irrégulières chez *Apis mellifica*. Insectes Soc 8:203–211. doi:10.1007/bf02224009
Owens CD, Taber S (1973) Size and shape of comb constructed by honey bees in 1.2 m^3 box during one season. J Econ Entomol 66:1234–1236
Pirk CWW, Hepburn HR, Radloff SE, Tautz J (2004) Honeybee combs: construction through a liquid equilibrium process? Naturwissenschaften 91:350–353
Pratt SC (2000) Gravity-independent orientation of honeycomb cells. Naturwissenschaften 87:33–35
Seeley T, Morse R (1976) The nest of the honey bee (*Apis mellifera* L.). Insectes Soc 23:495–512
Vanderberg JD, Massie DR, Shimanuki H, Peterson JR, Poskevich DM (1985) Survival, behaviour and comb construction by honeybees, *Apis mellifera*, in zero gravity aboard NASA Shuttle Mission STS-13. Apidologie 16:369–384
Wajnberg E, Acosta-Avalos D, Alves OC, de Oliveira JF, Srygley RB, Esquivel DM (2010) Magnetoreception in eusocial insects: an update. J R Soc Interface. doi:10.1098/rsif.2009.0526.focus
Wedmore EB (1929) The building of honey comb. Bee Wld 10:52–55
Yang MX, Tan K, Radloff SE, Pirk CWW, Hepburn HR (2010a) Hetero-specific queen retinue behavior of worker bees in mixed-species colonies of *Apis cerana* and *Apis mellifera*. Apidologie 41:54–61
Yang MX, Tan K, Radloff SE, Phiancharoen M, Hepburn HR (2010b) Comb construction in mixed-species colonies of honeybees, *Apis cerana* and *Apis mellifera*. J Exp Biol 213:1659–1664

Chapter 11
Energetics of Honey/Beeswax Conversion

Abstract By the mid-19th century consideration of the rates and costs of wax production by *A. mellifera* were developed using the balance sheet method. Moreover, it was known that brood, pollen, combs and queens affect wax production. A century later, sugar/wax conversion ratios were defined as the net amount of sugar consumed against wax produced. Taranov (1959) showed that the total amount of wax produced was linearly related to the amount of sugar consumed; others that comb construction was proportional to colony size and to nectar income. The experiments and observations of this period suffered from a failure to separate the costs of colony maintenance vis-à-vis the production of wax. However, two major factors remained in the cost equation: (1) the relative importance of colony age structure in wax production; and (2) the problems of heat production, colony size and the synthesis of wax itself. Subsequently, Hepburn et al. (1984) calculated the rate of sugar consumption (corrected for attrition), and sugar stored in the nascent combs, as well as the rate of comb construction. The real metabolic rate, averaged over time for bees of different ages, showed that a plateau was reached in bees at about 12 days old, figures that included an adjusted metabolic rate as a function of bee age. This trade-off or cost calculation comes into play at both individual and colony levels. Both wax secretion and construction rapidly decline in autumn and virtually cease during winter. It is not yet possible to adequately assess the relationship of wax synthesis and comb construction to the thermal conditions of a colony's nest.

11.1 Introduction

In the days when the great naturalists believed wax to be the product of flowers gathered by bees when foraging, the swineherd and beekeeper knew, as did his lord or abbot, what sort of ratio of honey to wax could be harvested after the skeps were taken off the sulphur pit (Ransome 1937; Galton 1971; Vernon 1979). In the year that Huber (1814) published his observations on bees, we find little in

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contemporary works other than yield figures. John Keys (1814) reported that a 2-year-old colony with a nest volume of three pecks (~27 l) would yield 25 pounds (~11 kg) of honey and not more than 2 pounds (~900 g) of wax.

A real interest in the rate of wax production (measured as combs) and the costs of construction (measured as the amount of sugar required) developed in parallel with agricultural chemistry in the 1830–1840 period. This was a time when the balance sheet method of Lavoisier came into widespread use among chemists (Holmes 1985) and was very effectively used to record food input and the corresponding recovery of a plant or animal product. The first experiments were expressly performed to find out how much sugar honeybees consume in the production of wax are given in a tortuous argument in the treatise of Gundelach who used old German units of measure, so that thirty-two loth equalled about half a kilogram (1842). He reported that 2765 bees produced 1.25 Loth of wax (~81360 individual wax plates, 18.25 g) requiring 27 Loth of honey (394.2 g) in 6.5 days. He concluded from his experiments, during an autumnal dearth in central Germany, that a colony requires about 20 measures of honey to produce one of wax. The experiments of Dumas and Edwards (1843) on confined bees were far more precise, but were concerned with the proof of fat synthesis and not conversion ratios. In any event, the thoroughness of their data handling using the balance sheet method allows us to calculate that their small colony of 1788 bees produced 17 g of wax in 11 days and consumed 411 g of honey, giving a honey/wax ratio of 24:1.

The next conceptual advance is contained in a note by Dönhoff (1854), entitled "*Kostet der Wabenbau Honig?*", who attempted to separate the cost of wax production from the energy necessary to support the other activities of bees. In the days when a sample size of one was acceptable experimental currency, Dönhoff (1854) arranged three skeps in his apiary one October. One skep was completely empty, in the next a large amount of comb was cut away, and the last contained fully drawn combs. Each contained a caged queen (to prevent egg-laying and brood care costs) and about 8000 bees. The bees of each skep were fed unspecified, but presumably equal, amounts of honey. After a week, Dönhoff (1854) determined that the bees of both wax-bearing skeps had built no additional comb but each had stored 1.25 kg of honey. The bees of the initially waxless skep had constructed 42 g of comb and stored 864 g of honey. The 344 g difference in honey stores was attributed to the cost of production, the rate of which would be 750 μg per bee per day with a honey/wax conversion ratio of about 8:1. He repeated the same basic experiments using Dżierzon hives instead of skeps, and obtained figures of 23:1 and 15:1, which is within the range of Gundelach (1842).

During the same period, recognition of other factors that could influence conversion ratios began to appear in the literature. For example, the renowned Dzierzon (1861), discussing the biology of wax production, suggested that pollen might well influence the amount of wax produced, although he did not advance any evidence to support this (correct) view. Over the next hundred years or so, numerous experiments were performed under a variety of circumstances and a number of variables that might affect wax production began to emerge. Thus, the presence or absence of brood, pollen, combs and queens were gradually recognised as having a direct affect

on wax production. Even so, curiously enough, it is implicit in the literature for the period 1840–1940 that, ultimately, a faultless solution would emerge. That is, that the definitive experiment could be done to provide *the* answer to the real cost of converting sugar into wax Hepburn (1986). Yet, conversion ratios ranging from 1.8:1 to 104:1 have been obtained experimentally (Table 11.1).

11.2 Cumulative Ratios

While it is undoubtedly true that these gross conversion ratios reflect real values obtained under varying conditions, the experiments of the various authors cannot be directly compared nor can an explanation for a particular result always be readily found. This difficulty is illustrated by a comparison of the work of Whitcomb (1946) with that of Tokuda (1955). Whitcomb intermittently fed unmerchantable honey to four queenright colonies over 70 days of summer and gave the bees frames of foundation on which to draw combs. His records show that in the first 10 days the ratio of sugar consumed to wax produced dropped from 104:1 to 7:1 and in the ensuing two months oscillated irregularly between 3:1 and 15:1. The running cumulative ratio gradually declined to 8.4:1 at the end of his observations.

Tokuda (1955) conducted a series of experiments on 22 queenright colonies during a good spring flow followed by a summer dearth. The control colonies were given empty drawn combs and the experimental ones frames with strips of wax foundation. The bees were allowed to forage and were fed sugar copiously. He measured the amount of sugar consumed and the amount of wax produced. Unfortunately, only three of his experiments had reasonable controls. In the analyses of Whitcomb (1946) and Tokuda (1955) the conversion ratios were simply defined as the net amount of sugar consumed against wax production. As such, their reports merely indicate the extent of wax production and sugar utilisation by different colonies during periods in which comb was or was not constructed. Their results do however show the extent of natural variation in comb construction, but give no insight as to how the energy assimilated by the bees might have been partitioned in the colony.

The manner in which energy might be related to wax production was further investigated in Moldavia by Taranov (1959) after a summer's flow had ended. He established 16 colonies, equalised for size (500 g) and age, each headed by a mated queen. The bees were given frames of combs from which a portion had been cut away. He fed them 50 % sugar syrup and replaced the combs 18 times over a 59 day period to preclude the rearing of brood. The colonies were grouped in pairs, and each pair was fed differing quantities of sugar-syrup every 24 h for the duration of the experiment. The average wax production obtained in this experiment in relation to the amount of sugar given to the different colonies is depicted in Fig. 11.1.

It can be seen that the total amount of wax produced in Taranov's colonies is linearly related to the amount of sugar consumed. This extremely interesting result

Table 11.1 Chronological and annotated list of studies on food conversion ratios (sugar/wax ratios) in the production of wax

	Author and summary	Sugar/wax ratios
1814	Huber. Three small, confined colonies fed sugar or honey, pollen; assumes all food converted to wax	5.7:1 and 12:1
1842	Gundelach. One small colony, broodless, with queen confined; fed honey in autumn dearth; assumes all sugar converted to wax	20:1
1843	Dumas and Edwards. One small confined colony fed honey, no pollen	24:1 and 36:1
1861	Dönhoff. Average colonies with queen confined; fed honey and allowed to forage in fall dearth; ratios based on consumption differences between colonies that built and those that did not build combs	8.2:1, 15.2:1 and 22.8:1
1873	von Berlepsch. Small confined colonies fed sugar or honey	No pollen, 19:1 and 20.5:1; with pollen, 13:1
1885	Viallon. Two colonies with drawn combs and two combless; bees allowed to forage; procedure then reversed; assumes differences in stores to be cost of wax	6.7:1
1886	Hasty. One average colony allowed to forage; attempted to measure food consumption through weight changes of colony; assumes all honey consumed converted to wax	2.9:1
1887	De Layens. As in Viallon, but with two broodless colonies in summer dearth	6.3:1
1901	Maupy. Minimal value based on theoretical calculations only	4:1
1905	Brünner. Using normal production colonies fed honey; assumes straight conversion of food into wax	6.8:1
1944	Rosov. Four average colonies allowed to forage in a greenhouse; attempted to separate costs of colony maintenance, brood rearing and comb-building	12.3:1 to 14.2:1
1946	Whitcomb. See text for full discussion	3:1 to 104:1
1955	Tokuda. See text for full discussion	1.8:1 to 8.2:1
1965	Weiss. See text for full discussion	3.3:1 to 13.2:1
1965	Horstmann. Theoretical calculations considering several biochemical pathways, none of which have been shown in bees	2.8:1 to 8:1
1984	Hepburn et al. See text for full discussion	4.3:1 to 26.3:1

is open to several interpretations, especially when it is compared to situations in which the bees were given sugar in sufficient excess that they were able to store it as ‘honey’ (e.g. Whitcomb’s (1946) experiment).

It is very tempting to conclude that there is simply an equal partitioning of the energy towards wax production (see Fig. 11.1) in Taranov’s experiment and that a fixed percentage is allocated for producing wax. Such a conclusion would be consistent with the results of Taber and Owens (1970), who found comb

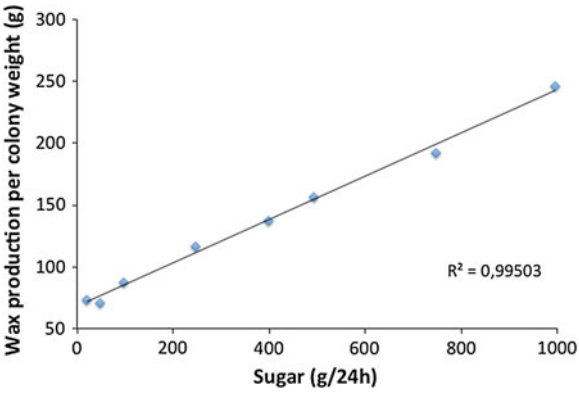

Fig. 11.1 The average wax production for 500 g colonies over a 59-day period. The amount of food, a 50 % sugar syrup, was fixed between 25 and 1000 g a day (based on data of Taranov 1959)

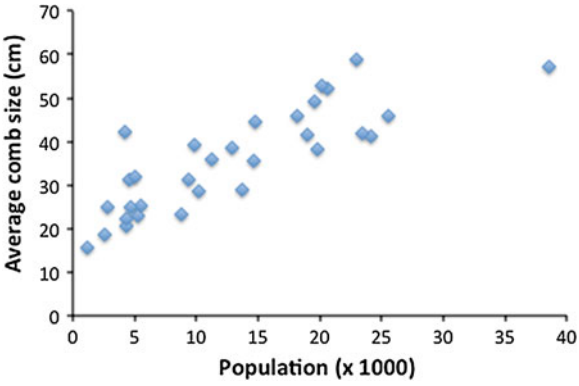

Fig. 11.2 The average size of the comb constructed as related to colony size (after Taber and Owens 1970)

construction to be proportional to colony size (Fig. 11.2), and with those of Florea and Malaiu (1961) that comb building is proportional to nectar income, even though both groups lacked a measure of energy flow in their studies. This would not be contradicted by the wide range of previously noted conversion ratios (Table 11.1), since the latter would reflect experiments in which foodstuffs were available in excess. In any event, although equivocal, the results of Taranov represent a first step towards an experimental solution to the partitioning of foodstuffs in the production of wax.

The experiments and observations during the period 1840–1940 principally suffered from a failure to measure the separate costs of colony maintenance on the one hand, and the production of wax on the other. Nonetheless, this early experimentation has borne much fruit in the recognition of some of the factors that impinge upon the biology of wax, even if the credits and debits for wax production cannot be balanced. The two major approaches used were those in the tradition of Dumas and Edwards (1843) and von Berlepsch (1873—cited from Taranov 1959), who worked, essentially in vitro, with confined colonies of bees and on the other hand those of Gundelach (1842) and Dönhoff (1854) whose in vivo bees were

allowed to forage away from the nest. Both approaches have their obvious advantages and disadvantages and both continue to be used today. They provide different kinds of information for the development of a general concept of the flow of energy in relation to wax production, which has gained momentum in the past two decades.

Weiss (1965) was the first to attempt a separation of the direct costs of wax production from the other combined activities of a colony under apiary conditions. He performed a series of three experiments on 'nigra' bees (an old local German term for *A. m. mellifera*), kept in field cages during summer. In the first trial he compared two colonies, made by division, each of about 1000 g bees and headed by young mated queens. One colony was given starting strips of foundation, the other fully drawn, but empty comb. For 15 days the bees were allowed to fly in the cage to a feeding site supplied with a 50 % sucrose (w/w) solution. At the beginning of the experiment the youngest bees in the colony were 3 days old. Weiss (1965) measured the amount of sugar consumed by the two colonies and the amount of wax produced by the colony that had been given starting strips of wax foundation. The colony that had been given drawn combs did not construct any additional comb.

Like Dönhoff (1861) before him, Weiss (1965) calculated sugar/wax ratios as the amount of sugar actually needed for wax production, based on the amount of sugar consumed by the experimental colony in excess of that consumed by the control. The analytical refinement made by Weiss (1965) was to compensate for the concentration of the sugar that the building bees had stored in their newly built combs, so that a more accurate measure of sugar consumption could be calculated. In this case, the experimental colony produced 105 g of wax and consumed 1585 g of sugar solution; the controls constructed no combs but consumed 1235 g of sugar. The excess 350 g of sugar divided by 105 g of wax gave a ratio of 3.3:1. In a second experiment using two different colonies (which probably had the same age composition as those in the first experiment), Weiss (1965) obtained a ratio of 3.5:1 over 16 days. These bees were then observed for another 8 days (at the beginning of this second period the youngest bees would have been 18 days old and past their normal wax-secreting prime), and the sugar/wax ratio for this period came to 11.4:1 after his original data were adjusted for production per unit time/day.

The same basic experiment was repeated a third time using Carniolan bees over a longer sampling period. At the outset the youngest bees would have been at least 2 days old; at the end of the experiment they would have been 22 days old and hence past their wax-secreting prime. The results of this experiment are shown in Table 11.2. Even though the relative composition of different age classes is not known, it is tempting to conclude that as the bees grew older the sugar/wax ratio increased; however, Weiss did not adjust for the attrition of bees in these experiments. The problem of attrition was clarified to some extent in a fourth experiment at Erlangen during autumn.

Weiss (1965) gave starting strips of beeswax foundation to five queenright colonies (the experimental group) while another five colonies (the control group) were given fully drawn but empty combs. Portions of the large body of data

Table 11.2 Sugar/wax ratios obtained from caged bees during summer (Weiss 1965)

Experimental period	Sugar consumed (mg/g bees)		Excess consumption (mg/g bees)	Wax (mg/g bees)	Sugar/wax ratio
	Building bees	Control bees			
1. 4 days	239	133	106	20.5	5.2:1
2. 4 days	420	109	311	21	14.8:1
3. 6 days	575	110	465	28.7	16.2:1
4. 4 days	487	113	374	24.7	15.1:1
5. 4 days	587	235	352	25.6	13.8:1

Table 11.3 Sugar/wax ratios in a fall experiment adjusted by attrition, Colony 1 is the same throughout all periods and is matched to the same control colony (Weiss 1965)

Experimental period	Colony number	Sugar consumed (mg/g bees)		Excess consumption (mg/g bees)	Wax (mg/g bees)	Sugar/wax ratio
		Building bees	Control bees			
10 days	1	471	279	192	54.4	3.5:1
	2	736	307	429	611.4	6.4:1
	3	846	296	434	81.9	5.3:1
	4	1031	412	619	86.3	11.2:1
	5	979	438	541	811.3	6.2:1
11 days	1	710	212	498	50.0	10.0:1
	2	1032	269	763	79.3	9.6:1
	3	1195	327	786	93.2	8.4:1
	4	1273	409	864	811.7	9.9:1
	5	1347	401	946	89.4	10.6:1
11 days	1	755	304	451	50.1	9.0:1
	2	1147	370	777	82.6	9.4:1
	3	1133	421	523	102.0	5.1:1
	4	1797	610	1185	89.5	13.2:1
	5	1675	578	1097	85.9	12.8:1

emanating from these experiments are important enough to reproduce in modified form (Table 11.3), because they show the extent of natural variation that can be expected of different colonies. These results also show that sugar consumption per gram of bees is higher in smaller than in larger colonies. Yet two major imponderable factors remain in the cost equation: (1) the relative importance of age structure in wax production; and (2) the problems of heat production as related to age, colony size and the synthesis of wax itself. Among other things, Weiss (1965) suggested that comb construction produces heat, and with it, greater activity in the colony. How should variables like conversion efficiency and foraging time, age structure and colony size, to name a few, should be considered?

11.3 Measures of Conversion Efficiency

Conversion efficiency will obviously depend on a host of factors, among them genetic background (e.g. the subspecies under investigation). This point was brought home very clearly in the comb production studies by Skowronek (1976). He compared four small colonies (about 2000 bees each) of each of three subspecies over three seasons. Pooling his data, we find that the amount of wax produced varied with race: the Caucasians averaged 41.4 mg/bee, the Carniolans 32.5 mg/bee and the native Polish bees 29.8 mg/bee; resulting in combs of 9.8 g of wax per dm^{-2}, 9.7 g dm^{-2} and 8.8 g dm^{-2} respectively. Over the 3 years the Caucasians had produced more wax per bee and had constructed heavier combs than did the other two strains. Similarly, in caged experiments, Jay and Jay (1983) found that American bees of European origin, produced just over twice the amount of wax as did African honeybees.

Combining the balance sheet method of Dumas and Edwards (1843) with the sophisticated instrumentation now available for measuring oxygen consumption and monitoring temperature, Hepburn et al. (1984) studied the relationships between wax and heat production, sugar consumption and metabolic rate, and age and in small queenright colonies (500 bees) of the African honeybee, *A. m. scutellata* (at that stage still called *A. m. adansonii,* only later work identified the populations in northern South Africa as *A. m. scutellata*, cf. Hepburn and Radloff 1998). Their colonies were made up of newly emerged bees from brood frames of several different colonies and which were combined at random to achieve a balanced genetic background to control for potential variability. In all the colonies the bees began the experiment when they were less than 1 day old, and all the variables mentioned were measured, including the daily rate of attrition over a 21 day period. The rate of sugar consumption (corrected for attrition) and sugar stored in the nascent combs, as well as the rate of comb construction, were calculated on a per bee basis at 3-day intervals as shown in Fig. 11.3.

The consumption of sugar increased over the first 12 days and then levelled off, even though the colony size had decreased. Similarly, the metabolic rates of the colonies were found to be parallel to that of sugar consumption. The core temperature of each of the colonies was also measured on an hourly basis over the 21 day period. Even though ambient temperature was kept constant in the environmental chamber, an initial and erratic core temperature was recorded for the first week. This was followed by the development of an extremely regular oscillation in core temperature with a morning low of about 30 °C and an evening high of about 32 °C (these details are discussed in Nijland and Hepburn 1985).

The real metabolic rate, averaged over time for bees of different ages, is shown on the left ordinate of Fig. 11.4 from which it is apparent that a plateau is reached in bees of about 12 days old. The same figure includes an adjusted metabolic rate as a function of bee age on the right ordinate. Since oscillations in metabolic rate did not occur in 3-day-old bees (nor did such bees secrete wax), the metabolic rate measured for these bees was taken as an approximate basal value for subsequently

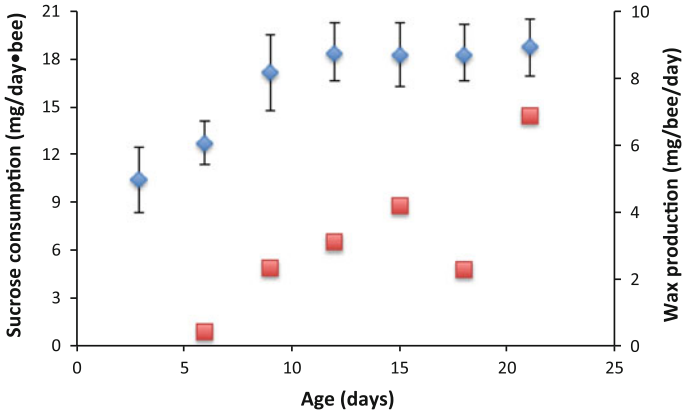

Fig. 11.3 Mean sucrose consumption and standard deviations are shown (*blue diamonds*) and the wax production (*red squares*) of small, queenright colonies of African honeybees (Hepburn et al. 1984)

estimating the cost of wax production. This is not to imply that all the energy expenditure above the 'basal' rate was diverted into wax production as such, because some expenditure would have been associated with the production of cluster heat in those bees more than 6 days old. The use of values of metabolic rate from 3-day-old bees thus provides only a partial compensation for energy expenditure in calculating a more exact energy budget for wax production.

Wax production was assessed as the wax which the bees had constructed as combs, as well as those scales which had fallen to the bottoms of the hives. The total wax production for five colonies was determined at the end of the experiment, on the 21st day; single values for the rates of production were obtained at 3-day intervals when five parallel colonies were killed. The total wax production per colony on a given day was the wax produced per bee, corrected for colony size. The value given for wax production per bee of a given age is absolute and independent of the prevailing size on a particular day. The metabolic cost of wax production per bee between the ages of 9 and 21 days was estimated to be about 6 ± 1 mW/g body mass for each milligram of beeswax produced and worked into comb; or in bee terms, about 70 μg of wax was produced per 420 μW of bee labour. These figures point to the interrelationships between metabolic rate, sugar consumption and wax production. The correlation coefficient between wax production and adjusted metabolic rate was 0.93 ($P < 0.005$); that between wax production and sugar consumption was also 0.93 ($P < 0.005$). Finally, the correlation between the adjusted metabolic rate and sugar consumption was 0.89 ($P < 0.007$). These relationships are shown in Figs. 11.4 and 11.5.

Just as the ability to thermoregulate develops with age, so does the ability of bees to significantly raise their metabolic rate change with age (Fig. 11.5—Allen 1959). Fine control over metabolic rate and the ability to thermoregulate go hand-in-hand in a mutually interdependent way. Because very young bees, 0 to 3 days

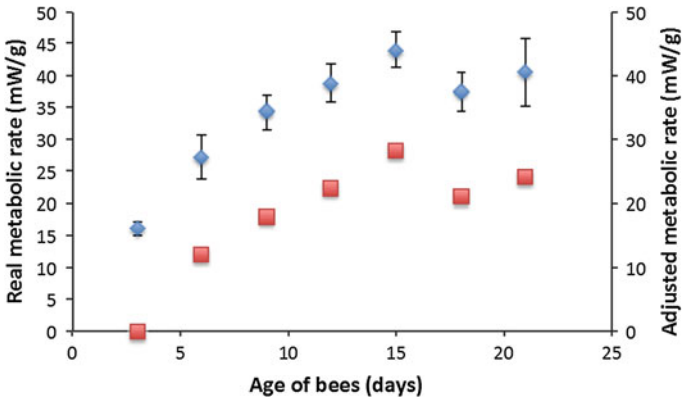

Fig. 11.4 The metabolic rates of African worker bees of different ages. The *blue diamonds* related to the *left* ordinate show the average mean real metabolic rate; *right* ordinate (*red squares*) show an adjusted metabolic rate used to calculate the cost of wax production (Hepburn et al. 1984)

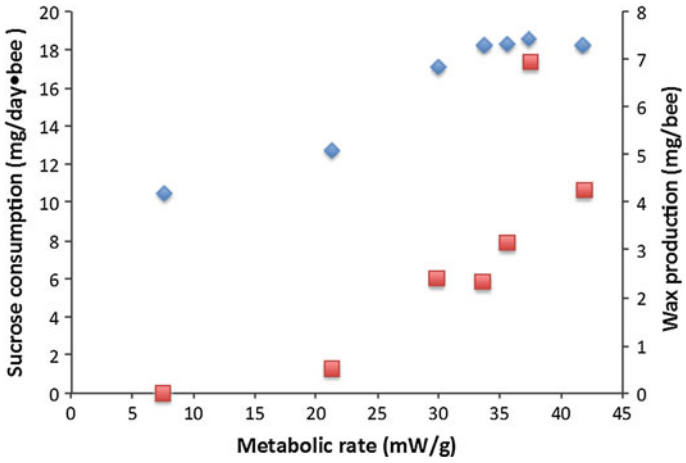

Fig. 11.5 Sucrose consumption (*blue diamonds*) and wax production (*red squares*) for queenright African bees as a function of metabolic rate (Hepburn et al. 1984)

old, have a reduced metabolic rate compared to older bees, they show a steady and unchanging cluster core temperature and do not secrete wax. The metabolic rate of these young bees was taken as basal, and used to approximate maintenance costs in older wax-producing bees. This simplification does not separate the cost of generating heat vis-à-vis production and comb-building per se. However, it is a telling point that the minimal level to which the metabolic rate fell in the older bees at night, was very similar to that of the younger bees which did not secrete wax (Fig. 11.6). It is highly suggestive, but has not as yet been shown, that the elevated temperature somehow facilitates, or is necessary, for wax secretion.

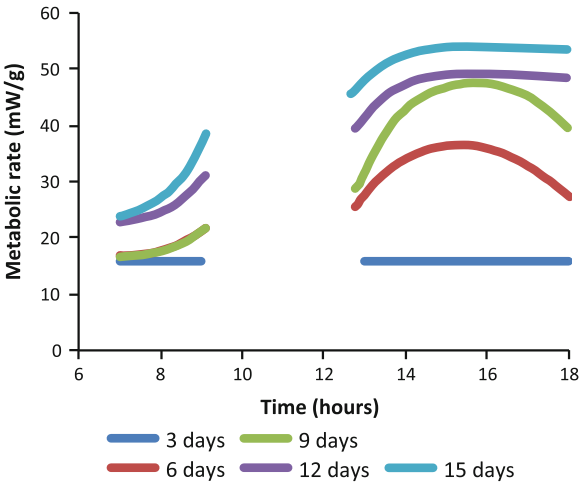

Fig. 11.6 The metabolic rate of queenright clusters of African bees of various ages in the course of a day. The gap indicates the absence of readings (Hepburn et al. 1984)

In trying to estimate the cost of wax production, many other considerations enter the equation. The progression of development in the workers that secrete wax and build comb had been previously observed by Rösch (1927), but the division of labour associated with age can easily be modified by manipulating the age structure of a honeybee colony; workers precluded from taking up a variety of activities, such as foraging, quickly change to other duties (Rösch 1925, 1930; Lindauer 1952). Under the confined conditions of the experiment of Hepburn et al. (1984), in which no foraging was allowed nor was there brood to tend, the metabolic expenditures were solely devoted to self-maintenance, temperature regulation, wax secretion and comb-building.

Among the minor factors affecting wax production is the provision of starting sheets of beeswax foundation. One small strip was attached to the top bar of a frame to define the locality of the cluster with respect to the thermocouple (Hepburn et al. 1984). In consequence, it is possible that the bees produced marginally less wax than they might otherwise have done, because, as Gillete (1900) discovered and Skowronek (1973) confirmed, bees produce marginally less wax when supplied with beeswax foundation than when not. The provision of wax starting strips represents a possible source of systematic error on the conservative side.

A major variable in the biology of wax is the relationship between production rate and colony size, established by Taranov (1959). A regression analysis of his original data showed that the amount of wax produced was directly correlated with the number of young wax-producing bees present in the colony, where the mass of bees ranged from between 0.5 kg (about 5000 bees) to 2.5 kg (about 25,000 bees). In his experiments wax was produced at a daily rate of 3 mg/bee. Extrapolation of the regression curve (for which the correlation coefficient for a straight line was 0.97) back to the size of the small colonies (0.35 kg, or 500 bees) used in the experiments of Hepburn et al. (1984), gave a predicted yield of about 150 mg of wax per day for the relationship to hold. Using 15 days of production time

Hepburn et al. (1984) obtained an experimental value of 3.2 mg per bee per day after adjustments were made for the changes in colony size due to death. The daily production rate of wax was calculated to be 141 mg per colony per day, which was within 5 % of the value expected from the analysis of Taranov's data.

Clearly a full analysis of the cost of wax production in a honeybee colony is exceedingly complex, and is made all the more difficult in the absence of measured rates of production for individual wax-secreting bees. Discounting the many variables which have been excluded by the form of these experiments, the observable differences in the amount and cost of wax produced varied greatly with the changing age structure of the colony. However, a general trend did emerge from the experiments; wax production appears to be a process which, for the honeybee colony, is akin to commercial amortisation. Within defined limits, it becomes cheaper for the bees to produce wax and to build comb as they become older. This trade-off or cost calculation comes into play on the individual level and also on the colony level.

The Asian dwarf honeybees, *Apis florea* and *A. andreniformis*, are the only species which salvage their old nest wax after absconding or migrating to a new nest site, thereby recycling the old wax (Hepburn et al. 2011). Although the recovered wax is of high energetic value, it is not the actual energy of the wax which makes it worth recovering, but rather the fuel costs to cover the distance by the workers to fetch it (Pirk et al. 2011). It is not the value of the resource but the time to recover it that is traded against the foraging time for nectar to replace the wax, explaining why this behaviour is only observed in *Apis florea*, (we do not know what *A. andreniformis* does), if the absconding range is within the foraging range of the new nest site, whereas in *Apis mellifera,* for example the absconding range is more than 6 km and the mean foraging range around 1 km (Hepburn et al. 2011; Pirk et al. 2011).

Colonies of *Apis florea*, which only abscond a short distance, and usually return to salvage old nest wax; but those colonies, and all other honeybee species, which go considerably further, do not. Wax salvage would clearly be counter-productive unless the energy input/energy yield threshold was profitable. There are two possible trade-offs in this scenario, the trade-off between the energy expended to recover the wax (recovering hypothesis), as against that of replacing the wax by new secretions (replacing hypothesis). In order to compare the two hypotheses, the fuel costs involved in salvaging wax on one return trip, the average flower handling time, flight time and relative values for substituting the salvaged wax with nectar were calculated. Moreover, the energy value of the wax was determined. Net energy gains for salvaged wax were calculated. The energy value of the salvaged wax was 42.7 J/mg, thus too high to be the limiting factor since salvaging costs are only 642.76 mJ/mg (recovering hypothesis). The recovery costs (642.76 mJ/mg) only fall below the replacement costs for absconding distances below 115 m, thus supporting the replacing hypothesis. This energetic trade-off between replacing and recycling, plus the limited absconding range of *A. florea* may explain why *A. florea* is probably the only honeybee species known to salvage wax. It parsimoniously explains the underlying reasons why *A. florea* only salvages wax from the old nest if the new nesting site is less than 100–200 m away-energetically, it pays off to recycle.

11.4 Temperature and Wax Production

While honeybees thermoregulate in the absence of a nest, it is seems that areas in which workers handle wax have higher temperatures (Pirk et al. 2004) suggesting that they regulate the heat to facilitate wax manipulation. Although colonies did not show any temperature preference for settling in the warmer section of an experimental nest cavity (Taber and Owens 1971), they are able to detect and distinguish small temperature gradients (Heran 1952; Basile et al. 2008). How wax synthesis and comb-building are constrained by thermal conditions is not well understood. There is only indirect evidence that bees cannot or will not sustain the costs of heat and comb production when both are very high. Both wax secretion and construction rapidly decline in autumn and virtually cease during winter. The onset of comb production in spring is well correlated with 11 °C for temperate honeybees and it has been suggested that sustained comb-building in practical apiculture occurs around 16 °C (Brünner 1905). That the production of comb apparently requires a minimum environmental temperature must be considered in juxtaposition to a regulated nest temperature. It has often been claimed (Philipp 1930; Büdel 1948; Weiss 1965), but never shown, that a nest temperature of 35 °C is essential for wax secretion and comb-building.

Indeed, Darchen (1962) recorded a range of temperatures around festoons varying from 30 to 34 °C, while Hepburn et al. (1984) recorded a maximum of just below 33 °C in the core temperatures of clusters of building bees. It is, simply, not yet possible to adequately assess the relationship of wax synthesis and comb construction to the thermal conditions of a colony's nest, much less how the microclimate of the nest is related to environmental conditions.

References

Allen MD (1959) Respiration rates of worker honeybees of different ages at different temperatures. J Exp Biol 36:92–101

Basile R, Pirk CWW, Tautz J (2008) Trophallactic activities in the honeybee brood nest—heaters get supplied with high performance fuel. Zoology 111:433–441

Büdel A (1948) Der Wasserdampfhaushalt im Bienenstock. Z Vergl Physiol 31:249–273

Darchen R (1962) Observation directe du developpement d'un rayon de cire. Le role des chaines d'abeilles. Insectes Soc 9:103–120

De Layens G (1887) New experiments to determine the proportion of honey used by bees in the production of wax. Glean Bee Cult 15:171–172

Dönhoff E (1854) Über den Übergang der Wachsblättchen in Wachslümpchen. Schweizerische Bienenzeitung 15:279

Dumas JB, Edwards HM (1843) Note sur la production de la cire des abeilles. Ann Sci Nat Paris 20:1–8

Dżieron J (1861) Rationelle Bienenzucht oder Theorie und Praxis. Falch'sche Buchruckerei, Brieg

Florea V, Malaiu A (1961) Research concerning wax production. Lucr Stiin Stat Cent Seruri Apic 3:201–210 (in Rumanian)

Galton D (1971) Beeswax as an import in medieval England. Bee Res Assoc Bee Wld 52:68–74
Gundelach FW (1842) Die Naturgeschichte der Honigbienen, durch langjährige Beobachtungen ermittelt. JJ Bohné Cassel, Kassel
Hasty EE (1886) The wax question. How many pounds of honey does a pound of wax actually cost us. Glean bee Cult 14:641–642
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer Verlag, Berlin
Hepburn HR, Hugo JJ, Mitchell D, Nijland MJM, Scrimgeour AG (1984) On the energetic costs of wax production by the African honeybee, *Apis mellifera adansonii* (= *scutellata*). S Afr J Sci 80:363–369
Hepburn HR, Radloff SE (1998) Honeybees of Africa. Springer, Berlin
Hepburn HR, Pirk CWW, Radloff SE (2011) Energetic aspects of flight. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 293–312
Heran H (1952) Untersuchungen Über den Temperatursinn der Honigbiene (*Apis mellifica*) unter besonderer BerÅcksichtigung der Wahrnehmung strahlender Worme. J Comp Physiol A 34:179–206
Holmes FL (1985) Lavoisier and the chemistry of life. University of Wisconsin Press, Madison
Huber F (1814) Nouvelles observations sur les abeilles [English translation, 1926]. Dadant, Hamilton
Jay SC, Jay DH (1983) Wax production by caged worker honeybees of European and tropical African origin. J Apic Res 22:229–231
Keys J (1814) A treatise on the breeding and management of bees to the greatest advantage. Lockington and Allen, London
Nijland MJM, Hepbrn HR (1985) Ontogeny of a circadian rhythm in the cluster temperature of honeybees. S Afr J Sci 81:100–101
Philipp PW (1930) Die Bautemperatur des Schwarms. Deutsche Bienenzeitung 48:166–171
Pirk CWW, Crous K, Duangphakdee O, Radloff SE, Hepburn HR (2011) Economics of comb wax salvage by the red dwarf honeybee, *Apis florea*. J Comp Physiol B 181:353–359. doi:10.1007/s00360-010-0530-6
Pirk CWW, Hepburn HR, Radloff SE, Tautz J (2004) Honeybee combs: construction through a liquid equilibrium process? Naturwissenschaften 91:350–353
Ransome HM (1937) The sacred bee in ancient times and folklore. Allen & Unwin, London
Rösch GA (1927) Über die Bautätigkeit in Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 6:265–298
Rösch GA (1930) Untersuchungen über die Arbeitsteilung im Bienenstaat. 2. Die Tätigkeiten
Rosov SA (1944) Food consumption by bees. Bee Wld 25:94–95
Skowronek W (1973) Development of wax glands in bees of different races. Pszczei Zesz Nauk 17:1-9 (in Polish)
Taber S, Owens CD (1970) Colony founding and initial nest design of honey bees, *Apis mellifera* L. Anim Behav 18:625–632. doi:10.1016/0003-3472(70)90005-9
Taber S, Owens CD (1971) Influence of temperature on location of clustering and comb building of honey bees. Ann Ent Soc Am 64:960–961
Taranov GF (1959) The production of wax in the honeybee colony. Bee Wld 40:113–121
Tokuda Y (1955) Experimental studies on beeswax production by the honeybee colonies. I. Beeswax yield by the honeybee colonies under sugar syrup feeding. Bull Nat Inst Agric Sci Series G (Animal Husbandry) 11:110–129
Vernon FG (1979) Beekeeping in 1269–1270 at Beaulieu Abbey in England. Bee Wld 60:170–175
Von Berlepsch B (1873) Die Biene und ihre Zucht mit beweglichen Waben. Mannheim
Weiss K (1965) Über den Zuckerverbrauch und die Beanspruchung der Bienen bei der Wachserzeugung. Zeit Bienenforsch 8:106–124
Whitcomb W (1946) Feeding bees for comb production. Glean Bee Cult 74:198–202

Chapter 12
Construction of Cells

Abstract Honeybee nests result from interactions among numerous bees performing different comb-building operations ranging from construction of new cells, shaving and thickening edges of cells, capping brood, and capping removals. The single major construction is comb-building. At the onset of comb-building, nascent cells are circular but soon after acquire a more crystalline structure; regular hexagons appear that are products of the physical properties of wax, equal pressure from adjacent cells, and the flow of the visco-elastic wax. The structure and formation of cells result from wax being a thermoplastic material while, the hexagonal structure is the result of the wax reaching a liquid equilibrium, changing from a crystalline state to an amorphous state at nest temperatures. The building 'instincts' of bees are labile and are supported by several possible subroutines in their total building programme; but the rather wide tolerances seen among cells show that bees cannot make precise measurements. In commercial beeswax foundation, both the cell base and the hexagonal rims of the cells have a pronounced taper to them. However, the natural outermost limits of cell patterns, and not the cell base, determine what pattern bees follow in cell construction. The antennae may play a role in maintaining tolerances on cell thickness because milling of the cell wall is controlled by individual workers at single sites, and antennectomy significantly increases wall thickness. The shape of the honeybee cell does not have its celebrated regularity; its economy is a teleological myth. The entire history of the honeybee cell in natural history, geometry and philosophy is the story of centuries-old misconceptions.

12.1 Introduction

Honeybee nests are the result of the interactions and interplay among numerous individuals performing various building and construction operations. The behavioural plasticity among individuals, paired with the various aspects of the construction of combs, makes for a fascinating and complex field of study. The end

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_12,

product, the comb, is used for storing resources, rearing the next generation of brood, and chemical and physical communication, which ensures the social cohesion of the honeybee colony.

12.2 Manipulation of Wax Scales

The first record on the handling of wax scales after they have been secreted is that of the famous 17th century work, *The feminine monarchie,* by Butler (1609) who wrote: "You may behold them (bees) working on the edges of their combs, and having blown their liquid and soft wax out of their mouths fasten and fashion it with their fanges and forefeet". In the 19th century different findings and observations to Butler's interpretations were proposed, particularly by Huber (1814), who noted that a worker removes a scale from the wax mirror with a hindleg and transfers it with a foreleg to its mouth. The scale is then thoroughly chewed and a frothy liquid added. He then mistakenly attributed the means for scale removal from the abdomen by the pollen press (the 'wax-pliers' of the old literature). Dönhoff (1854) disputed this, laid a counter claim for the basitarsal setae of the planta, but otherwise confirmed Huber's basic observations.

It is extremely difficult to decide how to apportion credit when it concerns fine details, even when careful comparisons are made of the different authors' works. While Huber (1814), admittedly, wrote the first extensive description of how bees handle wax scales, the most comprehensive account is by Casteel (1912), who meticulously followed the movements of bees in an observation hive with a binocular microscope. To briefly summarize, Casteel reported that wax scales are usually removed from the wax mirrors, passing them under the abdomen to the forelegs and finally to the mandibles, where they are chewed and then added to the comb. Lineburg (1924) extended Huber's observations on the mandibulation of wax, and confirmed the detailed observations of Casteel (1912). Rösch (1927) and Gwin (1936) confirmed both the origin and the mandibulation of wax scales.

12.3 Comb Operations

Honeybee nests are the result of numerous kinds of building operations performed by many individual bees; they range from the construction of new comb (Fig. 12.1), to cell-shaving and cell edge-thickening, capping brood and the removal of cappings when adults eclose (Fig. 12.2).

Meyer (1952) and Meyer and Ulrich (1952) published concise accounts of comb-building for which all constructions in the nest were divided into either major or minor building operations. There is one major construction, the overall building of combs which is the foundation of a honeybee colony (Fig. 12.1), enabling them to communicate, store food and raise brood. There are several

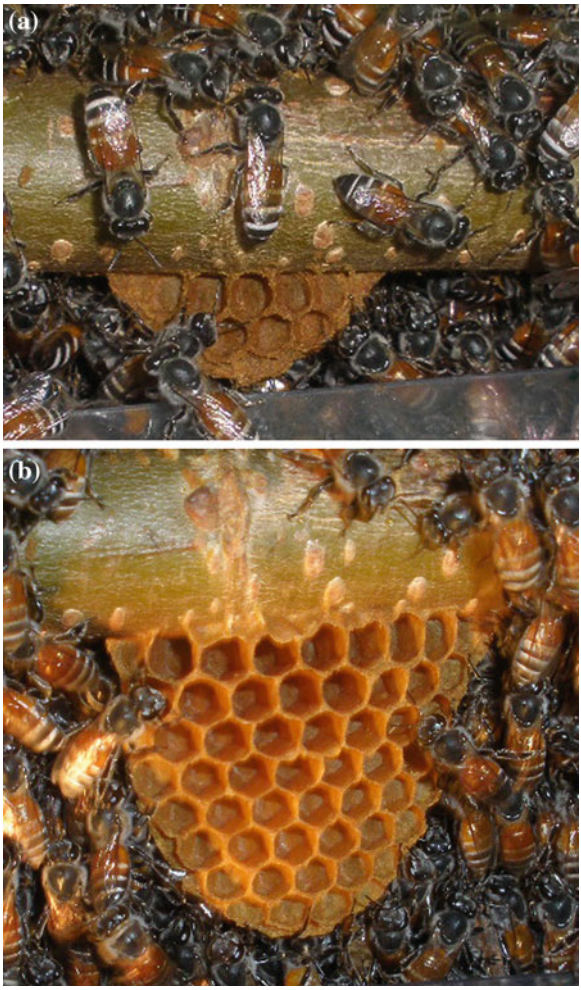

Fig. 12.1 Comb construction by *Apis florea* **a** 6 h; and **b** 12 h after settling on a twig

minor operations which tend to differ depending on whether they occur in areas of the brood comb or honeycomb (Figs. 12.2 and 12.3). Once the major task of constructing brood combs is completed, the cyclical rearing of multiple generations of brood occurs. Part of the cycle is when the larval cells are capped on the threshold of metamorphosis; the wax used for sealing these cells is generally recycled old wax, and not newly secreted wax. By using dyed waxes, Meyer and Ulrich (1952) reported that more than 60 % of the wax used to cap brood cells was salvaged from previously used brood cell cappings following the emergence of young adult bees. These minor constructions, based on recycling cappings wax (Lineburg 1923a, b), are generally performed by young bees, 3–9 days old, most of whom have not yet reached the peak of wax secretion (Rösch 1927). These nurse bees both feed the larvae and cap them in due course. Workers are able to

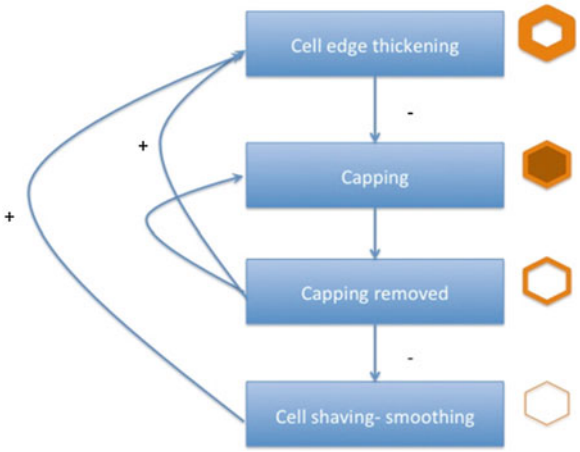

Fig. 12.2 Tasks related to minor comb-building operations in an *A. mellifera* colony proposed by Meyer and Ulrich (1952) and confirmed by Lau (1959). The *plus* and *minus symbols* next to the *arrows* indicate how the size of the cell rim changes

identify the age of the larvae mainly based on pheromones emitted by the brood (cf. Chap. 5).

After the adult bees emerge, workers smooth and shave the walls of the empty cells and collect the remains of the cappings. By removing the wax cappings, they also removed part of the silken cocoons which are attached to the inside of the cappings. This material is either used as cappings for larvae in close proximity, which are ready to pupate, or gets attached to the rims of nearby cells; however, the addition of this material results in the thickening of the edges of cells, which then has to be thinned by workers (Fig. 12.2).

The stored material on the rim can either be used to seal the cell if necessary, or may serve simply as a depot for the storage of capping material. Smith (1959) observed the capping activity of a single worker for an hour, and his results (Fig. 12.3) confirm that capping can be done either by a single or several bees (Meyer and Ulrich 1952).

The operation of capping is not restricted to brood, but is also performed on the honeycombs. It seems intuitively obvious that different stimuli trigger the bees to do these jobs in these two different areas. However, since the behavioural patterns involved in capping or uncapping cells are effectively the same as illustrated in Fig. 12.2, one might wonder if the stimuli are similar. An appreciation of the order of magnitude of the so-called minor operations can be gained from Lineburg's (1923a) study on the turnover of cappings wax. Let's assume that, in a colony in a standard Langstroth hive, half the cells are filled with brood at any given time. That would translate into workers having to cover an area with wax of about 800 cm^2 (equal to the area of $1\frac{1}{3}$ of an an A4 sheet of paper) with wax on a 3-weekly basis, and they, or the newly emerged workers would have to remove the

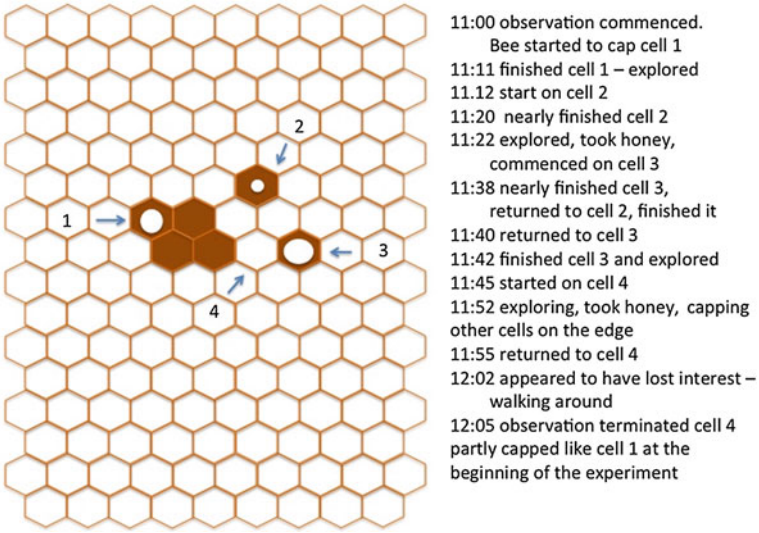

Fig. 12.3 Capping behaviour of an individual *A. mellifera* worker over the course of 1 h (Smith 1959)

same amount at the end of a brood cycle. Eight-hundred cm^2 converts to more than 10 g of wax, based on cappings of *A. m. scutellata*, or the production of nearly 1500 21-day old workers (Hepburn et al. 1984), which is built and removed every 3 weeks.

The huge turnover and shifting rate of wax is also evident given the fact that brood cell cappings in nests containing old and darkened combs are nearly as dark as the combs themselves. In the white combs of newly established swarms, the cappings are only slightly discoloured (Lineburg 1923a). The shifting of wax takes place within hours as was demonstrated by Darchen (1980) who showed that, by placing various radioactive labels within a colony and measuring their dispersal over 24 h the radioactivity spread to all the combs, although its intensity declined with increasing distance from the point of application of the tracer. Unfortunately, the passive transmission of label on the bodies of workers vis-à-vis real pieces of wax being moved could not be established.

12.4 Inception of the Nest

The processes that occur after a swarm settles at a new nest site are probably similar for cavity- and open-nesting *Apis* species when it comes to the inception of the comb or nest. One of the problems of studying comb-building has always been that of trying to see through a cluster of bees, often as much as 10 cm thick. Another problem with cavity-nesting species, like *A. cerana* or *A. koschevnikovi*, is

that nests in cavities are extremely difficult to observe. One can address the problem in two ways; either let the bees construct a bit of comb and remove it for recording, which is easier in open-nesting bees, like *Apis florea*; or force the bees to construct in a way that exposes their progress. Huber (1814) constructed an experimental design that forced the bees to build from the bottom upwards on a lath, a rare but natural form of building behaviour (Bone 1952).

When following the first approach, similar building activities were observed in Asian honeybees. Both dwarf honeybees, *A. florea* and *A. andreniformis,* usually have a single, exposed comb, typically situated on a single branch of a bush or tree, in a shady location (cf. Chap. 2). The structure of the comb has been described in some considerable detail (Akratanakul 1977; Mossadegh 1990; Phiancharoen et al. 2011). However, all of the published reports on comb structure in the dwarf honeybees were made on mature nests collected in nature or purchased at markets. In all the interpretations of dwarf bee comb structure, it is implicit that the comb is built top-down, continuously, in the vertical plane, a point not established by observations. Moreover building, using hexagonal cells, poses serious geometrical problems because it is not possible to encircle a regular cylinder, like a twig, with hexagons. Close examination of such combs revealed a combination of various other polygons (Phiancharoen et al. 2011), so that real solutions to the problem of comb geometry are yet to be determined. The actual inception of comb construction from scratch, and its subsequent development in real time, have not previously been reported and are described here for the first time.

In recently settled *A. florea* colonies comb-building probably commences almost immediately after landing on the twig and settling because a small row of about seven nascent cells were subtending the twig at 2 h (Fig. 12.4a). After 6 h the addition of a second lower row of cells appeared (Fig. 12.4b). It is evident that these nascent cells are not polygonal but virtually circular. After 9 h (Fig. 12.4c) there are four rows of nascent cells. The row at the base of the twig consists of truncated hexagons and 2 rows of crude hexagons. The third row is exactly the same as in the first row, circular burrows in the wax. Twelve hours (Fig. 12.4d) after settling the comb acquires a more crystalline structure as regular hexagons begin to form, which is most likely as a result of the wax flowing into shape (Pirk et al. 2004; Karihaloo et al. 2013), with equal pressure from adjacent cells to shape the forming hexagon in the centre of the developing comb (Bergman and Ishay 2007). As a rule, the first row of cells appears anomalously different from the hexagons of comb cells with which we are familiar. It generally consists of regular pentagons and circles; the site of support forms one side from which two vertical walls are suspended and then two oblique ones. Ordinarily, the growth of the comb then progresses at a faster downward than lateral rate, so the combs tend to be ellipsoid in the early stages of construction (Figs. 12.4, 12.5 and 12.6).

Huber's observations have been confirmed many times (Darwin 1856; Hubbe 1957; Lau 1959; Ulrich 1964; Darchen 1991). Figure 12.6 shows the development of an *A. florea* vertical, single comb nest over seventeen weeks once the swarm settled. By day 4 (b) the nest had already been partitioned into an area for honey (crown or top of comb), an underlying pollen layer, below which both capped and

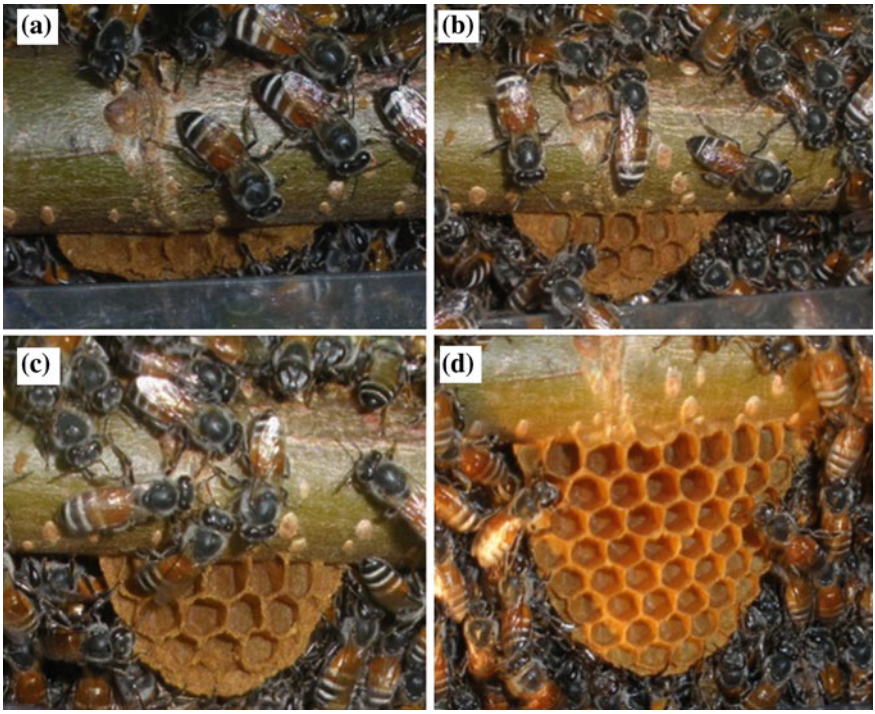

Fig. 12.4 Comb construction by *A. florea*: **a** 2 h after an *A. florea* swarm settled on a twig resulting in a single small row of about seven nascent cells below the twig; **b** 6 h; **c** 9 h; and **d** 12 h

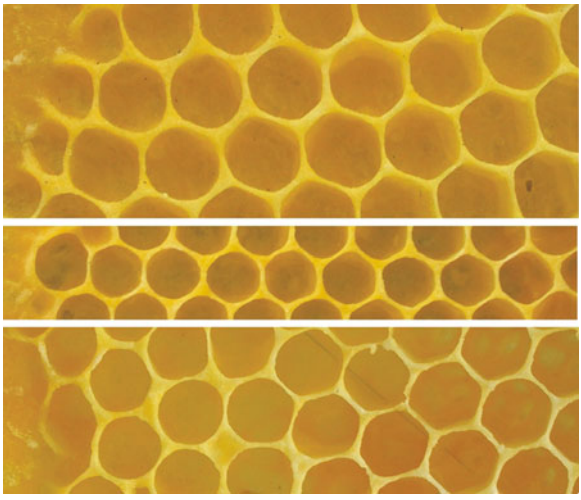

Fig. 12.5 Three samples of freshly constructed *A. mellifera* cells. The newest cell on the *left* (*round*) to the oldest one on the *right* (*hexagonal*) (Pirk et al. 2004)

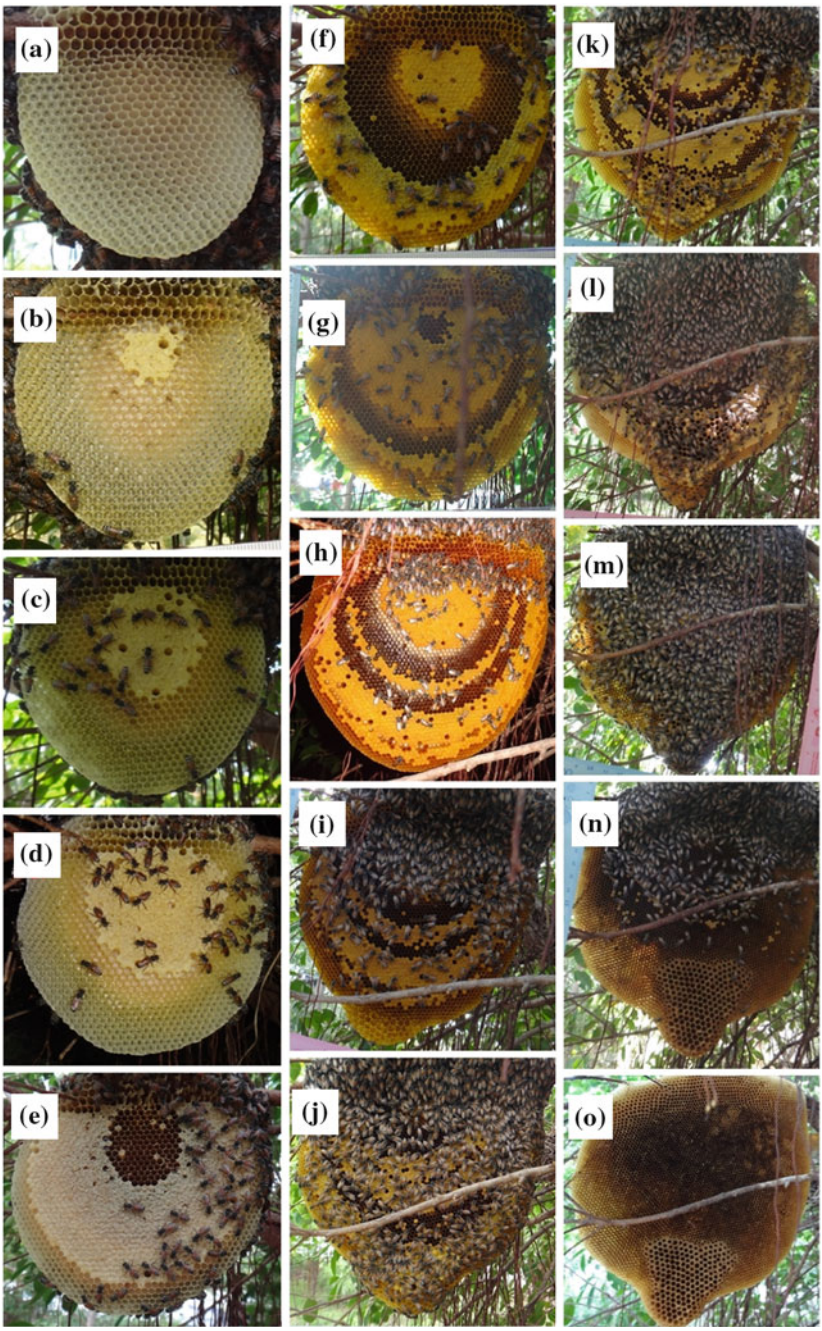

Fig. 12.6 Construction of an *A. florea* comb over 121 days. For details, see text. (Duangphakdee et al. 2013)

uncapped larval cells occurred. This basic pattern remained until the mature colony swarms some 4 months later. The sequence of photographs show: (a) on day 2, the darker wax honey crown was being developed above the brood area which contained eggs and larvae in a concentric pattern; (b) by day 4, a few brood cells had been capped with more eggs and larvae below, maintaining the concentric pattern; (c) on day 6, cell capping continued as did the expansion of the uncapped brood area; (d) by day 8, the concentric rings of capped and uncapped brood increased, workers were storing nectar in the crown; (e) on day 16, the first patch of brood emerged as adults and there was further extensive capping of brood cells (note that brood does not extend to the periphery of the comb); (f) on day 23, the empty cells of (e) contained capped brood from which the second generation of adults emerged, the cells in the surrounding area contained newly laid eggs, while the outer ring contained capped brood; (g–k) occurred sequentially between days 30 to 93, and the staggered distribution of concentric brood of various ages and generations are visible in each photograph, while drone cells were finally constructed by day 93; i) appearance of drone cells; (l) by day 100, drones emerged from their cells at the bottom of the comb; (m) on day 107, drones left the nest; (n) by day 114, there were no new eggs, no uncapped brood and only very few capped cells; (o) on day 121, the colony absconded (Duangphakdee et al. 2013).

The method of attachment of cells and the subsequent extension of the comb have been analysed in some detail for around 200 naturally drawn *A. mellifera* combs (Hubbe 1957); nevertheless the problems also persist in open-nesting species. Hubbe (1957) found that the irregular nature of cells may well extend from the first row downwards, for another five or six or sometimes more rows. Similarly, the bees may begin their work on a horizontal plane where even greater irregularity will be encountered. Eventually some regularity, or at least patches thereof, can be found in feral nests. The irregularities not only result from attaching comb to an irregular substrate, but also by including drone and worker cells and different orientations (Fig. 12.7).

Thompson (1930) analysed the orientation of cells in 267 pieces of comb with the following results: 123 combs contained cells in the horizontal mode, the cells were vertical in 131 others, one comb contained both, and 13 contained only oblique or tilted combs (Fig. 12.7). The problem is exacerbated by the fact that combs also contain drone cells which are larger hexagons than worker cells and therefore the transition of workers cells to drone cells has to be architecturally solved. This, in fact, can only be achieved with the addition of various non-hexagonal polygons (usually pentagons and heptagons—Fig. 12.8).

Not only does the type of the cell play a role in pattern formation but also in the queen-status; cells constructed by queenless bees are less regular than those constructed by queenright bees (Taber and Owens 1970). The different types of cells and variations in comb-building behaviour are a precursor for the introduction of dislocations in the geometry of combs. Alternatively, instead of accepting the dislocations, the following was noted by Darwin (1859): “it was really curious to note in cases of difficulty, as when two pieces of comb met at an angle how

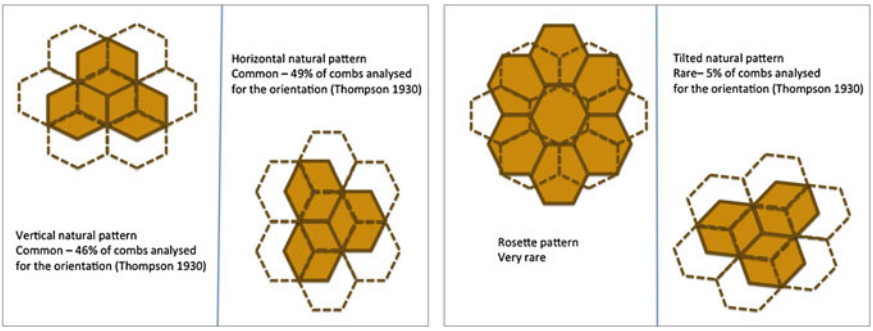

Fig. 12.7 Natural patterns of cells constructed by *A. mellifera.* The vertical and horizontal patterns dominate combs built without foundation, and occur with similar frequency (Thompson 1930)

Fig. 12.8 Transition of worker cells into larger drone cells of an *A. m. scutellata* comb

often the bees would entirely pull down and rebuild in different ways the same cell, sometimes returning to a shape which they had at first rejected." Therefore, when looking at the final comb many irregular cells (sometimes called 'transition cells'—Dadant 1946) may still be found, others are retouched and hidden from view in the final product which we see (Darchen 1954; Hepburn and Whiffler 1991).

The interpretation of the geometry of combs has quite a respectable history that dates from the 4th century BP with the writings of the Alexandrian, Pappus. He held that bees had a certain geometrical forethought by which the most economical container to be made of wax was, in fact, the hexagonal configuration. Mathematical arguments about the comb cell were later advanced by that giant of 17th century science, Kepler, and were also debated in the 18th century at the Royal Society of London by such notables as Maclaurin and Lhuiller, and in Paris by

Maraldi, Koenig, de Reaumur and Buffon. These mathematical discourses have been summarised by several authors over the years (Vogt 1911; Armbruster 1920; Thompson 1942; Meretz 1963).

Karl von Frisch (1974) emphasized the amazing level of precision in comb-building and that man could not undertake work of this nature without the use of specialised tools. Unveiling the underlying mechanism(s) of how bees are able to construct and measure with such apparent precision took place for centuries. However, Pirk et al. (2004) argued on theoretical grounds and provided hard experimental evidence that the structure of honeybee combs results from wax being a thermoplastic material, and the hexagonal structure is as a result of the wax reaching a liquid equilibrium. Tautz (2008) provided support for the liquid equilibrium hypothesis based on the physical properties of beeswax. From a physical viewpoint beeswax is not a solid, but a fluid that changes from a crystalline state to an amorphous state at temperatures of 25–40 °C. Pirk et al. (2004) hypothesised that the round wax cells might naturally form hexagonal shapes due to the mechanical tension between adjacent cylindrical cells in the amorphous state, as subsequently confirmed by Karihaloo et al. (2013).

Honeybees form cells with their mandibles while palpating the comb surface constantly with their antennae. The mandibles are used with a left/right movement of the head or in a repeated movement of the head upwards into the neck, thereby sliding across the cell walls. All these observations have been previously described (Lau 1959; Martin and Lindauer 1966). Besides these points, Karihaloo et al. (2013) developed two scenarios of how comb patterns emerge, both of which support the idea that "the regular pattern of rounded hexagons is a result of the progressive fusion of the circular walls induced by the flow of the visco-elastic molten wax…" (Fig. 12.9). Both models partially or fully support the idea of a liquid equilibrium (Pirk et al. 2004) process being involved in the production of hexagonal cells (Fig. 12.9). Furthermore, hexagonal cells can also form if equal pressure is applied to the sides of a group of cells, the central cell then becomes hexagonal (Bergman and Ishay 2007). A similar phenomenon can be observed in basalt rocks which form the Giant's Causeway located in County Antrim on the northeast coast of Northern Ireland (Thompson 1942).

When all is said and done, we concur with Vogt (1911), who performed the most exhaustive analyses of comb cells; that the shapes of worker and drone cells are, more or less, regular, hexagonal prisms. Each is closed by three quadrangular rhombs, the obtuse angles of which form a truncated pyramid which is the usual floor of a naturally built cell. As Vogt put it: "When judgement had to be passed on the way bees build, the metaphysical idea of perfection confused the issue for the great man (Darwin), impartial observer as he was, just as it had done for the eighteenth century teleologists. The shape of the bee cell does not have its celebrated regularity; its economy is a teleological myth. The entire history of the bee cell in natural history in geometry and philosophy is the story of a 200-year old mistake!"

Put another way, Vogt's pronouncement, coupled with the results of Darwin's second comb experiment and the measurements of Hubbe (1957), all point to the

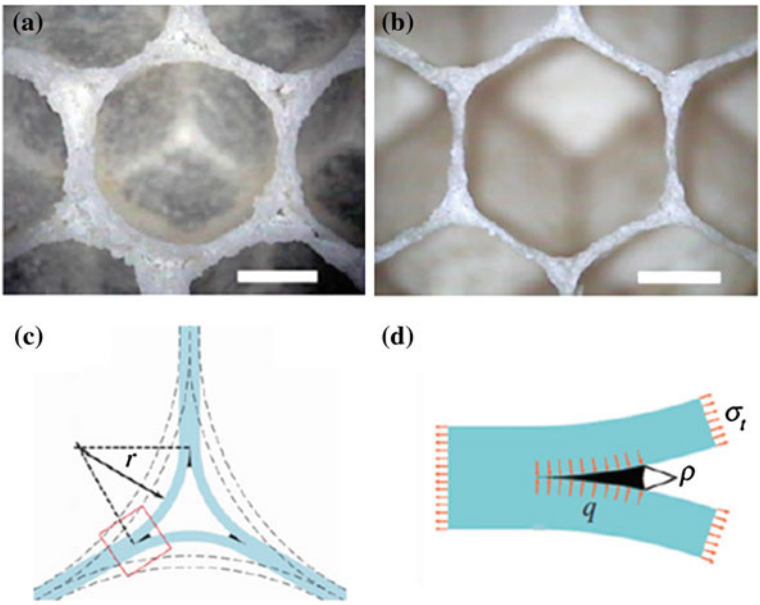

Fig. 12.9 The proposed mechanism for the transformation of a round cell to a hexagon in *A. m. ligustica* combs (Karihaloo et al. 2013)

concept of behavioural plasticity. Not only is the instinct of the bee labile, but it evokes the notion of several possible subroutines in the total building programme of bees, to use an apt analogy from Gould and Gould (1983). The experimentalist may wish to view things slightly differently. It could well be that the rather wide tolerances one observes in their constructions simply show that bees cannot make very precise measurements; they are victims of the limitations inherent in their own neurophysiology. The latter interpretation certainly sides with Vogt (1911), but rather than reach a conclusion, it is more interesting to consider what bees have done under various experimental circumstances.

12.5 Recognition of Cell Patterns

If one considers the cells of freely-built combs, it is apparent that they are not really as uniform as they tend to appear at first sight. Indeed, in one study of such combs, no two cells were found to be identical (Darchen 1956). Nonetheless, the size of worker cells fall within fairly narrow limits, with the average dimensions varying in different species and races (Vogt 1911; Alber 1953; Taber and Owens 1970; Hepburn 1983; Phiancharoen et al. 2011). Most of the cells that exceed two standard deviations of the mean actually occur in the basal few rows of cells from where the nest began.

Returning to Thompson's (1930) studies of the orientation of cells in three modes, a number of interesting questions emerge. For example, is there a genetic component to these different patterns? Do worker bees learn a cell type from whence they have come? Do workers learn, in the absence of cues, to work a particular pattern from those bees that come to a site with past experience? Oelsen and Rademacher (1979) considered these questions in an experimental way. In addition to vertical, horizontal, tilted (or oblique) cells, they also reported a rare form of a rosette pattern. All four of these kinds of cell patterns are shown in Fig. 12.7. On the assumption that newly emerged bees, deprived of their combs as reference cues, will demonstrate innate as opposed to learned behaviour, Oelsen and Rademacher (1979) reared bees in combs having vertical, horizontal or rosette cell patterns.

As the bees emerged they were collected and placed in a modified hive with space for the construction of three combs, to form small colonies of about 1000 bees. Each colony was given unembossed, pattern-free sheets of wax as sites to stimulate comb-building. The authors found that those bees bred from vertical cells constructed combs containing a mixture of vertical and oblique cells; bees reared from horizontal cells constructed a mixture of regular, vertical, oblique and horizontal cells; bees from rosette cells built a mixture containing all four patterns (Fig. 12.7).

To test whether bees learn to follow a particular pattern and thereby become behaviourally entrained to follow it, Oelsen and Rademacher (1979) caught newly emerged bees and again constituted them in small colonies, and allowed them to work in the absence of any bees that might have had prior experience in building a particular cell type. Each colony was provided with three forms of wax. In one case, a colony was given a frame with a full sheet of beeswax foundation embossed with the rosette pattern, one frame with a small strip of the same wax, and the other frame with a sheet of unembossed, pattern-free wax. Other colonies received the same permutations based on the vertical cell pattern. On recovery of the combs, the authors found that in each case the bees built true to the form of pattern with which they were supplied; however, when they worked the unembossed wax into combs, they constructed a mixture of vertical and horizontal cells.

Oelsen and Rademacher (1979) then asked whether the cell pattern in which a bee is reared affects her subsequent proclivities as to cell orientation in a comb-building situation. To test this, they formed four colonies of bees reared in the vertical mode, and four other colonies reared from the rosette pattern. The colonies were assigned wax sheets as follows: of the 'vertical' bees, one colony was given the vertical pattern, two were given the rosette, and one was given unembossed wax. The 'rosette' colonies of bees were allocated wax sheets in the same way. Each of the colonies that subsequently constructed combs did so according to the pattern given, regardless of the type from which they were reared. It so happened that none of the unembossed sheets were worked during the experiment. In an addendum to this experiment, some colonies of bees reared in vertical cells were given only small strips of the rosette pattern, and these bees built horizontal and oblique cells as well as a patch of the rosette type.

12.6 Assessment of Cell Size

Following Gontarski's (1935) experiments on how bees reacted to artificially enlarged cells, Hepburn (1983) analysed the tolerances in cell construction and the thresholds of acceptability for different cell sizes. He supplied colonies of the African honeybee, *A. m. scutellata*, with sheets of beeswax foundation manufactured in the laboratory of 170, 220, 275, 336, 390, 441, 462 and 522 cells/dm^2. In addition, commercially manufactured foundation of 476, 493 and 1022 cells/dm^2 were used. The foundation fashioned in the laboratory consisted of perfect hexagons with equilinear line segments, but the bottom of the cells were flat. In commercial foundation, the hexagons are very seldom equilinear and the cell bases consist of three rhombs. Six of the queenright *A. m. scutellata* colonies were tested on each of the different foundations. Each cell-type was tested using full foundation sheets given to the colonies nine times on a random basis. The resulting constructions (Figs. 12.10, 12.11 and 12.12) were divided into five different groups of building solutions.

170 cells/dm^2. The bees characteristically began building from the bottom of the frame upwards—the reverse of normal building. There was considerable dry-working of the foundation wax, but the bees did not destroy the hexagonal patterns as they so often do when reworking worker foundation into drone comb. The bees generally worked within the constraints of these large hexagons by filling them with rosettes of irregular 5- to 7-sided polygons (Fig. 12.10a). Building commenced on a cell base by drawing a line of wax from the centre point of one wall line segment to just short of the cell centre. The construction of this initial small cell determined the size of the adjacent cells within the large hexagon, and so on.

220 cells/dm^2. Although the cell bases in this foundation were smaller than those of 170 cells/dm^2 foundation, the bees were unable to provide a symmetrical solution either within, or above, the constraints of the given hexagon. Figure 12.10b shows that the original pattern had been almost totally destroyed, and the comb built on this now disrupted foundation consisted of only highly irregular cells.

275, 336 and 390 cells/dm^2. Here the construction solutions were uniformly the same. Each of the six corners forming the angles of the hexagons was used as the starting point for the construction of a new cell. As building progressed, a new, regular hexagonal pattern formed that was superimposed on, hence elevated above, the base pattern (Fig. 12.10c). In the finished comb all cells appear regular; however, there was a void in the centre, effectively a 'false' cell. The development of this false cell is shown in Fig. 12.10d from which it becomes apparent why the false cell cannot be easily detected in the finished comb.

The measurements of these new cells superimposed on the foundation cells are of considerable interest. Considering mid-wall to mid-wall diameters, it was found that these cells decreased in size in fixed proportion to the rate at which the foundation cell size decreased (Fig. 12.10). *A. m. scutellata* naturally drawn worker comb cells range in size from 4.37 to 5.39 mm, while those drawn on 275, 336 and 390 cells/dm^2 foundation were 5.15, 4.80 and 4.34 mm respectively. This last

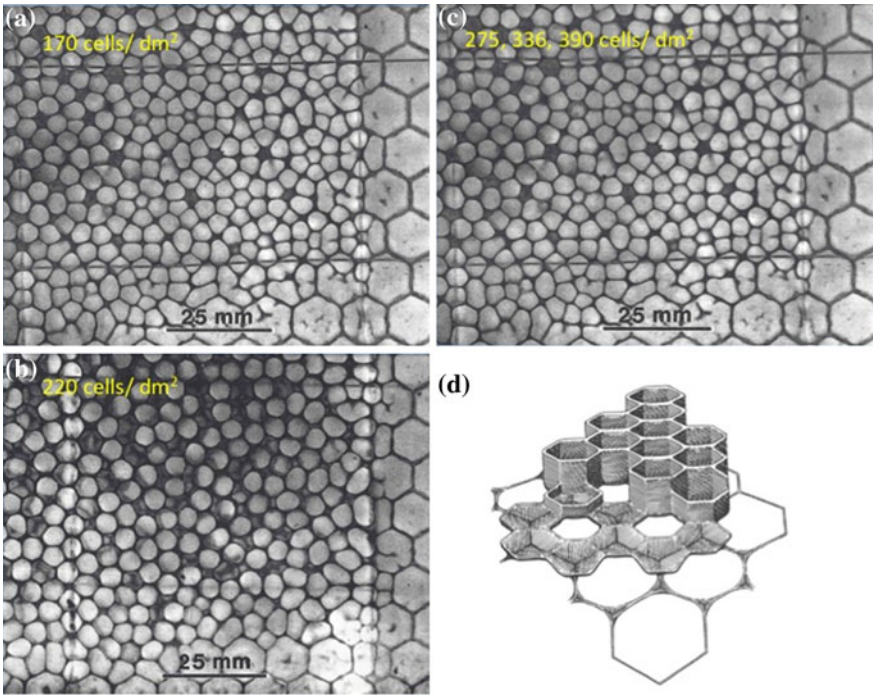

Fig. 12.10 Construction of cells by *A. m. scutellata* (formerly *A. m. adansonii*) on different sizes of beeswax foundation. **a** Is the result of 170 cells/dm^2; **b** 220 cells/dm^2; **c** 275, 336, 390 cells/dm^2; and **d** diagrammatic projection of a comb shows the origin of false cells (Hepburn 1983)

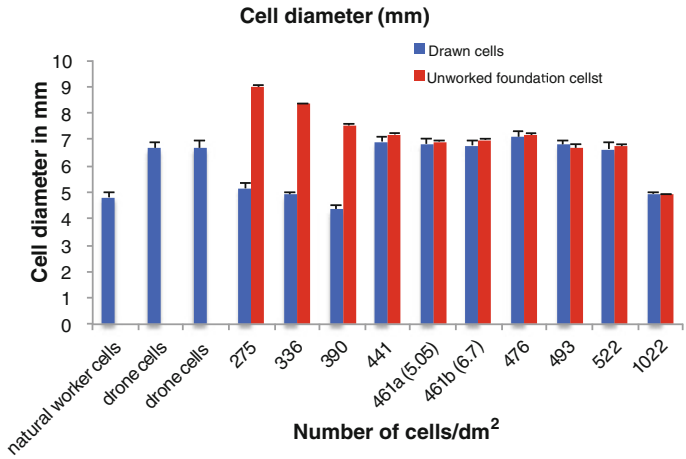

Fig. 12.11 The cell diameter of *A. m. scutellata* cells built naturally or on foundation, either fully drawn cells or unworked foundation cells. *461a* and *461b* differ in their basal diameter but the diameter was kept constant (cf. cell base below)

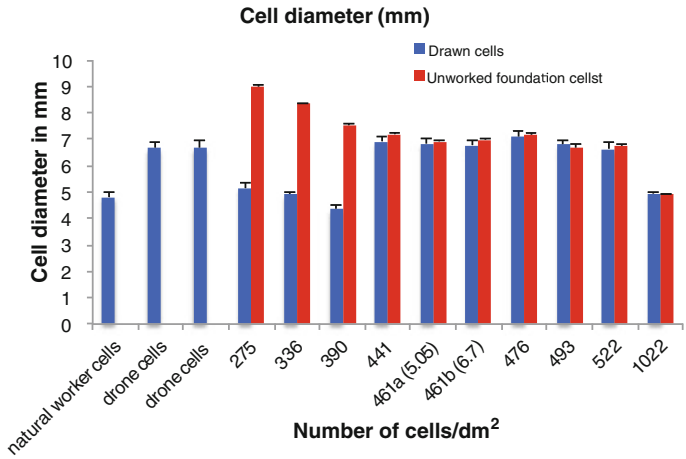

Fig. 12.12 The cell wall thickness of *A. m. scutellata* cells built naturally or on foundation. *461a* and *461b* differ in their basal diameters, but the diameter was kept constant (cf. Sect. 12.7, Cell Base)

dimension, 4.34 mm, is within 0.5 % of that occurring in the normal range of worker cells. This result implies that recognition and acceptance of a grossly large hexagon depends on the ability of the bees to superimpose a pattern of the cells which is within their natural working tolerances (Figs. 12.11 and 12.12).

441, 461 and 476 cells/dm^2. This series included cells which constituted the upper limits on cell size construction, on which one particular colony would work, but the other colonies would not. Only a single colony drew comb on foundation with 441 cells/dm^2; the other test colonies invariably began tapering the forming cells, and would then gap-fill the resulting voids. The 461 and 476 cells/dm^2 were treated in the same way as the 441 cells/dm^2 foundation; one colony would draw on them, the others would not. Just as the cells constructed on the 390 cells/dm^2 foundation resulted in a 0.5 % extension of the range of worker cell sizes, this foundation size defined the upper limits of drone cells in naturally-built combs, which vary in size from 6.18 to 7.24 mm, with a mean of 6.66 mm. Experimental foundation of 441 cells/dm^2 had a diameter of 7.20 mm, which was just within the limits of drone cell tolerances.

493 and 522 cells/dm^2. Foundation with 493 cells/dm^2 was the largest size which all six colonies would consistently draw into finished, regular combs. Workers easily filled these cells with honey and capped them, queens readily laid in them, and drones were reared from them. The appearances of such combs were the same as that of any comb drawn on commercially available foundation sheets, except that the cells were uniformly large. The cell walls were virtually identical to those of drone comb (Fig. 12.12).

Foundation of 522 cells/dm^2 was almost identical to that of combs in wild colonies and was treated accordingly by the bees. In the absence of building foundation, the cell sizes of European races of *A. mellifera* bees are notoriously variable (Vogt 1911; Darchen 1956), while African *A. mellifera* construct cells that range from about 4.8 mm to 6.7 mm in diameter or 500–1100 cells/dm^2. The reported lower limit on the cell size of African *A. m. litorea* from Tanganyika (presently, Tanzania) is 1243 cells/dm^2 (Smith 1960) and the upper limit close to 441 cells/dm^2 (Hepburn 1983). Considering drone and worker cells as separate entities that are recognised as such by bees, the variations in the tolerances of cell size approach some 40 %. Moreover, so-called intermediate or transitional cells are far from rare, as is shown when only a starting strip of embossed wax is given to honeybee colonies. The bees follow the pattern to the end of the strip, below which the cells become progressively larger. Over a distance of only 65 mm, the cells at the bottom of the comb were 20 % larger than those at the top, the rate of increase in cell size being about 3 % per mm (Hepburn 1983).

Against this background of seemingly immense variation there was, nonetheless, some control in comb-building. There were no strong correlation between cell diameter and cell wall thickness. Two groups appear with respect to these two variables (Hepburn 1986). One was a group of smallish cells centering around 5 mm in diameter with a mean wall thickness of about 0.25 mm, which were worker cells. In the other group, drone cells were around 6.8 mm, with an average wall thickness of about 0.43 mm. These two groups were discrete and there were no intermediate or bridging values between them (Figs. 12.11 and 12.12). The overall mean thickness of cell walls in naturally drawn combs is 0.36 ± 0.11 mm, whereas those drawn on foundation were marginally thicker, 0.41 ± 0.08 mm. However, the difference was not statistically significant. In view of the relatively large cell wall size from embossed foundation of about 0.60 mm (Figs. 12.11 and 12.12), it was significant that such large walls were planed by the bees and the thinning factor was about 30 % on average.

The limits on the acceptability of large cells by *A. m. scutellata* lie in the range of 441–493 cells/dm^2 and provide a basis for assessing whether bees consistently apply a set of working limits. Tests were conducted using the same six colonies of bees, and different types of foundation were placed in the same frame. Thus, 1 dm^2 sheets of foundation of one size were alternated with 1 dm^2 sheets of another size, in the following combinations: 441 + 390, 336 + 522, 493 + 441 and 493 + 390 cells/dm^2. The results of construction were astonishingly consistent, whatever the permutations of cell sizes, the bees consistently recognised 336 and 390 cells/dm^2 sizes and built their combs accordingly. Similarly, when they encountered 493 and 522 cells/dm^2 sizes, they built accordingly. The 441 cells/dm^2 size presented the same problem in mixed-size foundation frames as in the whole frame experiment—one colony would work it, and the other colonies resorted to tapering and gap-filling. There was no distortion of the foundation and the metrological abilities of the bees remained constant.

Table 12.1 Measurements of *A. mellifera* cells (Martin and Lindauer 1966)

	Worker cells	Drone cells
a Maximum diameter [mm]	5.7	6.9
b Minimum diameter [mm]	5.2	6.2
l Length of brood cell [mm]	12.0	15
α Cell angle	120°	120°

12.7 The Cell Base: Changing from Rhombus to Hemisphere

It is very difficult to assess the importance of the cell base in relation to the construction of the rest of the cell. Huber (1814) assigned great importance to it, but only a few studies address the issue (Hepburn 1983; Pirk et al. 2004; Hepburn et al. 2007). In the earliest study, Hepburn moulded the wax so that the foundation consisted of perfectly equilinear hexagons and the bases of the cells were flat and of the different sizes mentioned above. In professionally made commercial beeswax foundation, both the cell base and the hexagonal rims of the cells have a pronounced taper to them. To assess what the bees might have measured, base width or wall taper, foundation of 461 cells/dm^2 were manufactured in two different ways; cells were made in which the diameter was held constant at about 6.95 mm, but the bases varied. Type A cells had a base of 5.05 mm and type B cells with a basal diameter of 6.70 mm (Hepburn 1983). The results are shown in Figs. 12.11 and 12.12, from which it is evident that the finished cells differed, although not significantly, by about 0.1 %. One can conclude that the outermost limits of the pattern supplied to them, and not the nature of the cell base, determines what pattern the bees follow.

Therefore, the apparent regularity of comb cells derives from two sources: the abstractions of idealists (with the laudable exceptions of Vogt, Hubbe and Darchen), and the centuries-old use of beeswax foundation on which exact regularity has been embossed. The latter gives regularity to cells which masks the variability which one normally finds in feral honeybee nests. Based on that level of order, Martin and Lindauer (1966) thought that the diameter of cells must be measured by the bees in some way. The average measurements of the cells they studied (similar to others obtained by Taber and Owens 1970) are shown in Table 12.1.

Among the possible organs of measurement that came to mind were the interommatidial setae, whose function is unknown. These were excluded because Neese (1965) had shown that their removal had no measurable effect on comb construction. These setae, while not essential for comb-building, are not precluded from a role in comb-building, as Martin and Lindauer (1966) rightly pointed out. Similarly, Lau (1959) had shown that bees could build extensive and normal combs after amputation of the tarsi of the forelegs. But to consider the more obvious things, Huber (1814) was emphatic that not a parcel of wax is removed in

Table 12.2 Comparison of maximal and minimal cell diameters in combs built by normal and antennectomised *A. mellifera* workers (Martin and Lindauer 1966)

Experimental group	N	Maximal diameter [mm]	N	Minimal diameter [mm]
(A) Normal bees	27	5.7 ± 0.13	27	5.2 ± 0.17
(B) Apical segments removed—both antennae	37	5.7 ± 0.27	30	5.3 ± 0.22
(C) 2 or 3 Antennal segments removed—both antennae	40	5.7 ± 0.17	40	5.2 ± 0.15
(D) Right antenna removed	28	5.8 ± 0.22	28	5.3 ± 0.19
(E) 5–7 segments removed—both antennae	20	5.6 ± 0.25	20	5.2 ± 0.24
(F) Right antennae and 2 or 3 segments of the left removed	27	5.8 ± 0.23	45	5.2 ± 0.31
(G) Tips of both antennae treated with HNO_3	15	5.6 ± 0.28	15	5.2 ± 0.23
(H) 2–7 segments of both antennae cauterised and covered with wax	23	5.9 ± 0.48	25	5.1 ± 0.33

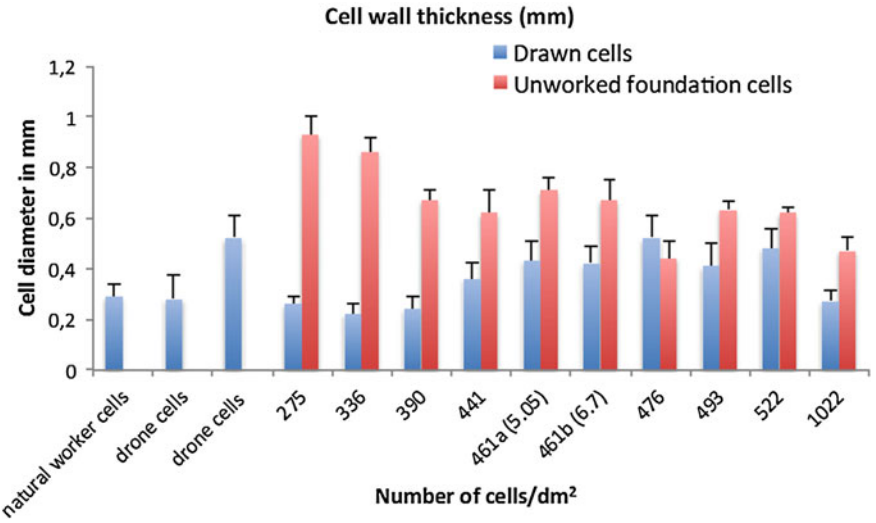

Fig. 12.13 Variation (*blue bars* in μm, *red bars* % increase) in the thickness of the cell walls built by antennectomised *A. mellifera* workers. Groups are as in Table 12.2. Group A (control) being significantly different from all other groups (Martin and Lindauer 1966)

cell thinning before the antennae have palpated the surface to be planed. Likewise, Lau (1959) had shown that combs built by antennectomised bees contained several structural aberrations. On these notes, Martin and Lindauer (1966) went on to evaluate the antennae in determining cell size and wall thickness.

Martin and Lindauer (1966) performed an incredible series of important experiments in which they removed either one or both antennae, as well as

different numbers of antennal segments from hundreds of bees (Table 12.2). The bees, despite the mutilations, constructed combs similar to the controls, providing negative results. However, returning to the palpations of the antennae, and because amputation did not prevent building (Lau 1959; Martin and Lindauer 1966), in future one just might have to re-define precisely the role of the antennae in comb-building. In both studies (Lau 1959; Martin and Lindauer 1966) it was noticed that the coping of the cell wall should show a deviation from the controls. The coping of cells built by antennectomised bees were wider and higher than those of the control groups (Martin and Lindauer 1966).

This suggests that the antennae play a role in maintaining tolerances on cell thickness. In building, the milling of the cell wall is controlled by a single worker working on a single side of the wall at a time. That the apical segment of the antenna is of great importance is shown by the effect of partially removing it, which increases the cell wall thickness significantly (Fig. 12.13). Martin and Lindauer (1966) observed that a building bee continuously executes planing movements with the curved edges of the mandibles during construction, while simultaneously 'monitoring' the forming or thinning wall with the antennae. As the mandibles are dragged over the wax, it is deformed. Presumably the controlled pressure on the mandibles could, in theory, be transmitted through the head capsule and onto the neck organ, whose response may inform the bee's brain of quantitative changes in the wax. Part of the problem with the results reported above is that these questions have to be answered experimentally. Otherwise, it is only conjecture as to what is measured and how, if indeed, anything is measured at all.

References

Akratanakul P (1977) The natural history of the dwarf honey bee, *Apis florea* F in Thailand. Thesis, Cornell University

Alber M (1953) Favi naturali. Apic Ital 20:55–58

Armbruster L (1920) Zum Problem der Bienenzelle: eine vergleichende Instinkt-Biologie des Nestbaues bei Bienen und Wespen. Theodor Fisher, Leipzig

Bergman DJ, Ishay JS (2007) Do bees and hornets use acoustic resonance in order to monitor and coordinate comb construction? Bull Math Biol 69:1777–1790. doi:10.1007/s11538-006-9190-9

Bone D (1952) A rare architectural work of bees: comb built upwards. GleanBee Cult 80:216–217

Butler C (1609) The feminine monarchie, shewing their admirable nature, and properties, their generation, and colonies, their Government, loyaltie, art, industrie, enemies, warres, magnamimitie, &c. together with the right ordering of them from time to time. Joseph Barnes, Oxford

Casteel DB (1912) The manipulation of the wax scales of the honey bee. US Department of Agriculture, Bureau of Entomology, Washington

Dadant HC (1946) The honeycomb. In: Grout RA (ed) The hive and the honeybee. Dadant, Hamilton

Darchen R (1954) Quclquer regulations sociales dans la construction chez les abcille. lnsectes Soc 1:219–228

Darchen R (1956) La construction sociale chez *Apis mellifica*. Insectes Soc 3:293–301. doi:10.1007/bf02224312
Darchen R (1980) La cire, son recyclage et son rôle probable à l'interieur d'une colonie d'*Apis mellifica*. Apidologie 11:193–202
Darchen R (1991) The construction strategy of bees. In: Proceedings of 3rd conference on Italian section of the IUSSI Ethol Ecol Evol 3:107–110
Darwin C (1856) The origin of species by natural selection. Murray, London
Darwin C (1859) The origin of species by natural election. Murray, London
Dönhoff E (1854) Über den Übergang der Wachsblättchen in Wachslümpchen. Schweizerische Bienenzeitung 15:279
Duangphakdee O, Hepburn HR, Rodim P (2013) Seasonal movements of the red dwarf honeybee, *Apis florea*. (In ms.)
Gontarski H (1935) Wabenzellmasze bei *Apis mellifica*. Z Vergl Physiol 21:682–698
Gould JL, Gould CG (1983) Can a bee behave intelligently? New Sci 98:84–87
Gwin CM (1936) Further developments concerning wax production by the honeybee colony. 1. A study of the production of wax scales and comb-building. J Econ Entomol 29:318–321
Hepburn HR (1983) Comb construction by the African honeybee, *Apis mellifera adansonii* (= *scutellata*). J Ent Soc S Afr 46:87–101
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR, Whiffler LA (1991) Construction defects define pattern and method in comb-building by honeybees. Apidologie 22:381–388
Hepburn HR, Hugo JJ, Mitchell D, Nijland MJM, Scrimgeour AG (1984) On the energetic costs of wax production by the African honeybee, *Apis mellifera adansonii* (= *scutellata*). S Afr J Sci 80:363–369
Hepburn HR, Muerrle T, Radloff SE (2007) The cell bases of honeybee combs. Apidologie 38:268–271. doi:10.1051/Apido:2007005
Hubbe W (1957) Beobachtungen zum Wabenbau der Honigbiene *Apis mellifica* L. Arch Geflügel Kleintier Kd 6:343–358
Huber F (1814) Nouvelles observations sur les abeilles. [English translation, 1926] Dadant, Hamilton
Karihaloo BL, Zhang K, Wang J (2013) Honeybee combs: how the circular cells transform into rounded hexagons. J Roy Soc Interface 10(86). doi:10.1098/rsif.2013.0299
Lau D (1959) Beobachtungen und Experimente über die Entstehung der Bienenwabe (*Apis mellifica* L.). Zool Beitr 4:233–306
Lineburg B (1923a) Conservation of wax by the bees. Am Bee J 63:615–616
Lineburg B (1923b) What do bees do with brood cappings? Am Bee J 63:235–236
Lineburg B (1924) Comb-building. Am Bee J 64:271–272
Martin H, Lindauer M (1966) Sinnesphysiologische Leistungen beim Wabenbau der Honigbiene. Z Vergl Physiol 53:372–404
Meretz W (1963) Die Wabenzelle der Honigbiene. Bull Math Biophys 25:95–110. doi:10.1007/bf02477773
Meyer W (1952) Die Kleinbauarbeiten unserer Bienen. Deutsche Bienenwirts 3:237
Meyer W, Ulrich W (1952) Zur Analyse der Bauinstinkte unserer Honigbiene. Untersuchungen Åber die "Kleinbauarbeiten". Naturwissenschaften 39:264
Mossadegh MS (1990) Nesting behaviour of *Apis florea* F (Hymenoptera: Apidae) in Khuzestan, Iran. Proc 11th Int Congr, IUSSI, New Delhi, pp 669–670
Neese V (1965) Zur Funktion der Augenborsten bei der Honigbiene. Z Vergl Physiol 49:543–585. doi:10.1007/bf00367159
Oelsen GV, Rademacher E (1979) Studies on the building behavior of the honeybee (*Apis mellifica*). Apidologie 10:175–209
Phiancharoen M, Duangphakdee O, Hepburn HR (2011) Biology of nesting. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 109–131
Pirk CWW, Hepburn HR, Radloff SE, Tautz J (2004) Honeybee combs: construction through a liquid equilibrium process? Naturwissenschaften 91:350–353

Rösch GA (1927) Über die Bautätigkeit im Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 6:264–298
Smith FG (1960) Comb foundation: its use for African honeybees. Bee World 42:235–240
Smith MV (1959) A note on the capping activities of an individual honeybee. Bee World 40:153–154
Taber S, Owens CD (1970) Colony founding and initial nest design of honey bees, *Apis mellifera* L. Anim Behav 18:625–630. doi:10.1016/0003-3472(70)90005-9
Tautz J (2008) The buzz about bees, biology of a superorganism. Springer, Berlin
Thompson DAW (1942) On growth and form. Cambridge University Press, Cambridge
Thompson F (1930) Observations on the positions of hexagons in natural comb-building. Bee World 11:107
Ulrich W (1964) Geometrie und Entstehung der Bienenwabe. Zeit Bienenforsch 7:62–71
Vogt H (1911) Geometrie und Ökonomie der Bienenzelle. Trewendt and Granier, Breslau
von Frisch K (1974) Tiere als Baumeister. Frankfurt am Main, Ullstein

Chapter 13
Conversion of Wax Scales into Comb Wax

Abstract The cyclical changes in cellular organelles and the chemical composition of beeswax precursors found in the haemolymph and gland tissues, closely coincide with age-related wax secretion rates. It is one of the divisions of labour, a coincidence of physiology and behaviour that parallels other polyethisms. The mechanical properties and crystal structure of wax change with chemical additions by honeybees. Intact wax scales contain some non-lipoidal components and differ from comb in lipid composition. The mechanical properties of scale and comb wax vary with temperature. There is a linear relationship between load and elongation in the tensile stress-strain curves to the maximum sustainable load, so that the yield stress coincides with the ultimate strength of the material. New comb wax is an isotropic plastic whose mechanical properties depend on temperature. Larvae introduce silk into the comb in a random array, the addition of which improves the load-carrying capacity of the combs. Over time, the combs become fibre-reinforced composite materials, with properties entirely different from the individual components. Wax scales form as the liquid wax fractions transude from the pore canals onto the surface of the wax mirror, where these small droplets coalesce to form thin layers of wax, and this process continues until a wax scale forms. The relatively crystalline scale is reduced to an amorphous state during cell construction; but, given the warmth of the colony, the physical manipulations of the wax by the bees gradually introduce an ordered texture.

13.1 Introduction

This chapter reviews the cycle of wax secretion in which the cyclical changes of cellular organelles and the chemical composition of beeswax precursors, found in the haemolymph and gland tissues, closely coincide with age-related rates of wax secretion (Rösch 1927; King 1928; Hepburn et al. 1991). It is one of the divisions of labour, and this temporal coincidence of physiology and behaviour parallels other polyethisms, such as colony defense (Whiffler et al. 1988; Breed et al. 1990)

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_13,

and brood care (Liu 1989; Crailsheim and Stolberg 1989); all are predictable activities correlated with age and cycles of glandular functions.

Some years ago, Robinson (1987) suggested that juvenile hormone was involved in the regulation of physiological processes that are associated with division of labor in honeybees, but the effects of juvenile hormone on behavior were not clear. He went on to test the hypothesis that, juvenile hormone affects worker age polyethism using a chemical analogue of the hormone, methoprene. On the basis of his results Robinson (1987), and subsequently Robinson and Vargo (1998) and Sullivan et al. (2000), claimed juvenile hormone was involved in the control of age polyethism, and that the hormone may regulate a colony's allocation of labour by altering the probabilities of response to tasks. Robinson and Vargo (1998) further stated that their experiments demonstrated that juvenile hormone acts as a "behavioral pacemaker," influencing how fast a worker grows up and makes the transition from nest to foraging activities. Unfortunately, the juvenile hormone analogue, methoprene, is a toxic, insect growth regulator, that was first registered as a conventional chemical pesticide in 1975, and remains so to date (US Environmental Protection Agency 2001). So in the circumstances, all of the attempts to define a role for juvenile hormone in the division of labour, based on methoprene, remain inconclusive.

Attempts to alter this cycle by increasing or decreasing the amounts of juvenile hormone and/or the addition or removal of the corpora allata, had no measurable effect on the onset of wax secretion, its duration, or the amount of wax actually produced for this age cohort (Muller and Hepburn 1994). Juvenile hormone III and the corpora allata do not play a role in regulating the age-related physiology of wax secretion in adult worker honeybees, as neither factor affected either the onset of wax production or the amount of wax produced. Allatectomy of newly eclosed workers did not affect wax production in adult worker honeybees. An experimentally increased juvenile hormone III haemolymph titre, as a result of either a single large injection or by implanting corpora allata from older workers into younger workers, did not affect either the onset of wax production or the mean amount of wax produced. No critical period could be established during which an elevated juvenile hormone III titre would affect the rate of wax secretion. Methoprene, on the other hand, significantly reduced wax secretion. This suggests that methoprene, applied pharmacologically as is done routinely in polyethism studies, is sublethal and poisonous to worker honeybees. Methoprene is a compound which mimics the action of an insect growth regulatory hormone and is used as an insecticide because it interferes with the normal development.

Hepburn and Muller (1988) performed experiments to determine the nature of the cycle of wax secretion. First, using bees of a precisely known age, it was shown that secretion was a continuous process, there was no diel rhythm. This led to a 2 year study of wax secretion in queenright colonies, from which the wax scales of some 11000 bees were recovered and weighed. Wax secretion was parabolically cyclic and related to age: secretion begins at about 3 days after emergence, reaches a peak between 9 and 12 days, and wanes between 18 and 21 days (Hepburn et al.

1991). These data finally filled the gap between the histological observations of Rösch (1927) and the physiology of the secretory cycles.

13.2 Wax Scales

The probable chemical changes in the conversion of wax scales into comb wax, first suggested by Hunter (1792), rest on two pieces of evidence. The first comes from Huber (1814), who found that wax scales dissolved quickly in turpentine, but fragments of comb failed to dissolve completely, leaving many particles of comb wax in the solvent. Moreover, fragments of comb disintegrated and fell to the bottom of a flask of ether, but scales preserved both size and shape and lost only their translucency. These two wonderful little experiments are easily repeated. The second arises, *en passant,* from the work of Lambremont and Wykle (1979), in which scale wax was analysed by thin layer chromatography and the resulting chromatograms lacked any activity in the diester position. However, the presence of this fraction in scale wax has since been confirmed (Schoening 1980; Kurstjens et al. 1985).

The components of the 'salivary' secretions of honeybees are poorly known, and those added to wax during comb-building are totally unknown. However, Heselhaus (1922) suggested that the salivary material added to wax might derive from the postcerebral gland, while others (Örösi-Pal 1957; Cruz Landim 1963, 1967) implicated the mandibular gland. Indeed, Cruz Landim claimed that the secretion of an isolated mandibular gland of some wild bees can dissolve wax. A ketone, 2-heptanone, has been identified from this gland in honeybees by Shearer and Boch (1966), which partially dissolves comb but does not affect scale wax at all.

13.3 Chemical Differences Between Scale and Comb Wax

While one can measure changes in the mechanical properties of wax that result from chemical additions by honeybees, allowance must be made for the changing crystal structure as well. Kurstjens et al. (1985) investigated permutations of scales and comb wax by separately varying crystal texture and chemical composition (to include or exclude a possible protein fraction in the wax—Kurstjens et al. 1990), and then tested these waxes under identical mechanical conditions. This led to two conclusions about the chemical differences of the waxes. First, intact scales must contain some non-lipoidal component. Secondly, wax scales must differ from comb wax in lipid composition as well (cf. Chap. 16).

Kurstjens et al. (1985) tested these inferences with standard techniques used for both the gross analysis of proteins and lipids, and found that both waxes contain proteins and also differ in gross lipid composition. Davidson and Hepburn (1986) then found that some of the changes in lipid composition were associated with the conversion of wax scales into comb wax. Although the total glycerol content of

both waxes remains constant, the diacylglycerols predominate in scales, but are reduced by nearly half in comb wax; on the other hand, the monoacylglycerol pool of comb wax is nearly double that of scale wax. However, this potential reduction in the stiffness of comb is ameliorated by a corresponding saturation of the acylglycerol fatty acids in comb wax. That these transformations enhance the stiffness of comb wax is evidenced by the fact that texturally isotropic and protein-free sheets of comb wax are significantly stiffer than similar specimens made of scale wax (Kurstjens et al. 1985).

The 'saliva' added to the wax scales by bees contains material with probable lipolytic activity that reduces the diglyceride pool of the scale wax with a corresponding increase in the monoglyceride fraction of the comb wax. The combined affects of the crytstallographic and chemical changes on the mechanical properties of the waxes are as follows: (1) scale wax is stronger than comb wax and, although the latter is twice as stiff as scale wax, it is less distensible than the former; (2) the energy to fracture comb wax (an index of the work bees must invest to shape it) is only half that of scale wax over the range of temperatures likely to impinge on the nests of honeybees; (3) the effects of mandibulation by the honeybees are to transform the texturally anisotropic scale wax into isotropic comb wax.

Although the mechanical properties of scales and comb wax vary with temperature (cf. Chap. 14), we have not considered the significance of temperature as such. It has been documented (Hepburn et al. 1983; Hepburn and Kurstjens 1984) that the physical effort required of bees and the mechanical performances of the nest are a superb compromise between bees and material at 35 °C. At the only slightly higher temperature of 40 °C, the mechanical properties of the nest are dangerously compromised, and the bees themselves begin to die in droves. On the other hand, were bees to work at lower temperatures, their construction costs would burgeon with decreasing temperature (cf. Chaps. 11 and 14). All things considered, one wonders why bees did not evolve a more heat-resistant wax. As it is, once the wax scale forms on the wax mirror, it has a melting point of about 65 °C, but the wax precursors were transuded through the pore canals at bee-body temperature, of only 35 °C. However, there is the intriguing reality that honeybee enzymes of begin to denature above 40 °C. The selective processes that led to this compromise must have been extraordinary.

13.4 Maturation of Newly Constructed Combs

Once the wax scales have been fashioned into pristine comb, many more physical and chemical transformations of the nest material occur. The first hint of such changes were observed by Huber (1814); that the very white wax of new combs seemed more brittle than that of the stronger and more pliable yellow combs. He also noted that bees add propolis (whose origin from plants he discovered independently of Hornbostel 1744) to wax, both in bulk and as a surface varnish, which he thought reinforced the combs. There is no dispute about the progressive

Fig. 13.1 Silken 'ghost' cells exhibit clear-cut rhomboids on the bottom outside of *A. m. capensis* cells (Hepburn et al. 2007)

changes in comb colour particularly associated with the rearing of several cycles of brood in the nest. The new, white cells progress through yellow, various shades of brown and finally become a very dark brownish-black.

The above is the usual sequence of colour changes in nests of all species of honeybees that have been examined. However, bees have been known to secrete pink or red wax; the pigment of candy floss collected by foragers in one case (S. Taber, pers comm.), and from dye, Sudan III, fed to bees in a vegetable oil carrier in another case (Örösi-Pal 1956). The blackness is said to derive partially from larval excrements and from propolis, but the chemical identity of such pigments has not been resolved (Chauvin 1962; Tischer 1962). The walls of brood cells also become thicker with continued use and include the exuviae and silken fibres spun by generations of larvae. Very old combs, from which the wax has been extracted, leave behind fairly substantial 'ghosts' of hexagonal cells (Fig. 13.1).

Through some simple chemical studies, Huber (1814) was able to show that the yellow principle was not likely to have come from propolis, a point subsequently confirmed by Jaubert (1927), who identified the component as 1,3-hydroxyflavone and named it chrysine. The origin of chrysine is totally obscure. Vansell and Bisson (1935) believed that the yellow colour of the wax arose through contamination by carotenoids from pollen, a view consistent with the report of Freudenstein (1962), that the combs of caged bees lacking pollen were white and those of bees with access to pollen were yellow. This is a commonplace occurrence. Alternatively, Philipp (1928) and Freudenstein (1932) suggested that it might be a glandular secretion. The occasional occurrence of bright yellow cells in the midst of new white combs with pollen nowhere near, also occurs.

What is the significance of these colour changes in comb? Reasonable hypotheses were formulated by Woog and Yannaquis (1935, 1936a, b) on the basis of their physical studies. They found that yellow and white wax taken from recently constructed combs had the same degree of crystal orientation, far greater than that of brown wax, which had only very slight, or no crystal orientation at all.

Table 13.1 Tensile properties of propolis and beeswax of the African honeybee, *A. m. scutellata* (Hepburn and Kurstjens 1984)

	Temperature (°C)	Yield strain (MPa)	Yield stress (%)	Stiffness (MPa)	Work to yield (MJm^{-1})
Propolis	25	0.26 ± 0.07	38	0.68	0.07
	30	0.16 ± 0.03	23	0.69	0.03
	35	0.12 ± 0.02	17	0.71	0.02
	40	0.08 ± 0.02	16	0.5	0.01
	45	0.03 ± 0.01	14	0.21	0.004
Beeswax	25	1.13 ± 0.08	30	3.77	1.2
	30	0.83 ± 0.06	14	5.93	0.5
	35	0.54 ± 0.08	12	4.5	0.23
	40	0.21 ± 0.03	4.2	5	0.04
	45	0.07 ± 0.01	4	1.75	0.01

However, in samples of white and yellow wax incubated at 38 °C for 3 days, the orientation of crystals in the latter was enhanced, and even more so after 50 °C for only 2 h. They concluded that chrysine somehow accelerates crystal orientation. Brown wax, in contrast, appeared totally different. There was no measurable crystallinity in these propolis-bearing waxes. Woog and Yannaquis (1935, 1936a, b) suggested that the wax-propolis mixtures were more plastic than either the white or yellow wax, and that the solidity of such constructions was augmented by the bees incorporating pupal exuviae into the cell walls. They further argued that such a fusion is highly dependent on good adhesion between the two, such forces being greater than surface effects that might otherwise have led to increases in crystal orientation.

The work of Woog and Yannaquis (1935, 1936a, b) provides ideas for a mechanism by which changes in the cell walls of combs may occur. In any event, other changes in comb have been explored by Hepburn and Kurstjens (1984) who compared the tensile properties of propolis (whose older name 'bee-glue' is actually more appropriate in view of its general use by bees), and of new white comb wax. They found that propolis exhibited an unusual behaviour on tensile deformation; there was a linear relationship between load and elongation from the origin of the curves to the maximum sustainable load. Thus, the yield stress coincided with the ultimate strength of the material. But propolis, like beeswax, is an entirely plastic material in the range of 25–40 °C, so that this linearity was not an elastic one. On yield, propolis was highly ductile and flowed about 200 % before the necking thread finally failed.

The tensile strength of propolis decreased eightfold over the range of temperatures tested, and the yield strain some threefold (Table 13.1). The stiffness remained virtually constant at lower temperatures with a major transition between 35 and 40 °C. The work to yield also decreased with increasing temperatures, as did the ductility of the substance. Over the range of temperatures tested, beeswax was at least four times stronger than propolis. Even so, the combs of bees in warm countries sometimes fail in hot weather (wax and honey were actually observed

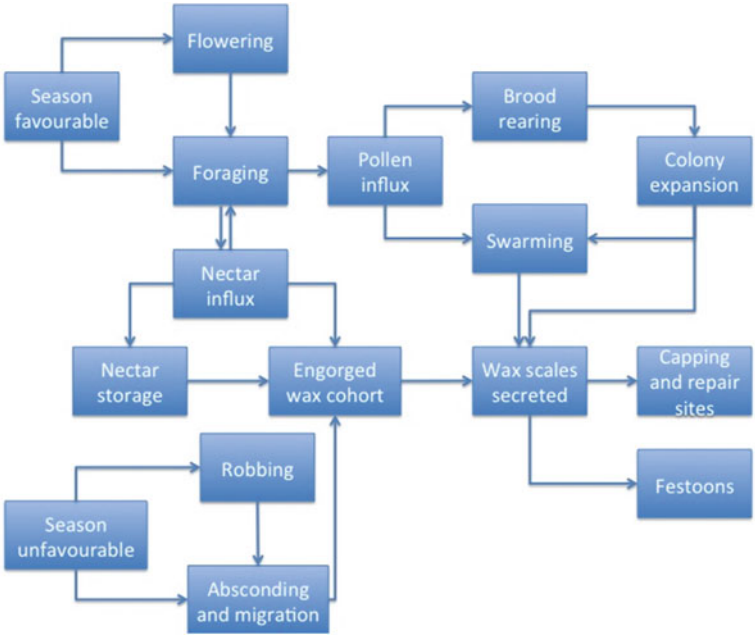

Fig. 13.2 Simplified flow diagram showing the steps involved in the conversion of newly secreted wax scales into new comb, followed by the events associated with the maturation and use of combs (Hepburn 1998)

flowing out of the entrance of hive exposed to the full sun in summer in South Africa (Hepburn unpubl. obs.). Propolis at its strongest is comparable to beeswax at its weakest over the range of temperatures tested that are likely to have an effect on honeybees' nests. These results are certainly consistent with the interpretation of Woog and Yannaquis (1935, 1936a, b) as to the possible effects of propolis in wax, but they do not constitute a direct test of the variables associated with the maturation of combs.

The modifications of comb performance by the presence of proteins and water can now be related to the material properties of combs as they evolve in the nest (Hepburn and Kurstjens 1988). In the course of its development, new comb wax is an isotropic plastic whose mechanical properties depend heavily on temperature. In time, generations of larvae introduce silk into the waxen structure in a random alignment to achieve equal properties in all directions. Thus with use, the comb becomes a fibre-reinforced composite material which exhibits properties entirely different to its individual components. The addition of silk greatly improves the load-carrying capacity of the combs (Hepburn and Kurstjens 1988). Although not a theoretically ideal stiff plate structure (Nachtigall and Kresling 1992), the mature comb is nonetheless a remarkable compromise between technical construction and the biological purposes it serves. A flow diagram showing the conversion of wax scales into comb is given in Fig. 13.2.

13.5 Wax Scales

The wax scales of honeybees are roughly pentagonoid but with rounded corners and are slightly convex in the surface plane. As such, they are of the same outline shape as the surfaces of the wax mirror cuticle on which they form (cf. Fig. 12.1); the most posterior of the four pairs of wax mirrors are somewhat smaller than the anterior ones (Huber 1814; Dreyling 1903). The wax scales vary in thickness, depending upon the time they have been developing on the abdomen of the honeybee (Huber 1814; Dönhoff 1854). They normally range in thickness from about 200 μm to 500 μm when used by bees (or fall from bees), but extremes of 1000 μm have been observed in *A. mellifera* (von Buttel-Reepen 1915; Jordan 1962) and *A. cerana* (HQ Zheng, University of Zhejiang, pers. comm. and photomicrograph). Whether scales are laminated has been a controversial point since Huber (1814) originally asserted that they are. Dreyling (1906) examined the fracture faces of thick scales that he had broken, and noted the jagged edges of distinct layers. But because the entire scale readily took up coloured dyes rather than penetrated the interstices of his apparent layers, he concluded that the scales were fused. The laminations of scales have also been shown by others (Gwin 1936; Baldaev 1968; Dietz and Humphreys 1970), but the suggestion that the layers are fused remains (Coggshall 1953), and, indeed, they are.

The formation of wax scales, as intimated by Huber (1814), have been observed microscopically by Philipp (1935), confirmed by Jordan (1962) and more recently by Cassier and Lensky (1995). The liquid wax fractions transude from the pore canals onto the surface of the wax mirror, where these small droplets coalesce to form a first layer of very thin wax. In the next phase of secretion, more droplets reach the surface of the mirror, lifting the first layer and become attached to it. So the process continues until several layers have been secreted to form a full wax scale. Scales usually consist of about three to six laminae by the time a bee uses them (Jordan 1962), and the older and thicker they become, the greater the extent of delamination at the edges. Dietz and Humphreys (1970) were able to resolve the laminae into finer sublayers of about 80 μm in thickness. It is very likely that the occurrence of real, but fused laminae, reflects 'pulsations' in the rate of wax secretion (Hepburn and Muller 1988). Indeed, the secretion of wax, at least in summer, follows a circadian rhythm (Baldaev 1968). As such, the layered nature of the scale merely reflects the supposition of Brewster (1815) that beeswax, like rubber, gum arabic and other substances, form by the successive deposition and induration of thin layers, a point confirmed by the observations of Philipp (1935) and Cassier and Lensky (1995).

Ever since Hunter (1792) observed that wax scales are translucent, but new comb is white and opaque, the idea that comb wax might be a mixture arose. Huber (1814) noted that worker bees chew and fragment wax scales and add a frothy substance to them. These facts assured that both mechanical and chemical changes occur in the conversion of scales into comb. The study of such changes and how they might come about has been slow and, experimentally, extremely

difficult. But, most importantly, studies of wax texture, or the arrangement of crystallites in relation to temperature, mechanical deformation, pressure and time, have provided much insight into how honeybees convert their minute scales of wax into combs.

Armed with a reasonable description of the gross changes in shape and in crystal texture that occur in the metamorphosis of wax scales into honeycomb, we are left to consider how these and other changes might come about and affect the wax combs as structural nest material. The roles of annealing, pressure, compressive and tensile deformation and time have been identified as important means by which the crystal texture of wax may change. The conceptual inter-relationships of these factors were developed in the papers of Woog and Yannaquis (1935, 1936a, b). They argued, from experimental analogies, that while the relatively crystalline scale is more or less reduced to an amorphous state during cell construction, given the warmth of the colony, the mandibulation of the wax by the bees, and the passage of time, the comb wax gradually becomes more ordered. Time and the warmth of the nest in tempering wax have also been considered by others (Hunter 1792; Kratky 1937; Schmidt 1941; Martin and Lindauer 1966). It is implicit in this argument that the more crystalline the structure, the stronger the material, an assumption that has now been tested experimentally.

13.6 Unnatural Building Materials

Recently, there have been several experiments and observations on the interspecific uses of beeswaxes. The different honeybee species share some homologous neutral lipids; but significant species-specific differences remain (Aichholz and Lorbeer 1999; Phiancharoen et al. 2011). Hepburn et al. (2009) analysed behavioural variation for wax choice in honeybees, calculated the Euclidean distances for different beeswaxes and assessed the relationship of Euclidean distances to wax choice among species. They tested *A. m. capensis*, *A. florea*, *A. cerana* and *A. dorsata* beeswaxes, the plant waxes, Japan wax, candelilla and bayberry and the mineral wax ozokerite. Foundation-like sheets of these waxes were produced and placed in *A. m. capensis*, *A. florea* and *A. cerana* colonies. *A. m. capensis* accepted the four beeswaxes, removed the Japan and bayberry waxes, and 'ignored' the candelilla and ozokerite waxes. *A. cerana* colonies accepted the *A. cerana*, *A. florea* and *A. dorsata* waxes but rejected or ignored the *A. m. capensis*, plant and mineral waxes. *A. florea* colonies accepted the *A. cerana*, *A. dorsata* and *A. florea* wax but rejected that of *A. m. capensis*. Unfortunately we had too little of the plant and mineral waxes to test on these bees in this experiment. In retrospect this might have been predicted on the basis of the Euclidean distances for the beeswaxes, which are also consistent with currently prevailing phylogenies for *Apis* (Raffiudin and Crozier 2007; Koeniger et al. 2011). Despite post-speciation chemical differences in the beeswaxes, they remain largely acceptable interspecifically, while

the plant and mineral waxes are not chemically close enough to beeswax for their acceptance.

This experimental approach was further extended by Hepburn et al. (2010) who worked on the basis that salvaging wax from an abandoned nest and reusing for the construction of a new nest is only known for absconding colonies of the red, *A. florea* (Pirk et al. 2011) and black dwarf honeybees, *A. andreniformis* (Duangphakdee and Wongvilas, pers. comm.). Hepburn et al. (2010) tested whether *A. florea* would preferentially choose to salvage wax from their own, original natal combs over other conspecific combs, and whether they would salvage wax from comb 'facsimiles' of *A. florea* combs fashioned from the combs of *A. cerana, A. dorsata* and *A. mellifera*. In the first experiments, *A. florea* preferences for their own natal combs were significantly greater than for non-natal combs. In the second experiment, *A. florea* did not collect wax from any of the heterospecific combs. It is evident that wax discrimination is very much context-dependent, and that there is considerable genetic variation for the wax-salvaging trait.

Gums, waxes and resins from plants are used as nest materials by many wild bee species (Michener 1974; Roubik 1992), but honeybees primarily restrict the building of their nests to beeswax and propolis. The popular literature occasionally lists the use of various paints, tars and asphalt collected by honeybees and these materials are sometimes recovered in pollen traps. It is also a comment on the flexibility of *A. mellifera* bees to note that, while they readily recover propolis from exposed and used frames and hives, they very rarely salvage wax beyond the confines of the nest in *A. mellifera* (Meyer 1954); however, wax salvage from abandoned nests is commonplace in *A. florea* (Pirk et al. 2011) and *A. andreniformis* (O. Duangphakdee and S. Wongvilas, pers. comm.). Moreover, the many unsuccessful attempts by man to get honeybees to accept foreign materials, such as raw plastics, as the base for their combs, attests to the reluctance of bees to work with unnatural materials (Johansson and Johansson 1971).

Thus it would be of interest, from an evolutionary point of view, to examine the extent of plasticity in honeybee behaviour regarding unusual materials. In this respect, we have the extraordinary results from the experiments by Perret-Maisonneuve (1927). In one instance, he gave *A. mellifera* colonies pieces of pure beeswax as well as dyed or coloured samples of ruberoid, modelling clay, ceresine (a purified ozokerite occasionally used as a substitute for beeswax), resins and beeswax, a mixture of carnauba wax, ceresine and beeswax and various permutations of these substances in different proportions. After a week, he found that comb had been drawn on all these variously coloured substitutes, and yet the piece of pure beeswax was virtually untouched. In a second experiment, Perret-Maisonneuve (1927) prepared a sheet of aluminium covered to a depth of 1 mm with a series of adjacent layers, each of which was composed of the same substitutes as well as the pitch fraction of propolis mixed with wax. Although the bees worked slowly, they constructed comb cells on all of the foreign bases, particularly worked on ruberoid, which they mixed with beeswax. These experimental results were met with some incredulity at the time, and were very soon repeated by Roussy (1929), who confirmed them. The extent to which these kinds of results support a notion of

plasticity in the choice of nest materials would probably be best assessed after the principles of similarity of these different materials to wax have been ascertained.

These observations obviously hold great interest and importance for their eventual application to studies of wax synthesis. It could be expected that the differences in the relative amounts of the major compound families in the waxes would be reflected in the physical and mechanical properties of the waxes (Buchwald et al. 2009). These authors recently reported the results of a comparative study of the mechanical properties of several different beeswaxes (*A. andreniformis, A. cerana, A. dorsata* and *A. mellifera*), and measured, among other things, the relative stiffness and resilience of the waxes. Because the mechanical properties of any structure result from both the intrinsic chemical nature of a material as well as its structural form, it is obviously desirable, but extremely difficult experimentally to work with whole comb specimens. So Buchwald et al. (2006) compromised by eliminating structure and simply measured the behaviour of wax cylinders under compression.

Although compression testing is not biologically appropriate for extrapolation to whole combs, (which are actually tension members with a relatively complex structure), the results of such measurements have heuristic value in trying to relate mechanical behaviour to differences in the major compound families of comb waxes given. Resilience represents the amount of energy required to deform the test material until it begins to fail irrecoverably. Stiffness is simply the rate of change of stress per unit strain. *A. dorsata* wax is significantly stiffer than those of other species. The waxes of *A. cerana* and *A. dorsata* do not significantly differ, but are significantly more resilient than that of the intermediate *A. mellifera,* which in turn is more resilient than that of *A. andreniformis*.

References

Aichholz R, Lorbeer E (1999) Investigation of combwax of honeybees with high-temperature gas chromatography and high-temperature gas chromatography—chemical ionization mass spectrometry I. High-temperature gas chromatography. J Chromatogr A 855:601–615

Baldaev K (1968) Activity of honeybee wax glands in winter. Uch Zap Fac Estestv Mariisk Gos Pedagog Inst 30:54-58 (in Russian)

Breed MD, Robinson GE, Page RE (1990) Division of labor during honey bee colony defense. Behav Ecol Sociobiol 27:395–401

Brewster D (1815) Experiments on the depolarisation of light as exhibited by various mineral, animal, and vegetable bodies, with a reference to the general principles of polarisation. Phil Trans R Soc Lond B 104:187–211

Buchwald R, Breed MD, Greenberg AR, Otis G (2006) Interspecific variation in beeswax as a biological construction material. J Exp Biol 209:3984–3989

Buchwald R, Breed MD, Bjostad L, Hibbard BE, Greenberg AR (2009) The role of fatty acids in the mechanical properties of beeswax. Apidologie 40:585–594

Cassier P, Lensky Y (1995) Ultrastructure of the wax gland complex and secretion of beeswax in the worker honey bee *Apis mellifera* L. Apidologie 26:17–26

Chauvin R (1962) Sur le noircissement des vieilles cires. Ann Abeille 5:59–63 (Paris)

Coggshall WL (1953) Secretion and coloration of beeswax. Am Bee J 93:287–289

Crailsheim K, Stolberg E (1989) Influence of diet, age and colony condition upon intestinal proteolytic activity and size of the hypopharyngeal glands in the honeybee (*Apis mellifera* L.). J Insect Physiol 35:595–602
Crailsheim K, Hrassnigg N, Gmeinbauer R, Szolderits MJ, Schneider LHW, Brosch U (1993) Pollen utilization in non-breeding honeybees in winter. J Insect Physiol 39:369–373
da Cruz Landim C (1963) Evaluation of the wax and scent glands in the Apinae (Hymenoptera: Apidae). J NY Entomol Soc 21:2–13
da Cruz Landim C (1967) Estudo comparativo de algumas glândulas das abelhas (Hymenoptera, Apidea) e respectivas implicacoes evolutivas. Arquivos Zool 15:177–290
Davidson BC, Hepburn HR (1986) Transformations of the acylglycerols in comb construction by honeybees. Naturwissenschaften 73:159–160
Dietz A, Humphreys WJ (1970) Scanning electron microscopy study of the structure of honey bee wax scales. J Ga Entomol Soc 5:1–6
Dönhoff E (1854) Ueber den Üebergang der Wachsblättchen in Wachstlümpchen. Schweiz Bienen-Ztg 15:279
Dreyling L (1903) Über die wachsbereitenden Organe der Honigbiene. Zoo1 Anz 26:710–715
Dreyling L (1906) Die wachsbereitenden Organe bei den gesellig lebenden Bienen. Zoo1 Jahrb Abt Anat Ont Tiere 22:289–330
Freudenstein K (1932) Das 'Hobein' der Bienen. Biol Zentralbl 52:343–349
Gwin CM (1936) Further developments concerning wax production by the honeybee colony. 1. A study of the production of wax scales and comb-building. J Econ Entomol 29:318–321
Hepburn HR, Kurstjens SP (1984) On the strength of propolis. Naturwissenschaften 71:591–592
Hepburn HR, Kurstjens SP (1988) The combs of honeybees as composite materials. Apidologie 19:25–36
Hepburn HR, Muller WJ (1988) Wax secretion in honeybees. Naturwissenschaften 75:628–629
Hepburn HR, Armstrong E, Kurstjens SP (1983) The ductility of native beeswax is optimally related to honeybee colony temperature. S Afr J Sci 79:416–417
Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax in honeybees. Apidologie 22:21–36
Hepburn HR (1998) Reciprocal interactions between honeybees and combs in the integration of some colony functions in *Apis mellifera* L. Apidologie 29:47–66
Hepburn HR, Muerrle T, Radloff SE (2007) The cell bases of honeybee combs. Apidologie 38:268–271
Hepburn HR, Radloff SE, Phiancharoen M, Duangphakdee O (2009) Interspecific utilisation of wax in comb building by honeybees. Naturwissenschaften 96:719–723
Hepburn R, Duangphakdee O, Phiancharoen M, Radloff SE (2010) Comb wax salvage by the red dwarf honeybee, *Apis florea* F. J Insect Behav 23:159–164
Heselhaus F (1922) Die Hautdrüsen der Apiden und vewandter Formen. Zool Jahrb Allg Zool Phyriol Tiere 43:369–464
Hornbostel HC (1744) Neue Entdeckung, wie das Wachs von den Bienen Komt. Hamburg Vermis Bibliothek 2:45–62 (orig Mellittophilus Theorebastus)
Huber F (1814) Nouvelles observations sur les abeilles. [English translation, 1926] Dadant, Hamilton
Hunter J (1792) Observations on bees. Phil Trans R Soc 82:128–196
Jaubert GF (1927) Sur l'origine de la coloration de la cire d'abeilles et la composition de la propolis. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 185:1134–1136
Johansson TSK, Johansson MP (1971) Substitutes for beeswax and comb foundation. Bee Wld 52:146–156
Jordan R (1962) Anomale Wachsplättchen. Bienenvater 63:299–301
King GE (1928) The larger glands in the honey-bee. A correlation of activity with age and physiological functioning, Thesis. University of Illinois, Urbana
Koeniger N, Koeniger G, Smith DR (2011) Phylogeny of the genus *Apis*. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 23–50

Kratky E (1937) Die Orientierung der Moleküle des Bienenwachses und die Festigkeit der Waben. Naturwissenschaften 25:239
Kurstjens SP, Hepburn HR, Schoening FRL, Davidson BC (1985) The conversion of wax scales into comb wax by African honeybees. J Comp Physiol 156:95–102
Kurstjens SP, McClain E, Hepburn HR (1990) The proteins of beeswax. Naturwissenschaften 77:34–35
Lambremont EN, Wykle RL (1979) Wax synthesis by an enzyme system from the honey bee. Comp Biochem Physiol 63B:131–135
Liu TP (1989) Juvenile hormone III induced ultrastructural changes in the hypopharyngeal glands of honeybee *Apis mellifera* L. (Hymenoptera: Apidae) without and with infection by *Nosema apis* Zander (Microsporidae: Nosematidae). Int J Insect Morphol Embryol 18:73–83
Martin H, Lindauer M (1966) Sinnesphysiologische Leistungen beim Wabenbau der Honigbiene. Z Vergl Physiol 53:372–404
Meyer W (1954) Beobachtungen ubd Versuche über den Transport und die Verwnedung von dargebotonem Wachs bei der Honigbiene. Z Bienenforsch 2:147–157
Michener CD (1974) The social behavior of the bees. A comparative study. Harvard University Press, Cambridge
Muller WJ, Hepburn HR (1994) Juvenile hormone III and wax secretion in honeybees (*Apis mellifera capensis*). J Insect Physiol 40:873–88181M
Nachtigall W, Kresling B (1992) Bauformen der Natur. Teil II: Technische Biologie und Bionik von Platten- und Falt-Konstruktionen. Naturwissenschaften 99:251–259
Örösi-Pàl Z (1956) Experiments on stimulating wax production by feeding oil to bees. Meheszet 4:87 [in Hungarian]
Örösi-Pàl Z (1957) The role of the mandibular glands of the honeybee. Bee Wld 38:70–73
Perret-Maisonneuve A (1927) Secretion et utilisation de la cire chez I'abeille (*Apis mellifica*). C R Hebd Seances Acad Sci Ser D Sci Nat Paris 185:1317–1319
Philipp PW (1928) Das Kittharz, seine Herkunft und Verwendung im Bienenhaushalt. Biol Zentralbl 48:705–714
Phiancharoen M, Duangphakdee O, Hepburn HR (2011) Biology of nesting. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 109–132
Philipp PW (1935) Neues vom Wachsschüppchen und der Wachszange. Rhein Bienen-Ztg 8:241–244
Pirk CWW, Crous KL, Duangphakdee O, Radloff SE, Hepburn HR (2011) Economics of comb wax salvage by the red dwarf honeybee, *Apis florea*. J Comp Physiol B181:353–359
Raffiudin R, Crozier RH (2007) Phylogenetic analysis of honey bee behavioral evolution. Mol Phylogen Evol 43:543–552
Robinson GE (1987) Regulation of honey bee age polyethism by juvenile hormone. Behav Ecol Sociobiol 20:329–338
Robinson GE, Vargo EL (1998) Juvenile hormone in adult eusocial hymenoptera: gonadotropin and behavioral pacemaker. Arch Insect Biochem Physiol 35:559–583
Rösch GA (1927) Über die Bautätigkeit im Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 6:265–298
Roussy L (1929) Biological experiments and a test of the thesis of Perret-Maisonneuve. Bee Wld 10:4–5
Roubik WD (1992) Ecology and natural history of tropical bees. Cambridge University Press, Cambridge
Schmidt WJ (1941) Polarisationsoptische Versuche mit Bienenwachs. Kolloid-Zeit 100:140–151
Schoening FRL (1980) The X-ray diffraction pattern and deformation texture of beeswax. S Afr J Sci 76:262–265
Shearer DA, Boch R (1966) 2-heptanone in the mandibular gland secretion of the honeybee. Nature 206:530
Sullivan JP, Jassim O, Fahrbach SE, Robinson GE (2000) Juvenile hormone paces behavioral development in the adult worker honey bee. Hormones Behav 37:1–14

Tischer J (1962) Über die Herkunft der gelben Farbstoffe des Bienenwachses. Hoppe-Seyler's Z Physiol Chem 267:14–22
US Environmental Protection Agency (2001) Pesticide fact sheet methoprene
Vansell GH, Bisson CS (1935) Origin of color in western beeswax. J Econ Entomol 28:1001–1002
von Buttel-Reepen H (1915) Leben und Wesen der Bienen. Friedr Viewweg, Braunschweig
Whiffler LA, Drusedau MUH, Crewe RM, Hepburn HR (1988) Defensive behavior and the division of labor in the African honeybee (*Apis mellifera scutellata*). J Comp Physiol A 163:401–411
Woog P, Yannaquis N (1935) Sur l'orientation des molélcules de la cire d'abeille. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 201:1400–1402
Woog P, Yannaquis N (1936a) Orientation des molécules de la cire d'abeille et répercussion sur la solidité der rayons. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 202:76–78
Woog P, Yannaquis N (1936b) Orientation des molécules de la cire d'abeille. Répercussion sur la construction des rayons. Arch Phys Biol 13:134–149

Chapter 14
Material Properties of Scale and Comb Wax

Abstract Although the honeybee nest begins with the conversion of wax scales into combs, these two materials differ in their chemistry, crystal structure, tensile strength and stiffness which, in turn, are modified by honeybee secretions during comb-building. The strength of wax scales is about the same at temperatures between 25 and 35 °C, but declines above 35 °C; in contrast, comb wax is weaker and progressively decreases in strength with increasing temperature. The relative workability of wax scale is about the same between 25 and 45 °C, but it is the converse with comb wax. Wax scales are stronger and more distensible, but less stiff than comb wax at 35 °C, and require more energy to work than comb. The reworking of constructed comb is significantly more cost-effective than starting comb-building from scratch. Salvaging old comb wax is also energetically advantageous. Differences in the mechanical properties of scale and comb wax show that comb-building involves chemical modification of the waxes. The relative amounts and kinds of lipids affect comb stiffness amongst species. Likewise, differing kinds and amounts of protein in the waxes affect their mechanical properties. Highly-textured scales are converted from an anisotropic into an isotropic state. Lipases added during chewing modify the lipid composition of the scale in which stiffness is lost, but regained with the addition of proteins in comb-building. Beeswaxes are crystalline, the crystallites in wax scales are aligned, some perpendicular to the surface, others between 62° and 65° to the surface. Their origin is probably due to a fusion of the liquid products reaching the surface from the different cells in the wax gland complex.

14.1 Introduction

There are many reciprocal interactions between honeybees and their nests such as providing dance platforms, allowing gaseous exchanges, heating and cooling, transmission of vibrations in communication, humidity control and the like. However, first and foremost, the nest, be it single or multiple combs, must serve as

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_14,

a structural framework adequate to bear the physical loads placed upon it (Hepburn 1998). Although the nest begins with the conversion of newly secreted wax scales into combs, these two materials are startlingly different in their chemistry, crystal structure, tensile strength and stiffness which, in turn, are modified by the addition of secretions during comb-building (Kurstjens et al. 1985). These properties are continually modified throughout the comb's life span because, just before pupation, honeybee larvae cover the walls of their cells with silk, which immediately increases the loading capacity of the combs. So there are variations in the mechanical properties (elastic modulus, tensile strength and strain at maximum loading of a cell wall) with age. Many of us have watched bees gnawing old comb and dry-working wax (Lineburg 1924; Darchen 1980; Phiancharoen et al. 2011). In physical terms, it is energetically less expensive to re-work old wax than to work with newly secreted wax scales. The promiscuous reworking of previously constructed combs has its origins in the mechanical data that describe the materials, and lie in the crystal and chemical changes that occur in fashioning comb from newly secreted wax scales.

Comb wax lacks texture because it has been structurally and chemically modified by the bees during the comb-building process. Like wax scales, naïve comb wax also contains a unique profile of proteins, probably derived from both the wax scales being carried over to the combs, as well as from some substance added to the scales during mandibulation and comb-building (Huber 1814; Lineburg 1924). There is also the matter of silk. Honeybee silk is an α-helical fibroin (Rudall 1962), the micelles of which form a four-stranded array of coiled-coils parallel to the fibre axis (Atkins 1967). Honeybee fibroin is crystalline, relative to other insect silks (Lucas and Rudall 1968), but the hydrated fibres are only half as stiff as dry ones although they are equal in strength (Hepburn et al. 1979). Fibroin is hygroscopic and when solvated is highly distensible, largely owing to its molecular conformation (Flower and Kenchington 1967; Lucas and Rudall 1968). These structurally undesirable properties of fibroin are largely suppressed by the cocoon-spinning larvae. Turning to wax, from two centuries of optical and one of X-ray diffraction studies, we know that sheets of wax form crystals perpendicularly orientated to two sheets of glass when pressure is applied to them. As such, beeswax belongs to a class of materials intermediate between hard and liquid crystals. In this Chapter only the material properties of the waxes are considered, while the silks are discussed in Chap. 18.

14.2 Temperature Effects

About 30 years ago, an intensive series of measurements were initiated to discover how scale and comb wax behave mechanically when deformed at temperatures a honeybee nest was likely to be subjected (Hepburn et al. 1983, et seq.). For these measurements, rectangular slices of cell wall wax were taken from newly constructed combs, free of silk, and were stretched under controlled conditions

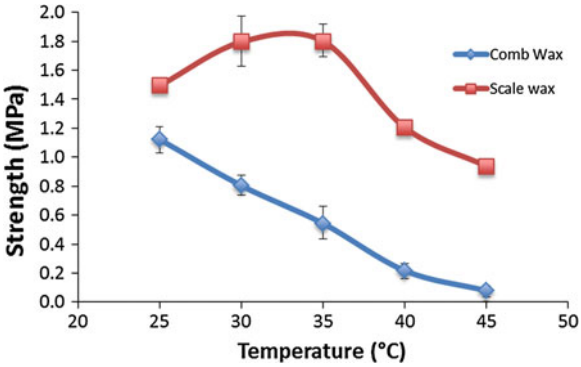

Fig. 14.1 Tensile strength of *A. m. scutellata* scale wax and newly constructed comb wall wax at different temperatures (Kurstjens et al. 1985)

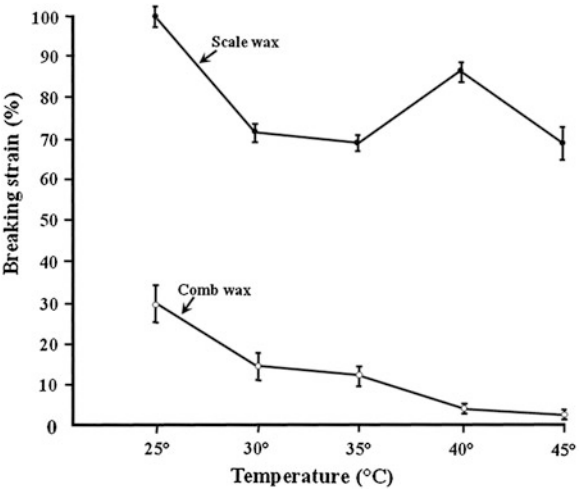

Fig. 14.2 Tensile breaking strain of *A. m. scutellata* scale wax and comb wall wax at different temperatures (Kurstjens et al. 1985)

(Hepburn and Kurstjens 1984). With some difficulty, Kurstjens et al. (1985) then sliced thin slivers of wax scales with razor blades, along the width of the scales, and stretched these specimens. Taking the scales first, their strength is more or less the same at temperatures between 25 and 35 °C; but, there is a major transition between 35 and 40 °C, over which strength declines very markedly indeed (Fig. 14.1). In contrast to this, comb wall wax is considerably weaker than scale wax over the whole range of temperatures, 25–45 °C, and steadily decreases in strength with increasing temperature.

The extent to which stretching wax samples to breaking point demonstrates that wax scales will literally flow three to six times more readily than comb wax below 35 °C, and above which there is about a 25-fold difference between the two waxes. This departure in the rate of change comes about because the scale wax stays in the range of 70–100 % elongation, while comb wax changes quite dramatically with increasing temperature from 30 % down to 2.5 % (Fig. 14.2).

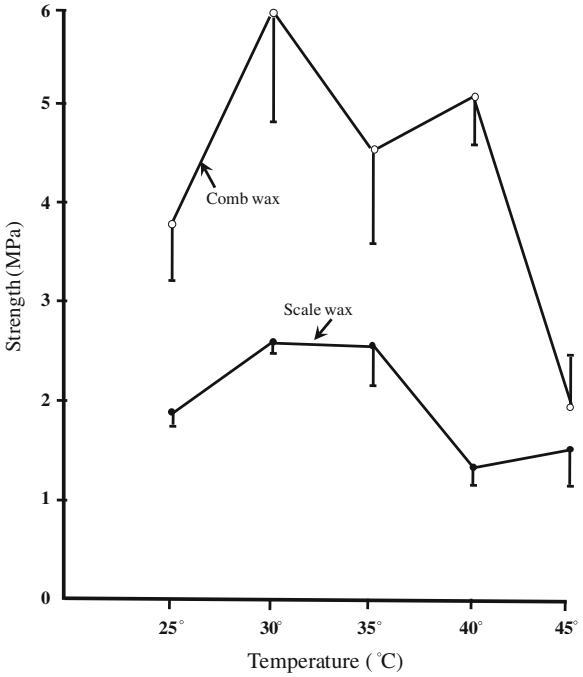

Fig. 14.3 Tensile stiffness of *A. m. scutellata* scale wax and comb wall wax at different temperatures (Kurstjens et al. 1985)

Neither the theory of plastics nor our current knowledge of wax chemistry is yet sufficiently robust to explain the basis of this behaviour. The stiffness (which is a measure of a material's resistance to deformation), of the two beeswaxes is shown in Fig. 14.3.

Comb wax is, on average, twice as stiff as wax scales. In the conversion of scales into comb, the bees must physically work the waxes. An indication of the work required to break a piece of wax is shown in Fig. 14.4. With the exception of the 40 °C value (which is simply anomalous and can simply be ignored), it is clear that the relative workability of the wax scale is more or less constant between 25 and 45 °C. Not so with comb wax; there is a quite dramatic decrease in the energetic cost of working comb wax at increasing temperature (Kurstjens et al. 1985).

To summarise, wax scales are stronger and more distensible, but less stiff than comb wax at a nest temperature of 35 °C. Wax scales require a greater initial input of energy to work than do cell wall wax. Thus, the promiscuous reworking of previously constructed comb in *A. mellifera* nests (Lineburg 1923; Darchen 1980; Hepburn and Whiffler 1991) and *A. cerana* nests (Phiancharoen et al. 2011), is a significantly more cost-effective and energetically parsimonious behaviour than starting any new building operation from scratch with scales. Likewise, salvaging old comb wax from recently abandoned nests by *A. andreniformis* (Duangphakdee and S Wongvilas, pers. comm.) and *A. florea* (Hepburn et al. 2010) is also

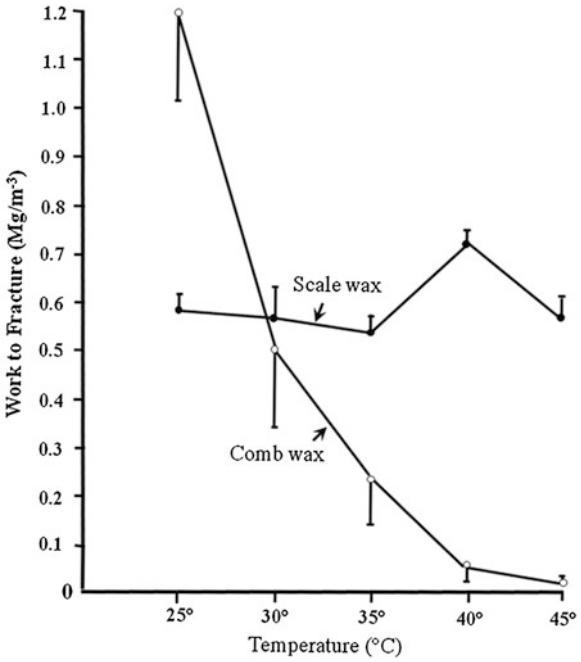

Fig. 14.4 Work to fracture of *A. m. scutellata* scale wax and comb wall wax at different temperatures (Kurstjens et al. 1985)

energetically advantageous (Pirk et al. 2011). The results of mechanical tests on scale and cell wall waxes are empirical in nature; they describe phenomena but do not explain how they come about.

14.3 Crystal Changes

The origins of the mechanical data on beeswax lie in the crystal and chemical changes that occur in fashioning comb from newly secreted wax scales (Kurstjens et al. 1985). A coherent picture of the material properties of the different waxes has been accumulating slowly over the years, beginning with Brewster (1815) and more recently by Kurstjens et al. (1985), Hepburn and Kurstjens (1988), Kurstjens et al. (1990) and Buchwald et al. (2006, 2009). There are orders of magnitude differences in the mechanical properties of wax scales and comb wax, and their texture-adjusted films clearly indicate that the process of comb-building involves chemical modification of the waxes (Kurstjens et al. 1985). Further analysis of the protein fraction revealed some 17 bands in the electrophoretograms, some unique to each wax (scale and comb), and others shared (Kurstjens et al. 1990). Two inferences were made from the data; two fractions common to both waxes are of similar molecular weight to other insect lipophorins and they may well be gland-to-surface transport proteins. In the mastication of wax scales, additional protein is

added, presumably lipases, because combs have a higher monoacylglycerol content than the diacylglyceride-richer wax scales (Davidson and Hepburn 1986). The effect of the latter is to increase the degree of saturated bonds in comb wax, thus contributing to better stiffness (Kurstjens et al. 1990).

Since lipolytic enzymes added by the bees to the wax require an aqueous medium to form reactions, a source of moisture needs to be present in the wax medium. Given an average relative humidity in a hive of about RH-50 (Simpson 1961), moisture is available as a by-product of worker bee respiration and thermoregulation as well as the dehydration of nectar (Ellis et al. 2010). Some means to deliver this moisture into the wax is also required. Donhowe and Fennema (1992) demonstrated that the water vapor permeance of beeswax films is sufficient to deliver 1.7 g of water per kg wax into the comb structure. They further pointed out that although beeswax is primarily hydrophobic, the esters, hydroxyl groups of free alcohols and the carboxyl groups of free fatty acids in beeswax are hydrophilic.

Finally, discoveries about comb chemistry have been made, but their significance is not yet apparent. Puleo (1991) summarized the details of the minor constituents of *A. mellifera* beeswax of and listed some 117 compounds derived from propolis commonly found in comb wax (cf. Chap. 16). Of these, 41 are specifically associated with wax aroma, which of course easily leads to discussions of kin recognition and colony odour. Similar reports on compounds derived from propolis have appeared elsewhere (Seifert and Haslinger 1989, 1991; Tomas-Barberan et al. 1993).

Although ubiquitous in the hives of *A. mellifera*, propolis, or 'bee gum' by its older name, is a collection of lipophilic plant exudates and resins that honeybees collect from resins of buds, bark and sap of plants; but otherwise whose origins have been intractably obscure (Nakamura and Seeley 2006). This, despite the fact that it has become a recent pharmaceutical commodity of no small importance in the Orient. Park (1946) provided an excellent account recalling that bees preferentially collect propolis during in late northern summer when the tacky substance is malleable. Many early accounts remark, unfavourably, on the propensity of different *A. mellifera* races to gather propolis. The way in which foragers collect propolis was described by Astor (1899), Betts (1921) and Alfonsus (1933).

An older view, never pursued beyond the time, was that there are actually two kinds of propolis; that derived from plant exudates, and another as chyme from the digestion of pollen (Philipp 1928). Philipp further observed that all cells in which eggs will be laid are first coated with this substance, a point confirmed by Chauvin (1962). The chemical, pharmacological and pharmaceutical uses for propolis have been reviewed often (Marcucci 1995; Bankova et al. 2000, 2006). However, the significance of propolis in honeybee hygiene, social immunity and medication have only recently been investigated and is thoroughly discussed by Simone et al. (2009) and Simone-Finstrom and Spivak (2010). While these matters are of considerable importance in honeybee biology, their purview is beyond the needs of the present text.

Fig. 14.5 Resinous sticky band of a twig extending towards a single comb *A. florea* nest (Phiancharoen et al. 2011)

The use of 'propolis' is thought to be unique to *A. mellifera* and is absent from any mention in the Asian honeybee literature (Hepburn and Hepburn 2011). Nonetheless, propolis in movable frame hives containing *A. cerana* has been observed at Kathmandu, Nepal (Hepburn, unpubl. obs.). However other species also use resins of one sort or another; the dwarf honeybees, *A. florea* and *A. andreniformis*, both utilize plant resins as nest material but not structurally (Duangphakdee et al. 2005a, b; Duangphakdee 2006). They apply a band of sticky resin around the twigs supporting the comb (Fig. 14.5). These bands are about 2.8 ± 2.1 cm wide with a range of 0.5–10.05 cm, and trap any small animals attempting to gain access to the colony (Seeley et al. 1982). A band is built on both sides of the comb, but there is a strong tendency for the band to be thicker on the side proximal to the tree trunk than on the distal side of the twig tip (Duangphakdee, pers. obs.).

The sticky bands of *A. florea* and *A. andreniformis* have clear-cut repellent properties against weaver ants, *Oecophylla smaragdina* (Duangphakdee et al. 2005b). Repair and re-enforcement of the sticky bands by *A. florea* is strongly correlated with invasions of weaver ants into the nest (Duangphakdee et al. 2005b). Duangphakdee (2006) performed some preliminary GC-MS analyses that give a rough idea of the chemical constituents of the sticky bands. The resins consist of more than 50 compounds (Fig. 14.6); the most abundant being a triterpene (amyrin—45.72 %) and steroids (30.32 %).

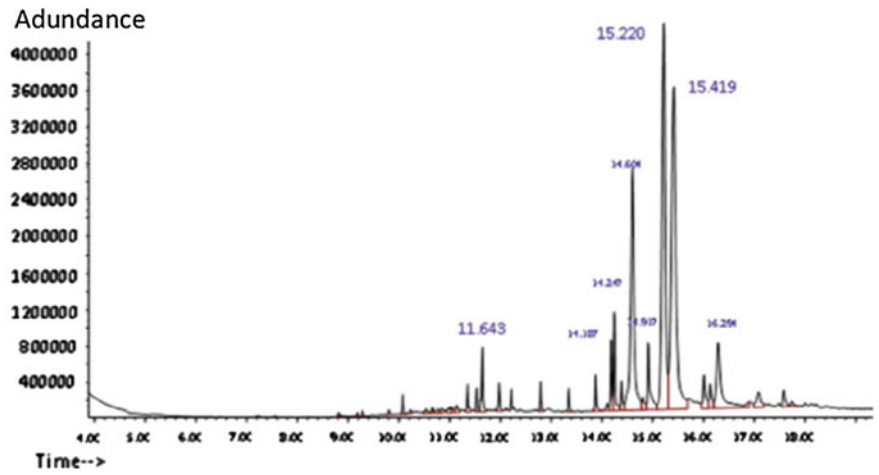

Fig. 14.6 Chromatogram of the sticky band material collected from *A. florea* nests. Preliminary identifications were based on the WILEY 7 N library database. To consider only the main constituents: $R_t = 11.643$, no satisfying library match; $R_t = 14.604$, triterpene (amyrin); $R_t = 14.917$, heptacosane; $R_t = 15.220$, steroid (cyclolanostenol; R_t = retention time) (Duangphakdee 2006)

The physical effects of wax hydration would include matrix swelling and an increase in the diffusion coefficient of the wax (Donhowe and Fennema 1992). The modifications of comb performance by the presence of proteins and water can now be related to the material properties of combs as they evolve in the nest (Hepburn and Kurstjens 1988). In the course of its development, new comb wax is an isotropic 'plastic' whose mechanical properties depend heavily on temperature. In time, generations of larvae introduce silk into the waxen structure in a random alignment to achieve equal properties in all directions (as in random mat fibreglass structures). Thus with use, the comb becomes a fibre-reinforced composite material which exhibits properties entirely different from the individual components. The addition of silk greatly improves the load-carrying capacity of the combs (Hepburn and Kurstjens 1988). Although not a theoretically ideal stiff plate structure (Nachtigall and Kresling 1992), the mature comb is nonetheless a remarkable compromise between its technical construction and the biological purposes it serves.

14.4 Tensile Properties

In their detailed analyses of combs, Zhang et al. (2010) noted that bees basically need to stiffen and strengthen their combs to avoid fragility, which they explained by performing a finite element analysis of the comb. They calculated the stress and

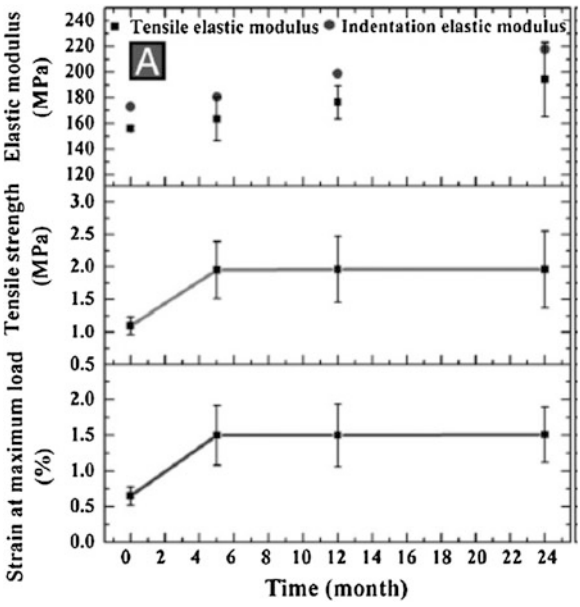

Fig. 14.7 Variations in the mechanical properties of an *A. m. ligustica* comb cell wall with age. Elastic modulus, tensile strength, and strain at maximum loading of a wall (with kind permission from Zhang et al. 2010)

strain fields in new and old combs using a linear elastic finite element model at 25 °C. For newly constructed comb, the maximum normal stress and the corresponding strain along the axis of the cell were found to be 72 kPa and 0.05 % respectively for the combined weight of honey and worker bees, which are well below the tensile strength (1.1 Mpa) and the corresponding strain (0.65 %) of the cell wall at 25 °C (Fig. 14.7). The computed maximum nominal out-of-plane shear stress (0.11 kPa) and the corresponding shear strain (0.04 %) in new comb are also below the nominal shear strength, results which explain how new comb can safely carry the weight of both honey and bees.

Zhang et al. (2010) further examined the effect of the viscoelastic nature of new beeswax on the stress and strain fields in the wall of new comb. The finite element method and an appropriate viscoelastic model were used to calculate the stress and strain fields in new comb at 45 °C. They found that, as a result of creep deformation, the maximum out-of-plane shear strain in a fully laden new comb reaches 1.9 % higher than the shear strain at the maximum load of new comb (1.5 %) at 45 °C. Thus, a temperature increase inside the combs from 25 to 45 °C would result in the collapse of fully laden new combs. That this does not actually happen is because the comb walls are continuously reinforced by silk cocoons during use (Hepburn and Kurstjens 1988). Old comb walls that contain 34 % silk cocoons by mass are practically insensitive to temperature fluctuations (Hepburn and Kurstjens 1988). The finite element calculations of Zhang et al. (2010) show that even if there is some decrease in the shear modulus and strain of older combs with increasing temperature, they will still have a sufficient margin of safety against collapse, in an engineering sense.

Differences in the relative amounts of the major families of compounds in the waxes could be expected to be reflected in the physical and mechanical properties. Buchwald et al. (2006) reported the results of a comparative study of the mechanical properties of beeswax samples from *A. andreniformis*, *A. cerana*, *A. dorsata* and *A. mellifera* and measured, among other things, the relative stiffness and resilience of the waxes. Because the mechanical properties of any structure result from both the intrinsic chemical nature of a material as well as its structural form, it is obviously desirable, but experimentally extremely difficult, to work with whole comb specimens. So Buchwald et al. (2006) compromised by eliminating structure and simply measured the behaviour of wax cylinders under compression.

Although compression testing is not biologically appropriate for extrapolation to whole combs, which are actually tension members with a relatively complex structure, the results of such measurements have heuristic value in trying to relate mechanical behaviour to differences in the major compound families of comb waxes. Resilience represents the amount of energy required to deform the test material until it begins to fail irrecoverably. Stiffness is simply the rate of change of stress per unit strain. Figure 14.8 shows that *A. dorsata* wax is significantly stiffer than the other *Apis* species tested. The waxes of *A. cerana* and *A. dorsata* do not differ significantly but are significantly more resilient than that of the intermediate *A. mellifera*, which in turn is more resilient than that of *A. andreniformis*.

Based on their results, Buchwald et al. (2006) showed that the combs of *A. dorsata*, the giant honeybees, which build single but very large combs, are indeed the stiffest and most resilient of all combs among the honeybee species. They must also sustain the weight of the honey stores and brood nest ($\sim$45 kg), and because the position of their nests are often on branches high up in trees, they are also exposed to possible wind damage. The multiple combs of the medium-sized bees (*A. cerana*, *A. koschevnikovi*, *A. mellifera*, *A. nigrocincta* and *A. nuluensis*), are usually constructed in cavities with multiple attachment sites so that the load of nest contents is widely distributed over several points of attachment. The dwarf honeybees make small, single combs which are seldom exposed to extreme weather. At the end of the day, it must be remembered that mature combs are not exclusively made of beeswax. The combs of all honeybee species are fibre-re-inforced, with increasingly more silk deposited with each successive generation of brood (Hepburn and Kurstjens 1988). And, while our knowledge of beeswax advances, that of the silk fraction (cf. Chap. 18) is thus far restricted to *A. mellifera* (Hepburn et al. 1979; Sutherland et al. 2011).

Just before pupation, honeybee larvae cover the walls of their cells with silk (Huber 1814; Arnhart 1906; Jay 1964), paying out the fibres randomly so that by the end of spinning the walls are covered by thin sheets in which the individual fibrils are readily discernible (Jay 1964). Subsequently the larvae produce a colourless, pollen-free material from the anus and then a yellow pollen-bearing one, both of which are applied in turn to the silk base (Verlich 1930; Jay 1964). Nothing further is known of these substances, but they invite the analogy of sizing in paper manufacture. Successive generations of brood apply more silk to the walls, the cell volume becomes reduced, and the mass ratio of silk to wax increases (Chauvin 1962).

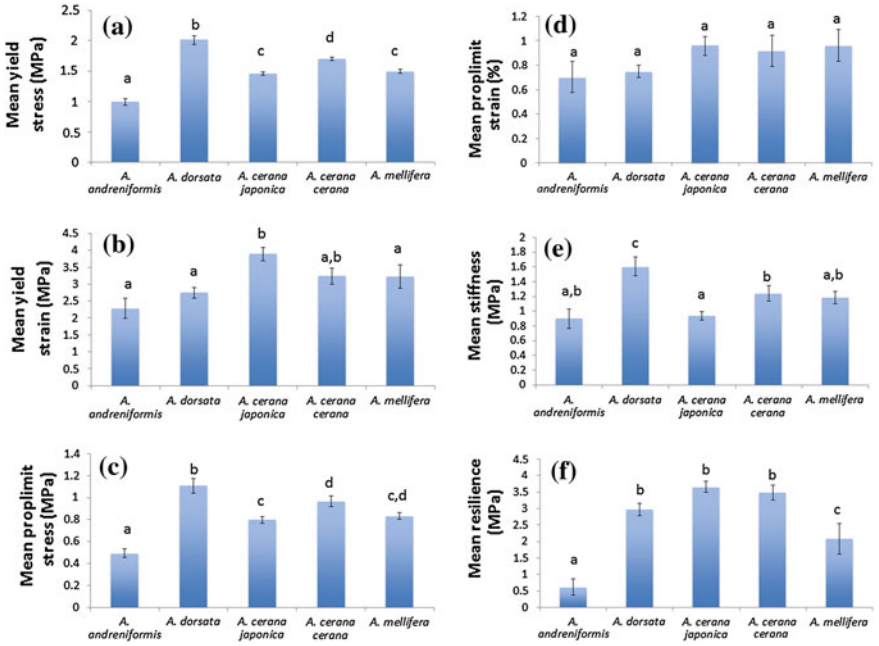

Fig. 14.8 Comparisons among the waxes of five honeybee species (*A. andreniformis, A. dorsata, A. cerana japonica, A. cerana cerana* and *A. mellifera*) for six mechanical measures: **a** yield stress, **b** yield strain, **c** stress at proportional limit, **d** strain at the proportional limit, **e** stiffness, **f** resilience (Buchwald et al. 2006)

Thus, old brood combs are heavily impregnated with silk which is inseparable from the wax except by fairly rigorous chemical and/or heat treatments. The development and maturation of brood comb proceeds from a single-phase material of pure white wax, to a coloured, fibre-reinforced, two-phase composite.

After a brief discussion of scale wax, the physical significance of these observations will be illustrated by comparing the properties of naïve fibroin, wax-free sheets of silk, silk-free wax, propolis and the final wax-silk composite. Wax scales are fused laminated structures (Huber 1814; Philipp 1935; Jordan 1962; Zhang et al. 2010), in which the well defined crystallites are vertically inclined to the plane of the scale. Thus, in uniaxial tensile tests in the plane of the scale, the crystallites have their c-axes normal and inclined to the direction of the load. The significance of this textural arrangement in the deformation of scales is demonstrated in part by comparing whole scales with chemically untreated but sheeted specimens made from molten wax scale. The results and tests for significant differences among them (Table 14.1) show that sheeted wax scale is both stronger and stiffer than naïve wax scales, but are of equal distensibility at fracture at 23 °C.

Because the scales are loaded normal to the c-axis of their textured crystallites, this implies that the crystallites probably flow passively in the amorphous matrix of the wax scale and contribute little to strength or stiffness. The increased strength

Table 14.1 Tensile strength, strain and stiffness of the wax preparations from *A. m. scutellata* deformed in the surface plane at 23 °C (mean ± SEM)

Wax sample	Strength (MPa)	Strain (%)	Stiffness (MPa)
Wax scales (untreated)	1.5 ± 0.2	61.3 ± 9.9	2.6 ± 0.3
Comb wax (untreated)	1.5 ± 0.5	35.6 ± 3.2	4.2 ± 0.4
Wax scales (sheeted)	2.1 ± 0.14	56.8 ± 1.8	3.6 ± 0.3
Comb wax (sheeted)	1.1 ± 0.07	23.6 ± 2.4	4.9 ± 0.4
Wax scales (soxhlet-extracted)	1.87 ± 0.04	85.6 ± 4.3	2.2 ± 0.1
Comb wax (soxhlet-extracted)	1.13 ± 0.09	36.0 ± 0.8	3.2 ± 0.3

For each value given, n = 6. All of these waxes were planar isotropic except for wax scales which could only be tested along its greatest length of sheeted wax (Kurstjens et al. 1985)

of sheeted scale wax could be due to the random re-orientation of the crystallites as shown by x-ray diffractograms (Kurstjens et al. 1985). In that case, some of the crystallites will have their molecular axes pointing more or less in the direction of loading. With a relatively low extension rate, it is probable that other crystallites become aligned to the load during deformation. This notion is supported by the development of texture in crude beeswax prepared by annealing between plates of glass (Brewster 1815; Schmidt 1941), film extrusion (Woog and Yannaquis 1936a, b) or by cold-rolling (Schoening 1980).

Although a full chemical analysis of wax scales has not been made, the presence of differing kinds and amounts of protein in both scale and comb wax might well contribute to their bulk mechanical properties. Kurstjens et al. (1985) assessed this possibility by comparing specimens of scale wax that had been soxhlet-extracted to obtain protein-free wax and then sheeted, with others that had only been sheeted, so that the only difference was the presence of a protein fraction in the scale wax. Their results showed that these two materials are indeed significantly different from one another (Table 14.1). The sheeted wax is significantly stiffer but less distensible than soxhlet-extracted sheeted wax. The implication is that the protein fraction in wax scales makes a positive contribution to the strength of this material.

In support of these interpretations, it is very gratifying to note that both wax scales and comb wax (produced under pollen-free conditions) were found to contain protein. Indeed, Kurstjens et al. (1985) found that, after 72 h of extraction, the protein content of the scale and comb wax was 2.2 μg protein/mg and 5.6 μg protein/mg, respectively. These results clearly demonstrate a more than twice greater mass of protein to lipid in finished comb than in scale wax. Moreover, the differences in rates of recovery of protein from the two waxes in a series of sequential extractions further indicated specific differences in the two protein fractions. It is also important to note that the comparison of mechanical measurements of soxhlet-extracted sheeted waxes with only sheeted waxes, tacitly subsumes the removal of protein in the extraction process. Indeed, this was experimentally confirmed by the presence of only trace amounts of protein in a Lowry assay (Lowry et al. 1951), performed on the soxhlet-extracted wax which had been subjected to the Folch procedure (Folch et al. 1957).

14.5 Crystal Texture

It is also necessary to consider the structure, composition and mechanical properties of comb beeswax vis-à-vis those of the starting material. It has been shown that comb lacks texture and it is known that the wax has been structurally and chemically modified by the bees in the process of comb construction (Huber 1814; Lineburg 1924; Hepburn and Kurstjens 1988). The results of the combined mechanical measurements and chemical extractions provide the means for analysing how the final properties of the comb have arisen. The possible variables are changes in texture, structure, chemistry and mechanical properties; the last a consequence of the former three, and is used to illuminate the importance of the other variables.

Unlike scale wax, comb wax lacks texture and is not laminated. It does, however, have a loosely particulate structure (Huber 1814), readily visible with polarizing microscopy (Schmidt 1941), and more clearly delineated in environmental scanning electron micrographs (Zhang et al. 2010). The significance of these differences is seen in a comparison of comb cell walls and sheeted comb wax. While both are planar isotropic and of equal strength and stiffness (Table 14.1), they differ significantly in extensibility. The greater extensibility of naïve comb wax suggests that there is an incomplete fusion of the new pieces of wax as they are added to the comb by bees during comb-building. This explanation, which is based on incomplete fusion, is consistent with what is known about the actual methods of comb construction (Casteel 1912; Lineburg 1924; Schmidt 1941). This is similar, by analogy, to the way in which sheets of comb wall wax placed on filter paper fragment along ‘glue lines’ when treated ‘chromatographically’ by applying pentane to the edge of the filter paper (Hepburn, unpubl. obs.).

14.6 Wax Proteins

Like wax scales, comb wax also contains a unique profile of proteins that probably derives both from the wax scales being carried over to the combs, as well as from some substance added to the scales during mandibulation and comb-building (Huber 1814; Lineburg 1924). The effect of the protein fraction in comb wax can be observed in a comparison of sheeted comb with soxhlet-extracted sheeted comb. While these two preparations are of about equal strength, untreated sheeted wax is significantly stiffer and less distensible than is the soxhlet-extracted equivalent (Table 14.1). Kurstjens et al. (1985) concluded that the presence of the protein fraction in comb wax improves its resistance to deformation, a result very similar to that observed for scale wax.

The differences in the mechanical properties of scale and comb waxes with respect to crystallographic texture and protein composition invite a consideration of their lipid compositions. That these waxes differ with respect to their lipid

profiles is shown by several comparisons. For example, a comparison of soxhlet-extracted, sheeted preparations of scale wax with similar preparations of comb wax (texture and protein being absent from both preparations), shows that while sheeted, soxhlet-extracted scale wax is stronger than its comb counterpart, its greater extensibility makes it less stiff. These differences in mechanical behaviour point to a substantial influence of the compositional variation in the lipids on the properties of the wax scales and the wax of finished combs (Kurstjens et al. 1985; Buchwald et al. 2009).

To further establish the effects of these probable chemical differences, a comparison of scale and comb waxes, both of which have been soxhlet-extracted and then sheeted, was made. In this case not only were texture and structure equalised, but possible contributions from non-lipoidal material were eliminated as well. The results (Table 14.1) show that the soxhlet-extracted scale wax is significantly stronger and more distensible than the corresponding soxhlet-extracted comb wax. The former is consequently not as stiff as the latter. The implication of this finding is that beeswax scales differ from comb wax in lipid composition; a fact subsequently confirmed by gross chemical analyses of the two waxes (Kurstjens et al. 1985; Davidson and Hepburn 1986).

The thin-layer chromatography plates of these two waxes, run against the appropriate mixed standards, showed that scale wax did not exhibit a monoglyceride fraction detectable by the method used, but gave a relatively large diglyceride pool. On the other hand, new comb wax contained detectable amounts of monoglycerides and a diglyceride fraction that was less intense than that of scale wax. These results show, pointedly, a gross difference in the monoglyceride and diglyceride compositions of scale wax and new comb wax (Kurstjens et al. 1985; Davidson and Hepburn 1986). The role of fatty acids in the mechanical properties of beeswax has been confirmed and further explored by Buchwald et al. (2006, 2009). They reported that the removal of fatty acids from beeswax results in diminished yield stress, resilience, stiffness, and proportional limit stress in beeswax samples (Fig. 14.9).

The total effects of the manipulation of wax scales by honeybees can now be summarised. In the process of mandibulation, the highly-textured scale is thoroughly masticated and is converted from a texturally anisotropic body into an isotropic one. At the time of chewing, the bees also add a salivary secretion to the wax that, at the very least, contains a lipase that modifies the lipid composition of the starting material; there is a marked reduction of the diglyceride fraction of the scale and a concomitant increase in the monoglyceride pool of the comb. Although full analyses of the protein fractions is not as yet available, it is evident that whatever protein is injected into the wax on chewing certainly acts to stiffen the final product. So, the stiffness of the scale that arises from its texture is lost on chewing, but is regained with the addition of proteins in comb-building.

In bee terms, the mechanical findings are of great significance. While wax scales are ideal moulding material due to their very high distensibility and relatively low stiffness, these properties make them unsuitable structural material. However, comb wax, which is modified scale wax produced by the bees during

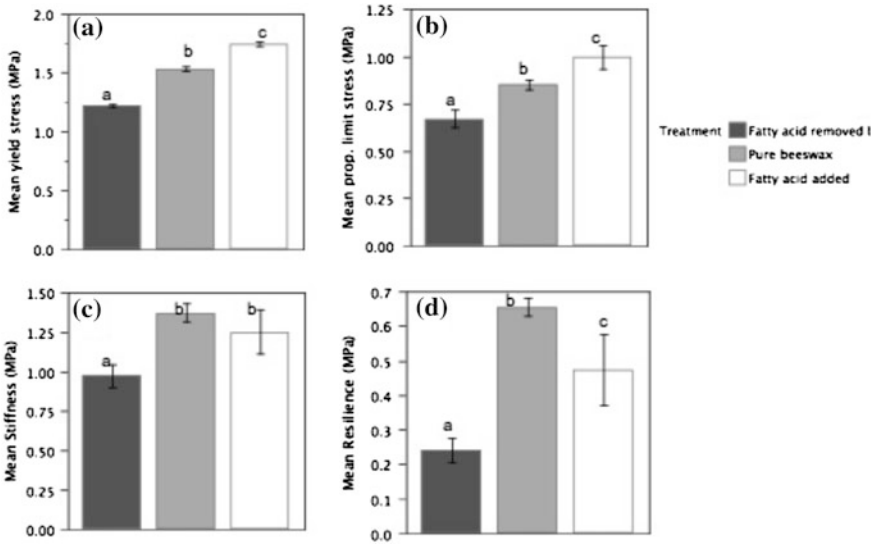

Fig. 14.9 The effects of fatty acids on the mechanical properties of *A. mellifera* beeswax. Values represent means and standard errors for beeswax with the free fatty acids removed, unmodified beeswax, and beeswax with added stearic acid. Four mechanical properties were examined: **a** yield stress; **b** stress at the proportional limit; **c** stiffness; and **d** resilience. N = 6 samples for each column. Matching letters above columns indicate no significant difference between the columns; differing letters indicate a significant difference (Buchwald et al. 2009)

comb-building, is a superior structural material. These are the conclusions that have been reached in recent studies of these waxes at ~23 °C, a temperature likely to be fractionally too low for comb-building. But, given a nest temperature of about ~35 °C, the process of chewing and building comb results in a final product that has twice the stiffness of the starting material, yet requires only half the ergonomic effort after it has been modified by mandibulation and the probable addition of a lipase.

14.7 α-Helical Silk

Honeybee silk is a α-helical fibroin (Rudall 1962), the micelles of which form a four-stranded array of coiled-coils parallel to the fibre axis (Atkins 1967). Honeybee fibroin is crystalline relative to other insect silks (Lucas and Rudall 1968), but the hydrated fibre is only half as stiff as dry ones, although they are equal in strength (Hepburn et al. 1979). The fibroin is hygroscopic and when solvated is highly distensible largely owing to its molecular conformation (Lucas and Rudall 1968). These structurally undesirable properties of fibroin are largely suppressed by the cocoon-spinning larvae. The fact that silk is impacted in the wax of the cell wall,

possibly aided by larval anal secretions, immediately checks the susceptibility of fibroin to solvation. Thus it is likely that inter-micellar friction is also enhanced (Warwicker 1960), and the conformational change restricted (Rudall 1962); effects which are consistent with good stiffness and reduced distensibility (Hepburn et al. 1979).

That silk fibres are spun and randomly arranged in the cell wall overcomes the basic anisotropy of the material; dewaxed sheets of cocoon silk are planar isotropic on tensile deformation. Natural variations in the temperature of honeybee nests invite a consideration of silk behaviour accordingly. The independence of the mechanical properties of sheets of honeybee fibroin deformed in tension at a fixed rate between 25 and 45 °C are given in Table 18.1. Sheets of silk maintain the same relative strength and distensibility. Consequently, changes in stiffness or the energy to fracture the sheet, an index of its relative workability, were not observed. The tensile properties of silk sheets over this range of temperatures are in sharp contrast to those of pure wax (Hepburn et al. 1983), propolis (Hepburn and Kurstjens 1984) and the wax-silk composite of brood combs (Hepburn and Kurstjens 1988).

14.8 Optical Studies

The first studies on the crystalline nature of the comb wax of the honeybee, *A. mellifera,* were those of Brewster (1815) who, in the early 19th century, investigated the reflection and refraction of plane-polarised light in different materials, the results of which now constitute Brewster's Law (Fig. 14.10). In a seminal paper, Brewster (1815) reported the results of experiments on the depolarisation of such diverse substances as spinel rubies, soap, ice, grape-skins and beeswax. He demonstrated that the cell walls of honeybee comb wax became transparent in Canada balsam and then depolarised light in every direction, lacking any neutral axis. The same was true of white comb wax that had been annealed between two sheets of glass. In both cases, Brewster had shown that, although beeswaxes appear amorphous, they are actually crystalline.

Brewster's discoveries of the laws of polarization of biaxial crystals, optical mineralogy, and double refraction by compression, remain major scientific achievements for that time. The crystalline nature of *A. mellifera* beeswax was rediscovered by Ehrenberg (1849); a principle subsequently confirmed many times for *A. mellifera* by optical methods in different circumstances (Ambronn 1892; Cesàro 1903; Gaubert 1910a, b; Schmidt 1924, 1941). Beeswax scales were first examined with polarisation microscopy by Dujardin (1850), who noted that, if a scale is crumpled or indented with a pin, the individual layers turn up at the edges and strongly depolarise light at an angle inclined to the depolarised plane. Thus, the crystals run more or less obliquely to the surface of the wax scale as was subsequently corroborated by Ambronn (1892).

Recognition of the precise arrangement of the crystallites in beeswax followed shortly after the Nobel laureate, Max von Laue, had developed the theory (1911):

Fig. 14.10 David Brewster (11 December 1781–10 February 1868) was a Scottish physicist, mathematician, astronomer, and inventor

that the distance between layers of atoms in crystals might be of the right order of magnitude for their measurement by the diffraction of X-rays. Indeed, this general principle was demonstrated experimentally in 1913 by W Friedrich, who obtained very regular patterns of spots from crystals of zinc sulphide, but only diffuse patterns from beeswax (von Laue 1913). The inclination of crystallites in both wax scales and in patches of comb cell wax were subsequently confirmed by X-ray diffraction studies (Woog and Yannaquis 1935), but, the chemical nature of the crystalline fraction had not yet been defined, despite the very large range of chain lengths already known to occur in wax (Halle 1931; Chibnall et al. 1934).

Many years later, Schoening (1980) reinvestigated wax and showed that it contains two crystalline components as well as an amorphous region. On the basis of the side spacings in his diffractograms, he identified component 'A' as a monoester fraction giving rise to needle-like crystals, and a component 'B' which he thought probably represented the diester and free acid fractions due to their long spacings. Shortly after, Kurstjens et al. (1985) extended Schoening's analyses of *A. m. scutellata* scale and honeycomb wax. The crystallites in wax scales are aligned, some perpendicular to the surface, with others between 62° and 65° to the surface plane. The comb wax did not show any clear arrangement of crystals. The arrangement of crystals in a wax scale is shown diagrammatically in Fig. 14.11.

14.9 X-ray Diffraction Studies

Brewster (1815) had observed texture in annealed wax, an effect further explored by Woog and Yannaquis (1935, 1936a). Using diffraction techniques, they showed that wax ribbons prepared by extrusion at different temperatures (as is routinely

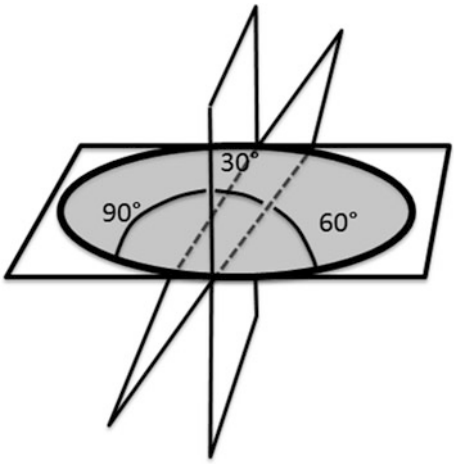

Fig. 14.11 Diagrammatic representation of the arrangement of crystallites in a newly secreted *A. m. scutellata* wax scale (Hepburn 1986)

done in the manufacture of sheets of beeswax foundation), showed enhanced texture at higher temperatures (38 °C) than at lower ones (17 and 29 °C), and the same was true of ribbons annealed at higher temperatures. New comb wax kept at 38 °C for 5 months was more crystalline than that stored at 15 °C. The former was more delicate and brittle than the latter, an effect quantified by measuring the loads required to break the ribbons. Wax kept at the higher temperature required 150 % of the load necessary to break than at lower temperatures. Thus, Woog and Yannaquis (1935, 1936a, b) showed that wax scales are more crystalline than comb wax, and that this texture is largely destroyed by bees when they chew them as reported by Casteel (1912). A diagrammatic interpretation of the arrangement of crystallites in a beeswax scale is shown in Fig. 14.11. Against this, the initial weakness of a newly constructed comb will be ameliorated in time by the warmth of the nest. The possibility that heat enhanced crystallisation was briefly addressed by Kratky (1937), and more extensively by Schmidt (1941). The significance of this effect is discussed below.

Several important remarks on the induction of crystal orientation in wax have appeared through the years. Following the note of Dujardin (1850) on wax scales, Gaubert (1910a, b) found that beeswax, like cholesteric salts and ammonium oleate, can form sheets of crystals perpendicularly orientated to two sheets of glass when pressure is applied. As such, beeswax was shown to belong to a class of materials intermediate between hard and liquid crystals. Similarly, the films of wax that Woog and Yannaquis (1935, 1936a, b) prepared by extrusion were more textured than comparably treated pieces of comb cell wax. Finally, the observations of Schoening (1980) are of great interest here because, using optical and X-ray techniques, he showed that the molecular axes of the crystals tended to be perpendicular to the axes of compression in deformed samples. Of equal interest, the molecular chain axis was not preferentially orientated along the tensile axis in specimens broken in tension.

Kurstjens et al. (1985) extended the earlier observations of Schoening (1980) with both wax scales and newly constructed combs produced by African honeybees, *A. m. scutellata.* Three different preparations of both wax scales and comb wax were investigated: (1) untreated sheeted; (2) chloroform/soxhlet-extracted and sheeted, and (3) untreated samples of comb cell wall and wax scales. The sheeted waxes were formed on a spreader blade coater, with siliconised release paper as the substrate. The molten wax was poured into the groove between the glass doctor and the release paper, which was pulled at a uniform rate. Constant layer thickness was obtained under conditions of maximal paper tension and minimal gape between substrate and glass applicator. These different sheets of wax were then used to produce test specimens. X-ray analysis was used to determine the presence or absence of crystallographic texture in all six preparations of wax. To obtain side-spacings, specimens were mounted on a goniometer and oscillated 10° about an axis perpendicular to the beam. Nickel filtered copper K $\acute{\alpha}$ radiation was used for these measurements. Transmission photographs were taken with the X-ray beam, both normal and parallel to the planar surface of the wax samples.

It is generally accepted that for long-spacings to be observed, molecules of a given length must predominate in the sample, because if the crystallites are composed of a mixture of chains of very divergent lengths, then no equally spaced planes can be formed and therefore no long-spacings will appear. If, however, the difference in chain lengths is not too great, mixed crystals may be formed and long-spacings will be observed. On the other hand, when the difference in chain lengths is too large, a mixture of crystals with different long-spacings may be observed. In this case, a compound such as beeswax, which is a complex mixture of different components, will exhibit more than one set of long-spacings, the reflections of some of which may be extremely faint. In the usual X-ray camera these reflections may be difficult to separate because of the relatively small radius of the camera.

In light of these considerations, Kurstjens et al. (1985) measured long-spacings on the diffractometer and special procedures were used in sample preparation. This involved melting the wax between two glass slides under slight finger pressure. Long-spacings were obtained using manganese filtered Fe K $\acute{\alpha}$ radiation. During the X-ray measurements the specimens were rotated around an axis normal to the specimen plane. To minimise systematic errors at low diffraction angles, diffraction peaks at both positive and negative diffractometer angles were obtained, and the angular difference between them measured. These measurements showed the presence of a strong reflection which was accompanied on its high angle shoulder by a weaker reflection.

14.10 Crystallites of Beeswax

In the work by Kurstjens et al. (1985) on the waxes of *A. m. scutellata* side-spacings for all specimens were easily observed in transmission and reflection. The crystal structures were monoclinic ($a \neq b \neq c$, $\acute{\alpha} = \gamma = 90°$, $\beta \neq 90°$) and

orthorhombic ($a = b = c$, $\alpha = \beta = \gamma = 90°$). If the c-axis is taken to be along the long axis of the molecule, then the side-spacings, d, are given by *hkO* reflections with the quadratic form: $1/d2 = h^2/a'^2 + k^2/b^2$, where a' is a in the orthorhombic crystal structure, and a' is a sin β in the monoclinic unit cell. The definitive crystallographic parameters of a sin β and b were obtained from a linear plot of $d^2 = h^2$ versus d^2k^2 using the equation: $d^2h^2 = -a^2$ sin2 β/b^2. $d^2k^2 + a^2$ sin^2 β (Fig. 14.12). The d values, Miller indices, intensities and crystallographic parameters of all six preparations of wax are given in Table 14.2.

The results from powder photographs taken with the X-ray beam parallel to the planar surface of the specimen are not included. In all cases, except for untreated scale wax, these diffraction patterns gave results similar to those obtained when the beam was at a normal angle. For the untreated scale wax, X-ray photographs were taken with the beam parallel to the plane. The existence of a crystallographic texture was obvious (Kurstjens et al. 1985). The a sin β and b parameters obtained for the side-spacings of all tensile specimens compared well with those previously obtained for monoesters (cetyl palmitate, a sin $\beta = 0.492$ nm, $b = 0.742$ nm) (Kohlhaas 1938). For all but the untreated scale wax samples, the powder photographs showed full concentric rings, and therefore indicate random orientation of the wax components. Untreated wax scales, on the other hand, have the molecular c-axis of their aliphatic components approximately perpendicular to the plane of the scales. Transmission with the beam normal to this plane shows continuous powder rings. With the beam parallel to the plane of the scale, photographs typical of an ordered molecular arrangement were obtained and the diffraction pattern then shows pronounced arcs (Fig. 14.13).

A comparison of the recorded long-spacings for untreated scale wax and untreated new comb wax are given in Table 14.3. However, as a result of possible chain inclination to the plane of reflection, as well as the possibility of two molecules joining end to end and thereby doubling the recorded chain length, a certain ambiguity arises. This difficulty was resolved by comparing the spacings with the results of Tulloch (1980). Subsequently a good correlation between both primary and shoulder reflections from the results of Kurstjens et al. (1985), and the diester component of the beeswax as reported by Tulloch, was obtained. The shoulder reflection is attributed to an inclined form (angle of inclination about 62° to 65°). Thus wax scales are textured as previously shown with polarised light techniques (Dujardin 1850; Schmidt 1924) and by X-ray diffraction (Woog and Yannaquis 1935).

The molecular c-axes of the crystallites are arranged perpendicular to and inclined at an angle of between 62° and 65° to the planar surface. New comb wax, and all of the variously treated waxes, exhibited no diffraction texture; therefore, this is interpreted to mean that they have a random crystallographic arrangement. Brewster (1815) had previously defined the comb cell walls as crystals, in which the neutral and depolarising axes of adjacent layers are not coincident. However, it should be noted that Woog and Yannaquis (1936a, b) reported the presence of very

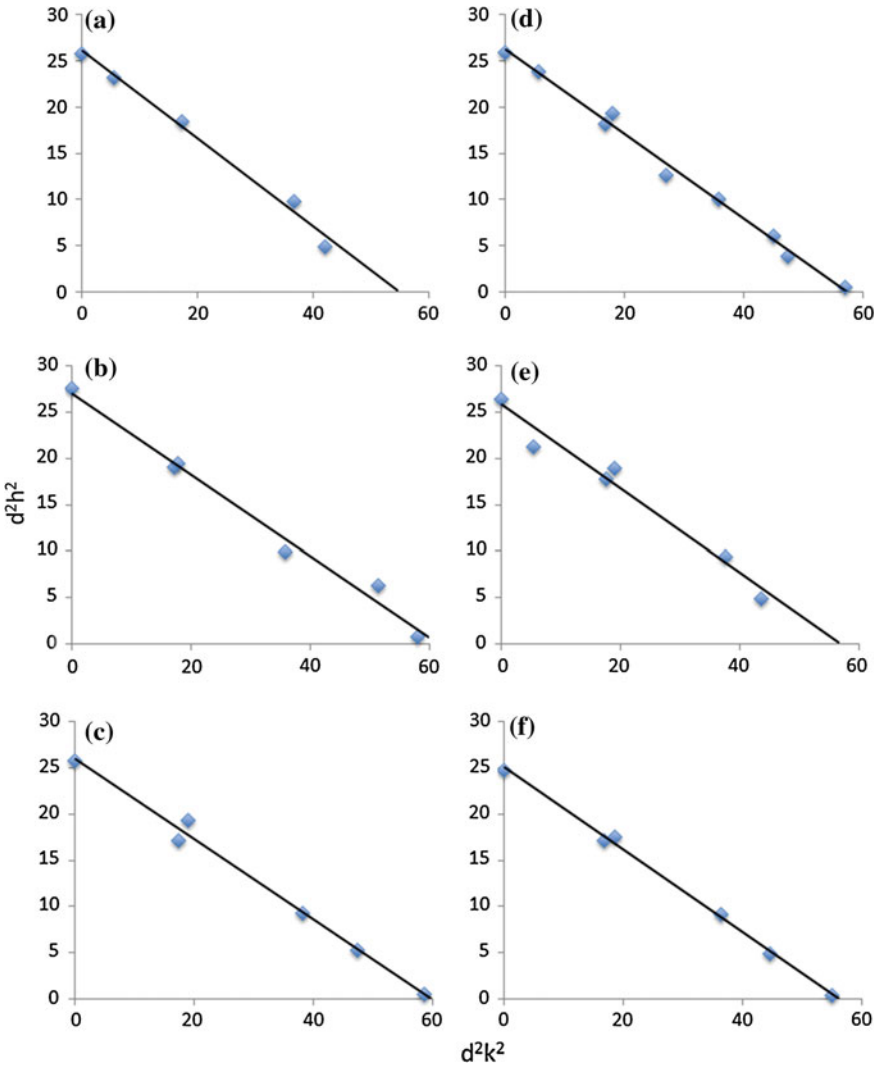

Fig. 14.12 d^2h^2/d^2k^2 plots for all six preparations of *A. m. scutellata* beeswax: **a** natural comb wax for which $\acute{\alpha}$ sin $\beta = 0.506$ nm, $b = 0.756$ nm; **b** sheeted comb wax for which $\acute{\alpha}$ sin $\beta = 0.503$ nm, $b = 0.772$ nm; **c** soxhlet-extracted and sheeted comb wax for which $\acute{\alpha}$ sin $\beta = 0.513$ nm, $b = 0.776$ nm; **d** beeswax scales for which $\acute{\alpha}$ sin $\beta = 0.502$ nm, b = 0.750; **e** sheeted scale wax for which $\acute{\alpha}$ sin $\beta = 0.519$ nm, $b = 0.781$ nm; **f** soxhlet-extracted and sheeted scale wax for which $\acute{\alpha}$ sin $\beta = 0.506$ nm, $b = 0.762$ nm (from Kurstjens et al. 1985)

weak X-ray reflections in the comb cell walls of *A. mellifera* samples of wax, which probably arose from patches of incompletely masticated scales, which are occasionally included in the comb as noted by Casteel (1912).

Table 14.2 The *d* values, Miller indices (hkl) and intensities (I) for all six wax preparations of *A. m. scutellata* (Kurstjens et al. 1985)

	hkl indices									
	?	110	020	120	200 030	210	130	220	?	140 230
Comb wax untreated d (nm)	0.474	0.425	0.382	0.306	0.254	0.239	0.217			
I	s	vs	s	w	w	w	w			
Comb wax sheeted d (nm)	0.45	0.423	0.381	0.302	0.252		0.24	0.21		
I	w	vs	s	w	w		w	w		
Comb wax soxhlet-extracted d (nm)	0.481	0.439	0.393	0.31	0.256		0.229	0.21		
I	w	vs	s	s	w		w	w		
Virgin wax untreated d (nm)	0.466	0.423	0.378	0.302	0.252	0.238	0.224	0.209	0.19	0.17
I	w	s	s	w	w	vw	w	w	vw	vw
Virgin wax sheeted d (nm)	0.47	0.43	0.386	0.309	0.256	0.23	0.22	0.213	0.19	
I	w	vs	s	w	w	w	w	w	vw	
Virgin wax soxhlet-extracted d (nm)		0.415	0.373	0.302	0.249		0.223	0.209		
I		vs	s	w	w		w	vw		

vs very strong; *s* strong; *w* weak; *vw* very weak

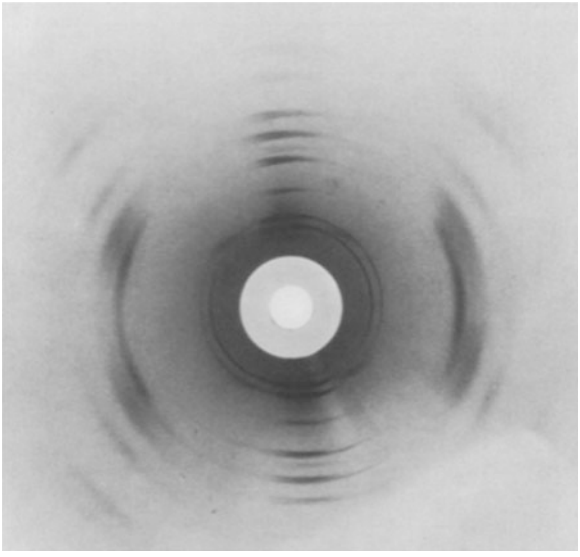

Fig. 14.13 An X-ray photograph of *A. m. scutellata* wax scale taken with the beam parallel to the planar surface of the specimen (from Kurstjens et al. 1985)

Table 14.3 A comparison of the long-spacing reflections of *A. m. scutellata* wax scales and comb wax (Kurstjens et al. 1985)

	Strong reflection	
	d (nm)	*d* (nm)
Comb wax	7.06 ± 0.05 (n = 9)	6.40 ± 0.05 (n = 6)
Virgin wax scales	7.07 ± 0.04 (n = 15)	6.22 ± 0.04 (n = 13)

14.11 Origins of Crystallites in Beeswax

Before assessing the above, it is worth considering the origin of the crystal texture of wax scales during their formation. That the scale is a fused, laminated structure, coupled with the fact that the surface of the wax mirror is 'wet' during secretion (Huber 1814; Philipp 1935; Cassier and Lensky 1995), jointly pointed to a probable fusion of the liquid secretions of different glands. This process has now been documented in the photomicrographs by Cassier and Lensky (1995). Their results, however, raise several questions. Does the arrangement of crystals occur after the proto-wax reaches the surface of the cuticle and only becomes textured during the hardening of the wax, or do the crystals reach the surface of the cuticle in a pre-orientated way (as do the α-helical protein precursors of honeybee silk before secretion—Flower and Kenchington 1968; cf. Chap. 18). The pore canal tubules, through which the wax passes, are in excess of 0.01 μm in diameter (Locke 1961), which is much greater than the chain length of the crystal constituents which have been identified.

Attempts at melting scales in situ on the surface of a wax mirror, letting them cool, and examining them for texture, have failed to obtain the same ordered diffractogram that one gets from wax scales (Kurstjens et al. 1985). However, Hallam (1967) removed wax from the leaves of *Eucalyptus* trees which, on recrystallisation, had a form very similar to that of untreated wax in situ. This suggests that, for at least some plant waxes, chemical composition may be of greater importance to the morphology of a wax than the particular surface on which it dries after secretion. There remains the intriguing possibility that the orientation of crystals in scale wax might well be the by-product of a slight compression of the wax as it increases in thickness between the plates of the abdomen. The affinity of beeswax to liquid crystals, noted by Gaubert (1910a, b), would certainly be consistent with such a possibility.

Towards the end of the 20th century, the distinction between physical chemistry and crystallography became increasingly blurred, but resulted in important additions to our understanding of beeswaxes. For example, in a detailed NMR investigation including differential scanning calorimetry and X-ray diffraction measurements of *A. m. scutellata* comb wax, Basson and Reynhardt (1988) showed that the average chain length in beeswax, determined by ebullioscopic

methods, is 40 carbon atoms. They also determined the liquid content of the wax as a function of temperature, a characteristic of great ergonomic importance in comb-building. More or less contemporaneously, Dorset (1983) began investigations on the crystallography of waxes, including beeswax. In a 1995 paper, Dorset noted that the most intense reflections obtained from beeswax resemble the electron diffraction patterns from the common plastic material, polyethylene. However, *A. m. scutellata* comb wax is much less ordered, even though it shares the same methylene sub-cell packing of most of the crystalline parts of harder waxes. Dorset (1997, 1999) suggested that beeswax cannot "fully separate into distinct lamellae, perhaps due to the presence of very long 'tie' molecules, and are therefore 'frustrated' crystal structures". Indeed, Kameda (2005) investigated the molecular structure of crude beeswax from *A. cerana* with solid-state NMR spectroscopy and showed that, although beeswax is composed of many chemical species, over 95 % of them consist of methylene units. Chemical shifts for at least three components indicate at least three differences in the crystal packing in crude beeswax. Kameda (2005) and Kameda and Tamada (2009) further found that the methylene carbon chains occur in two crystal forms, one orthorhombic and another triclinic or monoclinic, thus confirming the earlier interpretations of Kurstjens et al. (1985).

Dorset (1995) reported that when molten insect or natural plant waxes are recrystallized from the melt, they tend to form parallel arrays of polymethylene chains with little or no aggregation of the molecules into distinct layers. However, in an electron diffraction study of beeswax, he showed that the degree of molecular organization into lamellar structures can be enhanced by annealing (as previously shown by Dujardin 1850; Ambronn (1892) and more especially Woog and Yannaquis 1935). Nevertheless, the resultant layer structure in the annealed solid is not the same as that found in paraffin wax fractions, probably because of a small but significant fraction of a very long chain ingredient, the lamellar separation is incomplete, incorporating a number of 'bridging molecules' that span the nascent lamellar interface.

References

Alfonsus EC (1933) Some sources of propolis. Glean Bee Cult 61:92–93

Ambronn H (1892) Anleitung zur Benutzung der Polarisationsmikroskops bei histologischen Untersuchungen. (Cited from Schmidt 1941)

Arnhart L (1906) Die Zwischenräume zwischen den Wachsdrussenzellen der Honigbiene. Zool Anz 30:719–721

Astor A (1899) Notes d'un observation. Rev Int Apic 21:252–257

Atkins EDT (1967) A four-strand coiled-coil model for some insect fibrous proteins. J Mol Biol 24:139–141

Bankova VS, de Castro SL, Marcucci MC (2000) Propolis: recent advances in chemistry and plant origin. Apidologie 31:3–15

Bankova VS, Popova M, Trusheva B (2006) Plant sources of propolis: an update from a chemist's point of view. Nat Prod Commun 1:1023–1028

Basson I, Reynhardt EC (1988) An investigation of the structures and molecular dynamics of natural waxes. I. Beeswax. J Phys D: Appl Phys 21:1421–1428
Betts A (1921) Propolising. Bee Wld 2:131–132
Brewster D (1815) Experiments on the depolarisation of light as exhibited by various mineral, animal, and vegetable bodies, with a reference to the general principles of polarisation. Phil Trans R Soc Lond B 104:187–211
Buchwald R, Breed MD, Greenberg AR, Otis G (2006) Interspecific variation in beeswax as a biological construction material. J Exp Biol 209:3984–3989
Buchwald R, Breed MD, Bjostad L, Hibbard BE, Greenberg AR (2009) The role of fatty acids in the mechanical properties of beeswax. Apidologie 40:585–594
Cassier P, Lensky Y (1995) Ultrastructure of the wax gland complex and secretion of beeswax in the worker honey bee, *Apis mellifera* L. Apidologie 26:17–26
Casteel DB (1912) The manipulation of the wax scales of the honey bee. Circ US Bur Entomol No 161:l–13
Cesàro G (1903) Sur un curieux phénomene d'orientation par laminage. Bull Acad R Belg Sci, pp 432–441
Chauvin R (1962) Sur le noircissement der vieilles cires. Ann Abeille Paris 5:59–63
Chibnall AC, Piper SH (1934) The metabolism of plant and insect waxes. Biochem J 34:2209–2219
Darchen R (1980) La cire, son recyclage et son rôle probable à l'intérior d'une colonie d'*Apis mellifica*. Apidologie 11:193–202
Davidson BC, Hepburn HR (1986) Transformations of the acylglycerols in comb construction by honeybees. Naturwissenschaften 73:159–160
Donhowe IG, Fennema O (1992) The effect of relative humidity gradient on water vapor permeance of lipid and lipid-hydrocolloid bilayer films. J Am Oil Chem Soc 69:1081–1087
Dorset DL (1995) The crystal structure of waxes. Acta Cryst B Struct Sci 51:1021–1027
Dorset DL (1997) Crystallography of waxes—an electron diffraction study of refined and natural products. J Phys Lond D Appl Phys 30:451–457
Dorset DL (1999) Development of lamellar structures in natural waxes—an electron diffraction investigation. J Phys Lond D Appl Phys 30:1276–1280
Dorset DL, Ghiradella H (1983) Insect wax secretion the growth of tubular crystals. Biochim Biophys Acta 760:136–142
Duangphakdee O (2006) Biologically active compounds worker bees use to repel ants. Thesis, Chulalongkorn University, Bangkok
Duangphakdee O, Koeniger N, Koeniger G, Wongsiri S, Deowanish S (2005a) Ant repellent resins of honeybees and stingless bees. Insect Soc 56:333–339
Duangphakdee O, Koeniger N, Koeniger G, Wongsiri S, Deowanish S (2005b) Reinforcing a barrier—a specific social defense of the dwarf honeybee (*Apis florea*) released by the weaver ant (*Oecophylla smaragdina*). Apidologie 36:505–512
Dujardin F (1850) Sur l'étude microscopique de la cire appliquée de cette substance chez animaux et les végétaux. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 30:172–173
Ehrenberg F (1849) Mitteilungen über Resultate bei Anwendung der chromatisch polarisierten Lichter für mikrorkopische Vcrhältnisse. Ber Verh Preurs Akad Berlin (Cited from Schmidt 1941)
Ellis MB, Nicolson SW, Crewe RM, Dietemann V (2010) Brood comb as a humidity buffer in honeybee nests. Naturwissenschaften 97:429–433
Flower NE, Kenchington W (1967) Studies on insect fibrous proteins: the larval silk of *Apis, Bombus* and *Vespa* (Hymenoptera: Aculeata). J R Microsc Soc 86:297–310
Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
Gaubert P (1910a) Sur les crisstaux mous. Bull Soc Fr Mineral Cristallogr 33:326–335
Gaubert P (1910b) Sur les cristaux mous et sur la mesure de leurs indices de refraction. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 151:532–534

Hallam ND (1967) An electron microscope study of the leaf waxes of the genus *Eucalyptus* L'Heritier. Thesis, University of Melbourne, Melbourne. (Cited from Martin and Lindauer 1970)
Halle F (1931) Röntgenographische Messungen homologer normaler Polymethylenverbindungen in orientiert kristallisierter Schicht. Kollaid-Z 61:77–97
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR (1998) Reciprocal interactions between honeybees and combs in the integration of some colony functions in *Apis mellifera*. Apidologie 29:47–66
Hepburn HR, Hepburn C (2011) Bibliography of the Asian species of honeybees. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 473–657
Hepburn HR, Kurstjens SP (1984) On the strength of propolis (bee glue). Naturwissenschaften 71:591–592
Hepburn HR, Kurstjens SP (1988) The combs of honeybees as composite materials. Apidologie 19:25–36
Hepburn HR, Whiffler LA (1991) Construction defects define pattern and method in comb-building by honeybees. Apidologie 22:381–388
Hepburn HR, Chandler HD, Davidoff MR (1979) Extensometric properties of insect fibroins: the green lacewing cross-β, honeybee α-helical and greater waxmoth parallel-β conformations. Insect Biochem 9:69–77
Hepburn HR, Armstrong E, Kurstjens SP (1983) The ductility of native beeswax is optimally related to honeybee colony temperature. S Afr J Sci 79:416–417
Hepburn R, Duangphakdee O, Phiancharoen M, Radloff SE (2010) Comb wax salvage by the red dwarf honeybee, *Apis florea*. J Insect Behav 23:159–164
Huber F (1814) Nouvelles observations sur les abeilles. [English translation 1926] Dadant, Hamilton
Jay SC (1964) The cocoon of the honeybee, *Apis mellifera* L. Can Ent 96:784–792
Jordan R (1962) Anomale Wachsplattchen. Bienenvater 63:299–301
Kameda T (2005) ^{13}C solid-state NMR analysis of heterogeneous structure of beeswax in native state. J Phys D Appl Phys 38:4313–4320
Kameda T, Tamada Y (2009) Variable-temperature ^{13}C solid-state NMR study of the molecular structure of honeybee wax and silk. Int J Biol Macromol 44:64–69
Kohlhaas R (1938) Röntgenographische Untersuchung von definierten Einkristallen des Palmitinssaure-Cetylesters. Z Krist 98:418–438
Kratky E (1937) Die Orientierung der Moleküle des Bienenwachses und die Festigkeit der Waben. Naturwissenschaften 25:239
Kurstjens SP, Hepburn HR, Schoening FRL, Davidson BC (1985) The conversion of wax scales into comb wax by African honeybees. J Comp Physiol B156:95–102
Kurstjens SP, McClain E, Hepburn HR (1990) The proteins of beeswax. Naturwissenschaften 77:34–35
Lineburg B (1923) Conservation of wax by the bees. Am Bee J 63:615–616
Lineburg B (1924) Comb-building. Am Bee J 64:271–272
Locke M (1961) The cuticle and wax secretion in *Calpodes ethlius* (Lepidoptera: Hesperidae). Q J Microsc Sci 101:333–338
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
Lucas F, Rudall KM (1968) Extracellular fibrous proteins: the silks. In: Florkin M, Stotz EH (eds) Comprehensive biochemistry, vol 26. Amsterdam, Elsevier, pp 475–558
Marcucci MC (1995) Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 26:83–99
Nachtigall W, Kresling B (1992) Bauformen der Natur Teil I: Technische Biologie und Bionik von Knoten-Stab-Tragwerken. Naturwissenschaften 79:193–201
Nakamura J, Seeley TD (2006) The functional organization of resin work in honeybee colonies. Behav Ecol Sociobiol 60:339–349

Park OW (1946) Activities of honeybees. In: Grout RA (ed) The hive and the honeybee. Dadant Hamilton
Phiancharoen M, Duangphakdee O, Hepburn HR (2011) Biology of nesting. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 109–132
Philipp PW (1928) Das Kittharz, seine Herkunft und Verwendung in Bienenhaushalt. Biol Zentralbl 48:705–714
Philipp PW (1935) Neues vom Wachsschüppchen und der Wachszange. Rhein Bienen-Ztg 8:241–244
Pirk CWW, Crous KL, Duangphakdee O, Radloff SE, Hepburn HR (2011) Economics of comb wax salvage by the red dwarf honeybee, *Apis florea*. J Comp Physiol 181:353–359
Puleo S (1991) Beeswax minor components: a new approach. Cosmet Toil 106:83–89
Rudall KM (1962) Silk and other cocoon proteins. In: Florkin M, Mason HS (eds) Comparative biochemistry, vol IV. Academic Press, New York, pp 397–433
Schmidt WJ (1924) Die Bausteine der Tierkörpers in polarisierten Lichte (Cited from Schmidt 1941)
Schmidt WJ (1941) Polarisationsoptische Versuche mit Bienenwachs. Kolloid-Z 100:140–151
Schoening FRL (1980) The X-ray diffraction pattern and deformation texture of beeswax. S Afr J Sci 76:262–265
Seeley TD, Seeley RH, Akratanakul P (1982) Colony defense strategies in the honeybees of Thailand. Ecol Monogr 52:43–63
Seifert M, Haslinger E (1989) Über die Inhaltsstoffe der Propolis, I. Liebigs Ann Chem 1989:1123–1126
Seifert M, Haslinger E (1991) Über die Inhaltsstoffe der Propolis, II. Liebigs Ann Chem 1991:93–97
Simone M, Evans J, Spivak M (2009) Resin collection and social immunity in honeybees. Evolution 63:3016–3022
Simone-Finstrom M, Spivak M (2010) Propolis and bee health: the natural history and significance of resin used by honey bees. Apidologie 41:295–311
Simpson J (1961) Nest climate regulation in honey bee colonies. Science 133:1327–1333
Sutherland TD, Weisman S, Walker AA, Mudie ST (2011) The coiled-coil silk of bees, ants, and hornets. Biopolymers 97:446–454
Tomas-Barberan F, Garcia-Viguera C, Vit-Olivier P, Ferreres F (1993) Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. Phytochemistry 34:191–196
Tulloch AP (1980) Beeswax—composition and analysis. Bee Wld 1:47–62
Verlich AV (1930) Entwicklungsmechanische Studien an Bienenlarven. Z Wiss Zool 136:210–222
von Laue M (1913) Röntgenstrahlinterferenzen. Physikal Zeit 14:1075–1079
Warwicker JO (1960) Comparative studies of fibroins: II. The crystal structures of various fibroins. J Mol Biol 2:350–362
Woog P, Yannaquis N (1935) Sur l'orientation des molélcules de la cire d'abeille. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 201:1400–1402
Woog P, Yannaquis N (1936a) Orientation des molécules de la cire d'abeille et répercussion sur la solidité der rayons. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 202:76–78
Woog P, Yannaquis N (1936b) Orientation des molécules de la cire des d'abeille. Répercussions sur la construction des rayons. Arch Phys Biol 13:134–149
Zhang K, Duan H, Karihaloo BL, Wang J (2010) Hierarchical, multilayered cell walls reinforced by recycled silk cocoons enhance the structural integrity of honeybee combs. Proc Natl Acad Sci 107:9502–9506

Chapter 15
The Wax Gland Complex

Abstract The first correct descriptions of wax scales, their probable origin and uses, were made by Hornbostel (1744). In subsequent years, microscopists observed the synchronised rise and fall of the epidermis, oenocytes and fat body of honeybees and thought that these were highly suggestive of a direct involvement of all three tissues in wax production. In an attempt to prove a necessary relationship between wax secretion and the simultaneous development of the wax gland epithelium, fat cells and oenocytes, Graber (1872) noted that the adipocytes are interspersed with 'oenocytes' (Wielowiejski 1886), and Holz (1878) offered the first alternative to the 'wax-sweating' hypothesis. Detailed studies were conducted that provided circumstantial evidence to support this proposition. Indeed, the wax mirror epidermis belongs to the Type 1 class of glandular cells and indicates the reality of a system of microtubules to transport wax precursors from the fat body cells and oenocytes to the surface of the cuticle, where they solidify, and crystallise to become wax scales. Later studies of wax synthesis and secretion specifically identified sites for the origin of the hydrocarbon and fatty acid components within the wax gland complex, and established the necessary ultrastructural correlates of genesis and transport. The rates of wax secretion in honeybees of different ages have been measured, and the chemical composition of the tissues and ultrastructural changes corresponding with phases of wax production in relation to the division of labour, finally established.

15.1 Introduction

The beginning of an understanding of beeswax extends into prehistoric times in fashioning vessels and in early metallurgy (Crane 1999). Indeed, in the XII Book of the Odyssey, Homer made note of the plasticity of beeswax, and of its suitability as ear-plugs to escape the Sirens (dangerous and beautiful creatures who lured sailors with their enchanting music and voices to shipwreck vessels on the rocky coast of their islands). The Ancient Greek literature also abounds with small

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_15,

accounts of beeswax. Moving closer to our own times, Cowan (1908) recorded that in the reign of Alfred the Great, the passage of time was measured by marking beeswax candles into equal divisions. Throughout the medieval period, the system of fiefdom routinely required the payment of tribute, to the manor or monastery, in units of beeswax. The historical significance of beeswax during this period included using beeswax in incendiary devices, sealing waxes and seals, the lost-wax casting process, in paintings, writing tablets, as an adhesive, dyeing textile (batik), in pharmacy and cosmetics, for preserving human remains and various religious and liturgical procedures. Greater details of these examples are extensively covered in the comprehensive monograph by Crane (1999) in her *World History of Beekeeping and Honey-hunting*. Additional detailed information is contained in the works of Walker (1983), Ransome (1937), Bull (1959–1970) and in the archives of the Worshipful Company of Wax Chandlers (Dummelow 1973).

Just over a century ago, when Sir Joseph John Thomson was simultaneously appointed Director of the Cavendish Laboratory and Professor of Experimental Physics at Cambridge University, he described the available experimental equipment as "string and sealing wax", the latter of course being primarily composed of beeswax. This provides a small glimpse of mankind's reliance on beeswax. The ancient Orientals used beeswax (Luo et al. 2012), but we have no records of their thoughts on its origin (Crane 1999). The Occidental ancients viewed the origin of wax as derived from plants, and this view prevailed through the 17th century. A full historical perspective on classical, western ideas about beeswax is given in the monograph, Honeybees and Wax (Hepburn 1986).

By the 18th century considerable curiosity and argument attended the natural history of honeybees, particularly with regard to the origin of beeswax. By mid-18th century, the ancient notion of gathering wax had given way to the view that pollen had somehow been transformed into wax by bees. Oddly enough, the writings of three distinguished naturalists of that time, Swammerdam, Maraldi and de Réaumur, all suggested that bees make wax of pollen, yet none of them seem to have observed wax scales (Fraser 1931). Discoveries and claims for the origin of beeswax, as in so many other areas of apicultural history, are fraught with controversy. Walker (1909) documented the tortuous aspects of the story of the origin of wax and identified sources of confusion, plagiarism and other reasons (editorial excision) for historical obfuscation in the correspondence and publications of the latter half of the 18th century. Moreover, he has put to rest the charming, but tenuous claim, that a Lusatian peasant (reiterated in Huber 1814 and many other sources), discovered the origin of wax.

From the published evidence we must conclude, as did Walker (1909) and von Buttel-Reepen (1915) that the first correct description of wax scales, their probable origins and uses, were made by Hornbostel (1744). Hornbostel's published observations failed to spread beyond the world of the German language. In view of the primacy of Hornbostel's (1744) discovery, it is worth recording some of his observations from the original German. Thus, the worker bees "…. have small flaps under their bodies which lie on top of one another in the manner of fish scales, forming just as many compartments. In these compartments I once

accidentally found small, thin oval cakes of clear white wax, as many as there were compartments. These wax cakes in the bee were so robust that they protruded from the scales or flaps and became so noticeable that the bee appeared to be quite malformed".

"I touched these protruding cakes of wax with my fingernail and they fell out onto my hand. Just as the slivers of wax which one quite often sees lying under bee hives were well known to me, so I had no reason to doubt any longer that I had discovered the actual manner of how wax comes from bees". "Something remains which is impossible to find out and which will have to be found out only through conclusions and proper deductions. It is the question: by what manner do these wax cakes form and how did they get into the compartments? Only two manners can be thought of… either they are placed there as a previously prepared concoction by the bees themselves, or they come from the inside of the bees as a fluid mass gradually separated from the chyle of the bee so that the matter aligns itself in the compartments and remains there until they become so hard and thick that they can be removed again".

Having rejected the first hypothesis on the grounds that flowers do not contain a material remotely similar to wax, and that it would be anatomically impossible for bees to insert wax cakes into the compartments, Hornbostel further developed his surmise; "… these cakes of wax must of necessity come from the body of the bees and be laid down in the compartments. This is my opinion. The wax particles are mixed with the honey collected from the flowers, but are separated inside of the bees by digestion in such a way that the wax comes as a fluid material through the required vessels and is brought to the compartments through small passages. This separation occurs gradually until the wax cakes become so thick that the bees can take them out with the claws on their feet and are able to use them…"

In 1792 John Hunter independently provided a totally new account of wax production and showed that beeswax was really quite different from what his forbearers thought it to be. He restated the problem by noting that his predecessors held wax to be some form of transmuted pollen. But Hunter, like Hornbostel (1744) and Dobbs (1750) before him, observed that the pollen loads of bees were the same colour as the pollen in the flowers they had visited, and were not the colour of wax. Hunter went on to perform the first recorded experiments to test pollen for an oil base. Samples of pollen loads, which he held to a candle flame burned, but did not smell of burning wax; they actually smelled like samples of pure, hand-collected pollen when burned!

He confirmed his suspicions, that scales might be wax, by holding them to a candle where they melted and immediately formed a round globule (like molten wax). Hunter also noted that the pollen loads of bees were of many colours, but that newly built comb was usually white. Moreover, pollen was collected more avidly by established colonies than by founding ones—just the opposite of what one would expect were pollen the basis of wax. He wrote that founding colonies gather very little pollen during the first few days after they have settled, having no storage capacity for it, but that they do secrete wax and build combs. He adduced more circumstantial evidence against pollen as a precursor of wax from the fact

that, when the weather had been too cold or wet for the bees to forage, they constructed as much new comb as they would have in fair weather: bees do not need pollen to make wax. (This dissociation of pollen from wax led in turn to the idea that only sugar is needed to produce wax, a view which was expanded by Huber (1814) and dominated much of 19th century thought).

Hunter went on to record direct observations made with glass hives: "The wax is formed by the bees themselves; it may be called an external secretion of oil. It is formed in doublets beneath each scale but is not attached to the bee's body". He assumed that pollen loads were for the feeding of brood and not a source of wax. He recorded intact wax scales and tattered fragments on hive floors, as well as the absence of wax scales on the bees outside the normal building period. Furthermore, Hunter specifically tried to observe bees handling scales and making combs of them but failed to do so. He was nonetheless convinced of a scale-comb relationship, but since the thickness of comb exceeded that of scales he hedged, proposing that bees possibly added either pollen or silk to increase the bulk of the combs.

Natural wax is white but becomes yellow when rendered from old comb. Hunter speculated that the yellowness might arise from staining by honey, larval excrements or beebread. He steeped some white combs in honey, boiled others with pollen and yet others with pieces of old, darkened combs, but the original white wax did not acquire a deeper yellow hue. When bleached, wax returned to its natural colour (white), which proved that the yellow derived from a mixture of wax and some other substance. This notion of mixtures becomes extended: Hunter (1792) suggested that the substance used for attaching combs to surrounding hive parts was not common wax, but was softer, tougher and resembled cell cappings. He concluded that the material was probably a mixture of pollen and wax.

Also, the first new combs of the nest are almost white, but became yellow by the end of a season. In describing the structure of cells, Hunter often implied that wax was mixed with other, if unspecified, substances. He had a feeling for the physical properties of the building materials of bees, and linked their workability to the heat of the bees; the warmth generated by a colony kept the wax warm and soft enough for ease of modelling. Hunter's was the first substantial document on beeswax. By observation and experiment he showed that the scales on the bellies of bees were wax, and provided a reasonable, if still somewhat equivocal case that wax was not transmuted pollen but was secreted only by worker bees. The works of Hornbostel (1744) and Hunter (1792) never really gained currency in the development of ideas or hard knowledge on the biology of beeswax. Hunter, incidentally, was an eminent surgeon and anatomist and his place in the history of science hardly lies with honeybees. Nonetheless, his only paper on bees, the last he published before his death, is a quite remarkable document. It forms, coupled with that of Hornbostel, the basis for a modern biology of beeswax that has been developing, slowly, over the past two centuries.

15.2 Source of Secretion

A major figure in the history of the study of beeswax was the blind Swiss naturalist François Huber (1814), who observed bees through the eyes of his assistant François Burnens. This collaboration was succinctly described by the novelist, Sara George (2002), quoting correspondence from de Candolle to Burnens, "…yours was the sight and his the vision". They noted that wax scales are more or less pentagonal as is the surface of the cuticle, the wax mirrors, on which they form (Fig. 15.1). Huber tried to identify the origin of the liquid secretion by dissection, but failed to find any channels connecting the epidermal cells to the exterior surface, and surmised that the wax was somehow 'sweated out'. Then, in an extensive study of wax secretion, Claus (1867) observed that the wax gland epithelium in bees actively secreting wax is larger than in foragers, and concluded that the wax glands were simply a specialised region of the epidermis, subtended by a fat layer, the adipocytes, that might be involved in wax production.

These observations were confirmed by Graber (1872), who further noted that the adipocytes are interspersed with 'oenocytes' (Wielowiejski 1886). As an alternative to the 'sweating' hypothesis, Holz (1878) observed fatty tissue attached to the epidermis of bees actively secreting wax, and its absence in queens and drones. He interpreted the 'striped' appearance of the epidermis as tubes that convey the wax secretion to the surface of the wax mirror. Thus, even at this early stage ($\sim$1850) there was the general inference that the wax complex of the worker honeybee consisted of a specialized cuticle, epidermis, fat body, oenocytes and a tracheal air supply as well as a proposal that beeswax was a product of secretion.

15.2.1 The Cuticle

In the first electron microscopical study of the honeybee wax gland complex, Reimann (1952) noted that the cuticle was fully formed in the pharate adult, but that the procuticle of the mirror lacked an endocuticle, which was present on the adjacent non-mirror portion of the same sternite. The mirror cuticle is about 3 μm thick and does not increase in thickness, as do other regions of the cuticle with the ageing of a bee (King 1928; Menzel et al. 1969). Locke (1961) subsequently showed that the wax mirror cuticle consisted of an outer epicuticle of oriented lipid, and an inner epicuticle, but lacked a cement layer (Fig. 15.2). The inner epicuticle was penetrated by 'wax canal filaments' (Locke 1961) and was subtended by a lamellate procuticle (Neville et al. 1969; Cassier and Lensky 1995).

The procuticle of the wax mirror differed markedly from other regions of the honeybee cuticle, and also from other insects, because the 'pore canals' which extend up to the inner epicuticle, were tightly packed with filaments of about 0.01–0.03 μm in diameter, and were of the same dimensions as the wax canal filaments seen in the epidermal cells beneath the cuticle (Fig. 15.2). The pore

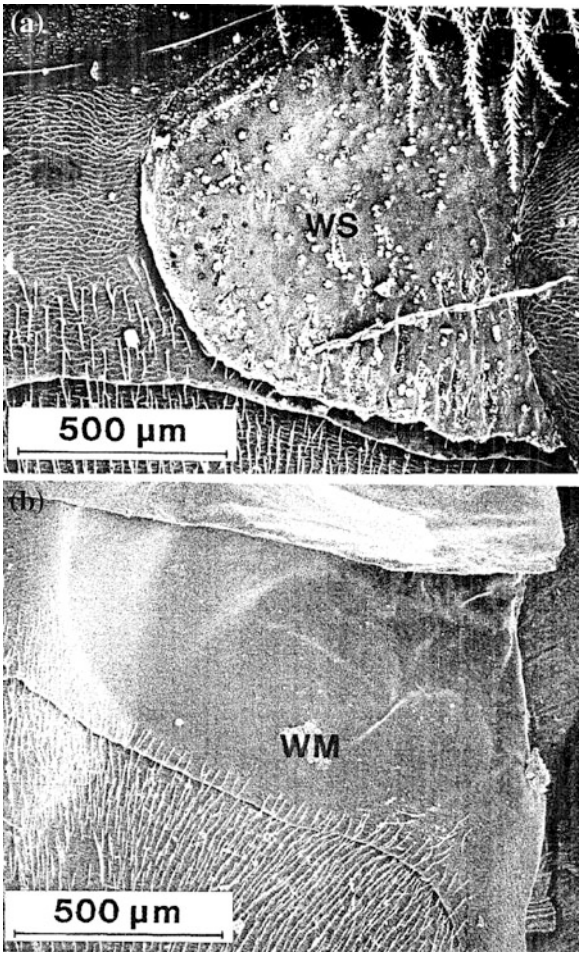

Fig. 15.1 Scanning electron photomicrograph of **a** a wax scale (*WS*) in situ; and **b** the surface of the mirror (*WM*) after removal of the scale from an *A. m. scutellata* worker (Hepburn 1986)

canals also formed a distinct layer between the procuticle and epithelium and were filled with dense material (Reimann 1952; Locke 1961; Cassier and Lensky 1995). The wax canal filaments project through the cells in bundles of microtubules and into the cuticle (Locke 1961).

All of the filament-like structures of the epicuticle, procuticle and epidermis are in the 0.01–0.03 µm range. Those passing through the cells average about 75–100 filaments or tubules per bundle, and range from 0.15 to 0.30 µm in diameter. Those passing through the pore canals are of the same dimensions, but there are fewer tubules in each pore canal than in the cellular bundles. The possible involvement of these structures, described by Reimann (1952), Locke (1961) and Sanford and Dietz (1976) in wax transport, long remained moot. Whorls of apparent tubules enter the cuticle from the cell, traverse the procuticle and terminate at the surface of the outer epicuticle (Hepburn 1986; Cassier and Lensky 1995) (Fig. 15.3). It has

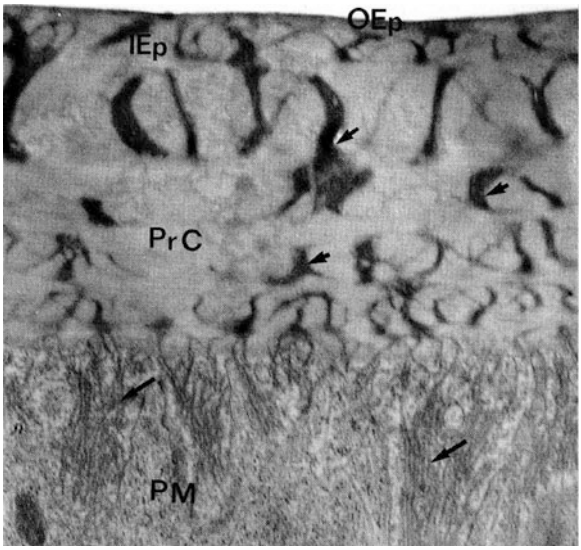

Fig. 15.2 Electron photomicrograph of the outer portion of the wax mirror cuticle of an *A. m. ligustica* worker, showing an outer epicuticle (*OEp*) subtended by a dark-staining inner epicuticle (*IEp*). In the body of the photograph, what are now thought to be wax canal tubules (*PrC*) and plasma membrane (*PM*), can be seen as twisted hanks within the pore canals (*dark patches*), microfilaments (*arrows*) linked to the folds of the apical plasma membrane form the pore canal system (*bottom arrow*) of the wax plate (Cassier and Lensky 1995, with kind permission, Apidologie)

subsequently been confirmed that this system of microtubules transports wax precursors from the fat body cells and oenocytes to the surface of the cuticle, where they solidify and crystallise to become wax scales (Cassier and Lensky 1995).

15.2.2 The Epidermis

The epidermis of the wax gland complex was first recognised as such by Claus (1867), while cell nuclei, nucleoli and membranes were reported by Carlet (1890) and illustrated by Mayer (1892). The epidermis below the wax mirror cuticle is associated with the oenocytes and fat body, and they collectively constitute the 'wax gland' tissue of the honeybee (Fig. 15.4). All three undergo dramatic changes during development and between periods of glandular activity. Histologically, the wax gland epidermis forms a continuous sheet of cells under the mirror cuticle of young bees, while in older bees past their prime for wax secretion, the epidermis reverts to a squamous epithelium (Dreyling 1906).

The solution to the problem of distinguishing bees whose wax glands were either in the ascending or descending phase, was provided by Rösch (1927).

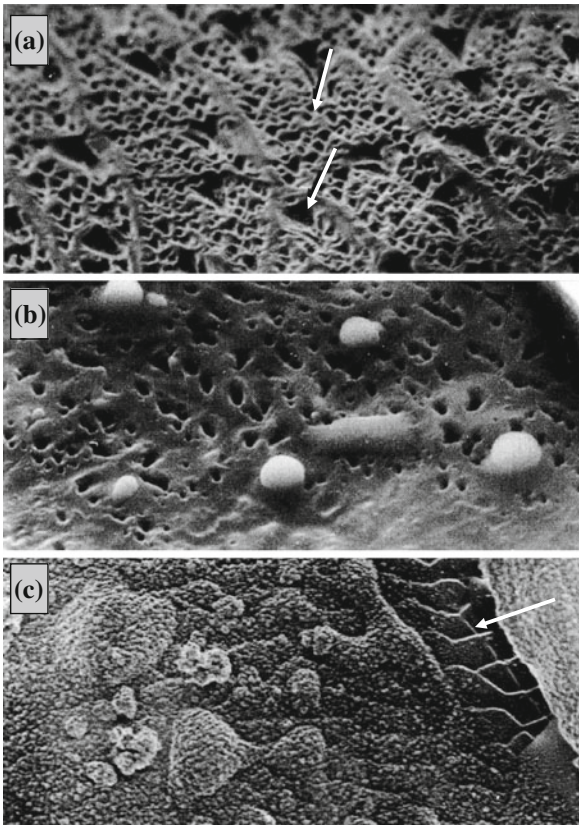

Fig. 15.3 SEM of honeybee, *A. m. ligustica,* wax mirrors. **a**. Outer surface of a cleaned wax mirror showing the cuticular pattern. Each unit shows numerous *holes* and *pits* (*arrows*, × 1,800). **b**. Extrusion of globular droplets of wax through the *holes* of a wax mirror, (× 2,500). **c**. Droplets of wax fuse and form irregular puddles which mask the cuticular pattern (arrow, × 750) (Cassier and Lensky 1995, with kind permission, Apidologie)

In very young bees, the epidermal cells are cuboidal and abut one another. At the first sign of development, intercellular spaces begin to appear in the epidermis (Fig. 15.5), and the cells are elongated. At peak development, the epidermal cells are narrow-waisted and are partially separated by intercellular spaces. In the rising phase of activation and secretion, cell membranes, nuclei and protoplasm are well defined. In the descending phase, cell height decreases and membranes, nuclei and protoplasm are far less defined; the cells become squamous and gradually deteriorate into a flat sheet (Fig. 15.4).

Electron microscopical studies (Fig. 15.6) have shown that the epithelium underlying the wax mirror is supported by a basement membrane (Reimann 1952). The cytoplasm of the cell contains numerous pleomorphic mitochondria, a rough endoplasmic reticulum, polyribosomes and microtubules (Sanford and Dietz 1976; Hepburn et al. 1991) that are common to most cells. The smooth endoplasmic reticulum and a Golgi apparatus, normally regarded as essential for protein secretion but thought to be absent from these cells, were finally observed in detail

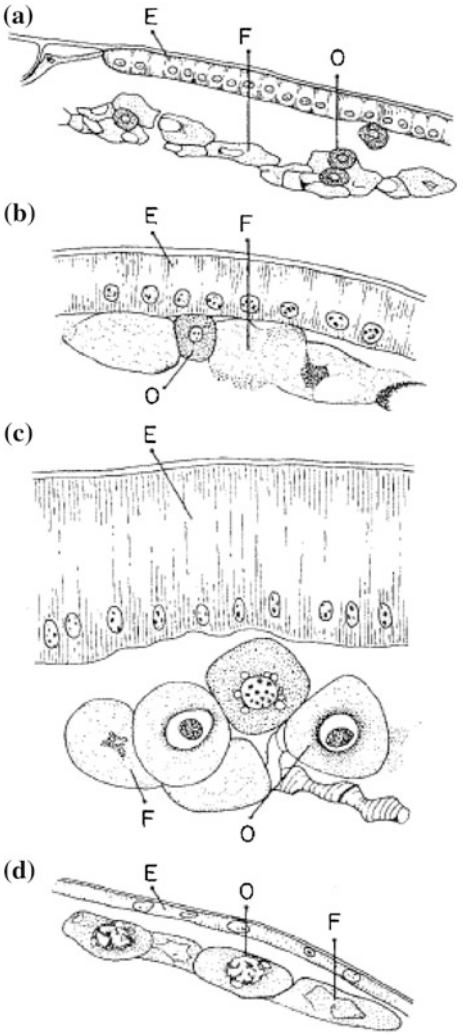

Fig. 15.4 Changes in the ascending and descending phases of the wax gland system as documented by Dreyling (1906), Rosch (1927, 1930) and Boehm (1965). **a** newly emerged bee; **b** a young bee at the onset of development; **c** the wax gland system at the peak of glandular activity and wax secretion; **d** the degenerate glands in an older forager. *E* epidermis, *F* fat body, *O* oenocytes (after Boehm 1965)

by Cassier and Lensky (1995). The abundant tracheae ramify into tracheoles that extend three or four cell widths and terminate either intra- or extracellularly in the tissues of the wax gland complex (Reimann 1952; Sanford and Dietz 1976).

15.2.3 Fat Body and Oenocytes

In the heyday of histological studies, Koschevnikov (1900) and Hollande (1914) finally forged a link between the oenocytes, the fat body and wax secretion. Moreover, using specific staining techniques, Koschevnikov also established

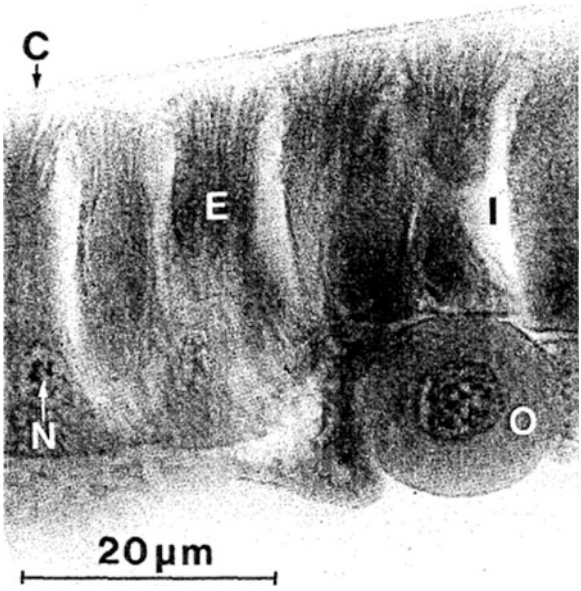

Fig. 15.5 Light photomicrograph of the wax gland complex of a 9-day-old African honeybee, *A. m. scutellata.* The epidermal cells (*E*) below the cuticle (*C*) have become elongated, and hyaline intercellular spaces (*I*) occur between the tubular cells. The epidermal cells have ellipsoid nuclei (*N*) which are characteristic of an active epithelium. On the *lower right*, an oenocyte (*O*), closely appressed to the epithelium, is in position to discharge its contents into the epithelium (Hepburn 1986)

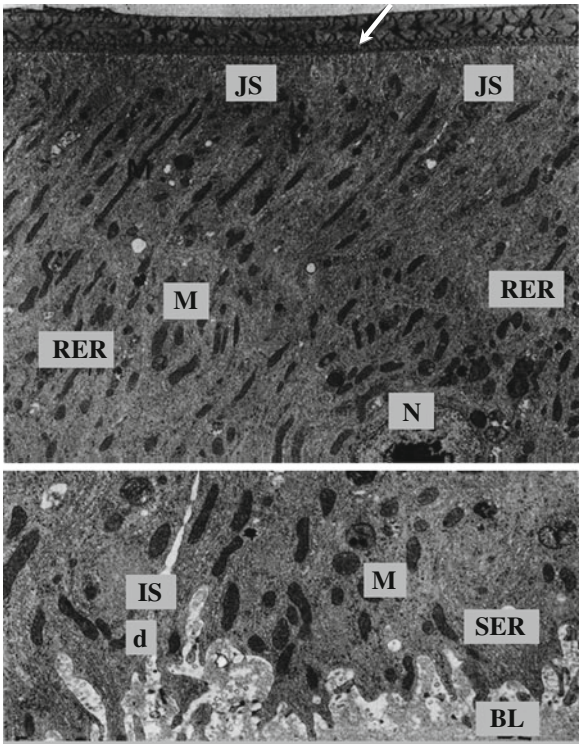

Fig. 15.6 Transmission electron microscopy. The epithelial cells of *A. m. ligustica* with the apical part of cell just below the wax mirror cuticle (*arrow*) in the *top* photomicrograph. The pore canal system is well developed. *JS* junction system, *M* mitochondria, *N* nucleus, *RER* rough endoplasmic reticulum, (× 14,000). In the basal part of the cell (*bottom* micrograph) *BL* basal lamina, *d* desmosomes, *IS* intercellular space, *SER* smooth endoplasmic reticulum (× 9,000) (from Cassier and Lensky 1995, with kind permission, Apidologie)

functional differences between the two intimately related tissues. Pursuing this lead, Rösch (1930) found that the cell membranes of the epithelium seem to dissolve at the places where a fat cell or oenocyte is in apposition to it, and the contents of the fat body cells appear to escape into the wax gland epithelium (Fig. 15.6). Rösch (1930) eventually found histological sections in which the oenocytes were being disgorged into the epidermal cells. After communicating with the epithelium, the nuclei of both oenocytes and the fat body eventually dispersed into chromatin granules. Rösch (1930) concluded that the fat body and oenocytes make a major contribution to wax secretion. Furthermore, they not only reach their greatest sizes at the peak of wax secretion but also simultaneously decline following secretion, observations subsequently confirmed by Reimann (1952), Boehm (1965) and Hepburn (1986).

The synchronised rise and fall of the epidermis, oenocytes and fat body are highly suggestive of a direct involvement of all three tissues in wax production, but does not constitute direct proof of the hypothesis. Nonetheless, by 1965 it had become a tenet of insect cell biology that the fat body plays a major role in the storage and transformation of fats, protein and carbohydrates and as a major organ of intermediate metabolism (Chino and Gilbert 1965). So armed, Boehm (1965) repeated the work of Dreyling (1906) and Rösch (1927, 1930) and extensively described the rise and fall of the wax gland epithelium, oenocytes and fat body of the wax gland complex. Thus, in the conversion of a newly emerged bee into a full-blown wax producing bee, there is a strong correlation between the increase in the size of the oenocytes and the epidermis, previously suggested by Freudenstein (1960) and confirmed in considerable detail by Boehm (1965) (cf. Fig. 15.7).

15.2.4 Synchronising Cellular Activity

In an attempt to prove that there was an essential relationship between wax secretion and the simultaneous development of the wax gland epithelium, fat cells and oenocytes previously claimed by Rösch (1930), Boehm (1965) conducted detailed studies that provided a reasonable amount of circumstantial evidence to support this proposition (Figs. 15.7 and 15.8). However, solutions to two general questions were still required: (a) what are the functions of the respective tissues thought to be involved in wax synthesis? and (b) once the wax or wax precursors have been formed, how do they physically reach the cuticle to form wax scales?

These problems were considered by Rösch (1930) who interpreted his histological sections as follows. The cell membranes of both oenocytes and fat cells appeared to ‘dissolve’ into the epidermis. By using differential stains, he observed that the otherwise non-staining epidermis gradually acquired the stained material of the oenocytes as they emptied their contents into the epidermis; the same happened to the fat cells, but in a less pronounced way. However, the fat cells were markedly smaller after having given up their secretions, than were the oenocytes. The nuclei of both the epidermis and oenocytes deteriorated more quickly than the

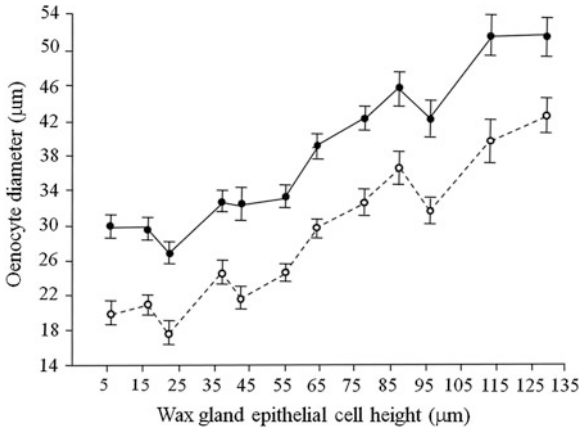

Fig. 15.7 Relationship between the diameters of the oenocytes and the height of the wax gland epithelium of an *A. mellifera* worker. The curve joining the *closed circles* is based on the largest oenocytes seen, and that joining the *open circles* represents the smallest oenocytes seen (Boehm 1965)

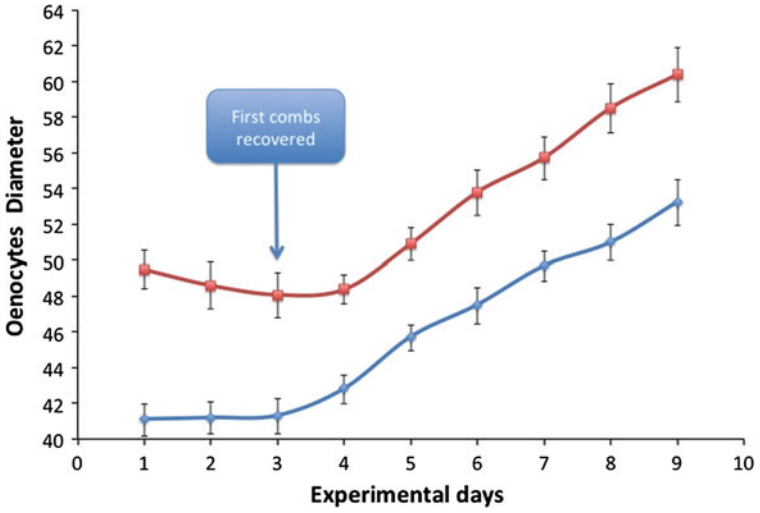

Fig. 15.8 Increase in the diameter (μm) of oenocytes of old *A. mellifera* field bees that had been induced to reactivate their wax glands, secrete wax and build combs. The average and standard deviation of the smallest oenocyte (*blue*) and the biggest oenocyte (*red*), are shown. Each average is based on 12 workers (Boehm 1965)

fat cells, the contents of both cells passing into the epidermis. This hypothesis of Rösch (1930), that the fat cells and oenocytes contribute wax precursors to the epidermis (which turned out to be correct), certainly ran counter to the prevailing physiological opinion of the day.

The surmise that the oenocytes grow at the cost of the fat cells, coupled to her exhaustive histological study of the wax organs, subsequently led Boehm (1965) to postulate a working hypotheses relating the putative wax organ complex: (1) the oenocytes stimulate the development of the wax gland epithelium with substances that are sequestered from the fat cells and then liberated in the haemolymph; or (2) the oenocytes stimulate the fat body cells which in turn affect the epidermis. In either case, the fat cells would be the driving force for development of the wax epithelium. Alternatively, (3) the oenocytes might themselves produce wax precursors.

15.2.5 Ultrastructure of the Organelles of Wax Gland Cells

The wax gland epithelium had long been thought to lack several organelles—Golgi apparatus and associated vesicles and granules and a smooth endoplasmic reticulum regarded as indispensable for secretion (Boehm 1965; Sanford and Dietz 1976, Hepburn et al. 1991); however, these organelles were subsequently confirmed (cf. Fig. 15.5). Indeed, the wax mirror epidermis belongs to the Type 1 class of glandular cells (Noirot and Quennedey 1974). This strongly indicates the reality of a system of microtubules to transport wax precursors from the fat body cells and oenocytes to the surface of the cuticle where they solidify and crystallise to become wax scales (Cassier and Lensky 1995).

Searches for the means by which synthesized wax within the abdomen actually reaches the surface of the wax mirrors, strongly suggests that it passes through the pore canal system of the epidermis and cuticle (Locke 1961; Hepburn 1986; Hepburn et al. 1991; Cassier and Lensky 1995). Yet the means by which the precursors are transported from as yet unidentified points of origin, remained elusive. Hepburn et al. (1991) conducted studies of wax synthesis and secretion to specifically identify sites for the origin of the hydrocarbon and fatty acid components within the wax gland complex, and to establish the necessary ultrastructual correlates of genesis and transport. They also measured the rates of wax secretion in honeybees of different ages, to assess how well chemical composition of the tissues and ultrastructural changes correspond with phases of wax production, in relation to the division of labour.

In newly enclosed honeybees the SER of the oenocytes is barely discernible, but by day 4 the volume and density of these organelles is elevated (Table 15.1; and cf. Hepburn et al. 1991).

Likewise there is a large increase in oenocyte volume as previously noted by Boehm (1965), which remains elevated throughout the secretory phase (Table 15.1). By day 18, both the oenocytes and SER begin to decrease with the

Table 15.1 Volume changes in the wax gland oenocytes of Cape honeybee workers, *A. m. capensis*, with age (Hepburn et al. 1991)

Feature	Bee age (days)								
	0	4	6	9	12	14	16	18	21
Whole cell[a]	8 ± 4	36 ± 13	54 ± 16	39 ± 10	45 ± 13	40 ± 14	52 ± 22	32 ± 10	30 ± 12
SER[b]	65 ± 10	83 ± 14	82 ± 6	83 ± 24	81 ± 9	82 ± 10	8 ± 12	85 ± 12	74 ± 15
Mitochondria[b]	10	9	8	8	8	9	7	5	7
Glycogen granules[b]	23	7	8	9	9	7	6	7	15

[a] Oenocyte volume ($\times$ 1000 μm^3). Bees aged 0, 4 and 6 days were significantly different ($P < 0.05$) from each other and the groups 9- to 18-day olds. There were no significant differences within the 9- to 18-day-old groups. Similarly, bees 18 and 21 days-old did not differ significantly from each other but were different ($P < 0.05$) from the 9- to 18-day-old groups

[b] Organelle volume density expressed as a percentage of mean oenocyte cytoplasmic volume occupied by organelle. For SER, bees aged 0- and 21-days-old were significantly different ($P < 0.05$ from bees aged 4- to 18-days. There were no significant differences between bees of the 4- to18-day-old groups

Table 15.2 Volume changes in the wax gland adipocytes of Cape worker honeybees, *A. m. capensis*, with age (Hepburn et al. 1991)

Feature	Bee age (days)								
	0	4	6	9	12	14	16	18	21
Whole cell[a]	65 ± 26	110 ± 52	130 ± 100	117 ± 48	123 ± 52	120 ± 87	143 ± 65	95 ± 37	97 ± 32
RER[b]	2	8	5	4	4	3	3	2	2
Mitochondria[b]	5	10	8	8	10	9	11	12	13
Lipid droplets[b]	61	24	22	21	17	25	18	17	21
Protein granules[b]	–	10	19	22	7	1	6	–	–
Glycogen[b] granules	5	3	14	6	10	5	7	10	7

[a] Adipocyte volume (x 1000 μm^3). Bees of age 0 d were significantly different ($P < 0.05$) from those of 4- to16-day groups but there were no differences within the 4- to 16-day-old group. Similarly, bees 18 and 21-days-old did not differ, but both significantly differed ($P < 0.05$) from the 4- to16-day-old groups;
[b] volume of organelle

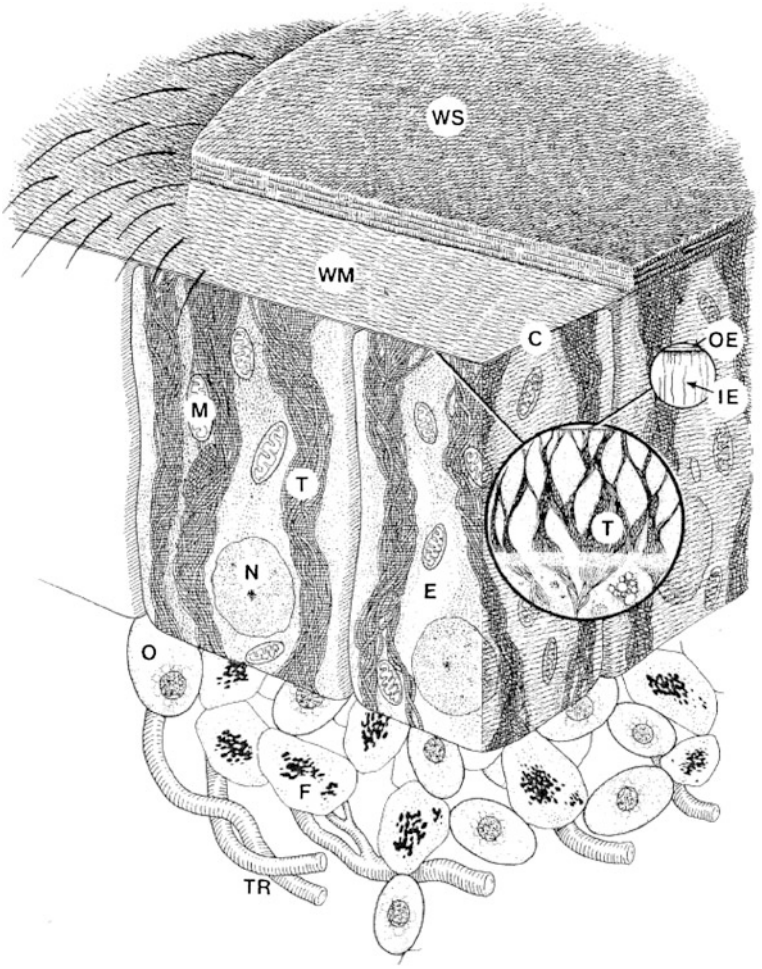

Fig. 15.9 The wax gland complex of an *A. m. scutellata* worker illustrating: wax scale (*WS*), wax mirror (*WM*), cuticle (*C*), outer epicuticle (*OE*), inner epicuticle (*IE*), wax canal tubules (*T*), epidermal cells (*E*), nuclei (*N*), mitochondria (*M*), oenocytes (*O*), fat body adipocytes (*F*) , tracheole (*TR*) (original artwork by CP Richards; Hepburn 1986)

simultaneous appearance of primary lysosomes and autolytic vacuoles. Lipid and protein droplets were never observed in the oenocytes and no other organelles showed cyclical changes associated with wax synthesis (Table 15.2).

During wax synthesis glycogen stores are notably large, and the reticular system of the organelles remains unchanged or show small decreases in size (Table 15.2). Adjacent adipocytes within the fat body tissue are separated by a gap of about 0.25 μm, which is filled with material of the basal lamina. There are many hemidesmosomes between each adipocyte and its basal lamina. In places where

adjacent adipocytes are <0.05 μm, they are joined by desmosomes and gap junctions. The basal laminae of neighbouring oenocytes are separated by a gap of 0.15 μm, and like the adipocytes, are attached by hemidesmosomes to their basal laminae. Similarly, where oenocytes and the fat body cells are closely applied to the basal lamina of the epidermis, particularly during synthesis and secretion (Hepburn 1986), only hemidesmosomes are present. During synthesis and secretion the epidermal cells and fat body cells are not connected by any junctions.

In earlier studies, Sanford and Dietz (1976) and Hepburn et al. (1991) both reported that smooth endoplasmic reticulum (SER) is absent from wax-secreting workers, and concluded that the epidermis mainly provides an elaborate system for wax precursor transport (Reimann 1952; Locke 1961; Hepburn 1986). However, in further electron microscopical studies of the wax gland complex, Cassier and Lensky (1995) reinvestigated the possible role of the epidermis and its transport modalities. They were able to show that there are indeed large cisternae of SER and that they are probably involved in the transport of wax precursors from the oenocytes to the pore canals, as well as carrying apolipophorins from the haemolymph to the wax mirrors. Although the entire discussion in this chapter is based on studies of *A. mellifera,* it can be noted that a brief paper on the ultrastructure of the wax gland of *A. cerana* confirms that this species is conformal with the details given here (Du and Li 1991).

Finally, we include a reconstruction of the combined elements of the wax gland complex of *A. m. scutellata* (Fig. 15.9). This drawing was prepared by examining a large number of serial sections from electron micrographs.

References

Boehm B (1965) Beziehungen zwischen Fettkörper, Oenocyten und Wachsdrüsenentwicklung bei *Apis mellifica* L. Zeit Zellforsch Mikrosk Anat 65:74–115

Bull R (1959–1970) Vom Wachs. Hoechster Beirage zur Kenntnis der Wachs. Hoechst, Frankfurt

Carlet G (1890) La cire et ses organes sécréteur. Le Naturaliste 12:149–151

Cassier P, Lensky Y (1995) Ultrastructure of the wax gland complex and secretion of beeswax in the worker honey bee, *Apis mellifera* L. Apidologie 26:17–26

Chino H, Gilbert LI (1965) Lipid release and transport in insects. Biochim Biophys Acta 98:94–110

Claus C (1867) Über die Wachbereitenden Hautdrüsen der lnsekten. SB Ges Beford Naturwiss Marburg. (Cited from von Buttel-Reepen 1915)

Cowan TW (1908) Wax craft. Sampson Low, London

Crane E (1999) The world history of beekeeping and honey hunting. Duckworth, London

Dobbs A (1750) A letter from Arthur Dobbs Esq: to Charles Stanhope Esq; F.R.S. concerning bees, and their method of gathering wax and honey. Phil Trans R Soc 46:536–549

Dreyling L (1906) Die wachsbereitenden Organe bei den gesellig lebenden Bienen. Zoo1 Jahrb Abt Anat Ont Tiere 22:289–330

Du ZL, Li N (1991) The ultrastructure of the wax gland cell of the worker honey bee, *Apis cerana*. Acta Ent Sinica 34:289–291 [In Chinese]

Dummelow J (1973) The wax chandlers of London. Phillimore & Co, London

Fraser HM (1931) Beekeeping in antiquity. University of London Press, London

Freudenstein H (1960) Einfluss der Pollennahrung auf der Bauvermögen, die Wachrdrüsen und den Fettkörper der Honigbiene (*Apis mellifera* L.). Zoo1 Jahrb Allg Zool Physiol Tiere 69:95–124
George S (2002) The beekeeper's pupil. Headline Book Publishing, London
Graber V (1872) Vorläufiger Bericht über den propulsatorischen Apparat der Insekten. Sber Akad Wiss Wien 65:189–204
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax in honeybees. Apidologie 22:21–36
Hollande S (1914) Les cérodécytes ou oenocytes des insectes. Arch Anat Microsc Morphol Exp 16:l–66
Holz H (1878) Das Organ der Wachsbildung. Bienen-Ztg 34:l83–l184
Hornbostel HC (1744) (orig Melittophilus Theosebastus) Neue Entdeckung, wie das Wachs von den Bienen Komt. Hamburg Vermis Bibliothek 2:45–62
Huber F (1814) Nouvelles observations sur les abeilles (English translation, 1926). Dadant, Hamilton
Hunter J (1792) Observations on bees. Phil Trans R Soc 82:128–196
King GE (1928) The larger glands in the worker honey-bee. A correlation of activity with age and with physiological functioning. Ph.D. thesis, University of Illinois, Urbana
Koschevnikov GA (1900) Über den Fettkörper und die Oenocyten der Honigbiene (*Apis mellifera* L.). Zoo1 Anz 23:337–353
Locke M (1961) The cuticle and wax secretion in *Calpodes ethlius* (Lepidoptera: Hesperidae). Q J Microsc Sci 101:333–338
Luo W, Li T, Wang C, Hiang F (2012) Discovery of beeswax as binding agent on a 6th-century BC Chinese turquoise-inlaid bronze sword. J Archeol Sci 39:1227–1237
Mayer P (1892) Zur Kenntnis von *Coccus cacti*. Mitt Zool Stn Neapel 10:505–518
Menzel R, Wladarz G, Lindauer M (1969) Tagesperiodische Ablagerungen in der Endokutikula der Honigbiene. Biol Zentralbl 88:61–67
Neville AC, Thomas MG, Zelazny B (1969) Pore canal shape related to molecular architecture of arthropod cuticle. Tissue Cell 1:183–200
Noirot C, Quennedey A (1974) Fine structure of insect epidermal glands. Ann Rev Entomol 19:61–80
Ransome HM (1937) The sacred bee. Allen & Unwin, London
Reimann K (1952) Neue Untersuchungen über die Wachrdrüse der Honigbiene. Zoo1 Jahrb Abt Anat Ont 72:147–188
Rösch GA (1927) Über die Bautätigkeit im Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 6:265–298
Rösch GA (1930) Untersuchungen über die Arbeitsteilung im Bienenstaat. 2. Die Tätigkeiten der Arbeitsbiencn unter experimentell veränderten Bedingungen. Z Vergl Physiol 12:l–71
Sanford MT, Dietz A (1976) The fine structure of the wax gland of the honeybee (*Apis mellifera* L). Apidologie 7:197–207
von Buttel-Reepen H (1915) Leben und Wesen der Bienen. Vieweg, Braunschweig
Walker HJO (1909) The discovery of the origin of beeswax. Br Bee J 37(31–34):42–45
Walker P (1983) Beeswax publications of historical interest, vol 34. IBRA Bibliography, London
Wielowiejski H (1886) Über das Blutgewebe der insekten. Z Wiss Zoo1 43:512–536

Chapter 16
The Chemistry of Beeswax

Abstract Publications on the physical constants for the comb waxes of Asian and European beeswaxes first appeared a century ago. It was soon shown that carbon chain length was, on average, shorter in the Asian beeswaxes than in *A. mellifera,* which explains the lower melting points of the former. The Asian waxes are more similar to one another than to *A. mellifera.* In Asian beeswaxes, the amounts of C_{31} and C_{33} in the pool of free fatty acids are reduced, but C_{25} hydrocarbons are increased compared to that of *A. mellifera.* The major compound families in beeswax are alkanes, alkenes, free fatty acids, monoesters, diesters and hydroxymonoesters, while fatty alcohols and hydroxydiesters are minor constituents. There are notable species-specific differences in the beeswaxes among honeybee species, but all share a complex mixture of homologous neutral lipids. The amounts of acylglycerols are the same in scale and comb wax, but diacylglycerols dominate the former and monoacylglycerols the latter. There are more double-bonded fatty acids in comb than in scale wax, and a greater saturation of fatty acids in comb wax. Beeswaxes analysed with high temperature gas chromatography yielded a characteristic elution pattern for waxes of each honeybee species. A parsimonious, unweighted, pair-group analysis based on the distribution of the chemical constituents for 82 elution peaks of the derivatized comb waxes of six species of honeybees. The Euclidean distances of the beeswaxes present a picture very similar to those obtained from morphometric, behavioural and DNA sequence analyses. The wax glands and the products of their secretions were highly conserved features during honeybee evolution.

16.1 Introduction

In this chapter, discussions on the chemistry of beeswax are restricted entirely to honeybee wax scales and comb wax in a biological context. Investigations of both the chemical composition and physical properties of beeswaxes of *A. mellifera* have been pursued for centuries, and these earlier works have been documented by Grün and Halden (1929). Preparations for and practical uses of beeswax have also

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_16,

Table 16.1 Composition of beeswax derived from *A. mellifera* combs (Tulloch 1980)[a]

Constituent fractions	Number of components in fractions		
	Percentage %	Major	Minor
Hydrocarbons	14	10	66
Monoesters	35	10	10
Diesters	14	6	24
Triesters	3	5	20
Hydroxy monoesters	4	6	20
Hydroxy polyesters	8	5	20
Acid esters	1	7	20
Acid polyesters	2	5	20
Free acids	12	8	10
Free alcohols	1	5	?
Unidentified	6	7	?
Total	100	74	210

[a] Major components are those forming more than 1 % of the fraction; for minor components only estimates are given (Tulloch 1980)

been documented (Cowan 1908; Coggshall and Morse 1995), and the commercial industrial aspects of beeswax have been exhaustively monographed (Büll 1977); thousands of publications have appeared on these topics since then. However, the very first studies of Asian beeswaxes appeared only a century ago (Hooper 1904; Bellier 1906; Büchner 1906; Hooper and Büchner 1906; Ueno 1915; Roberts and Islip 1922; Ikuta 1931, 1934), who between them recorded the physical constants (specific gravity, melting point, acid and saponification values, etc.) of the comb waxes of *A. cerana, A. dorsata, A. florea* and *A. mellifera.*

As our knowledge of the hydrocarbon, alcohol and acid fractions of beeswaxes developed, two points of importance to honeybee biology emerged. Firstly, Phadke (1961) re-examined the physical constants of *A. cerana, A. dorsata, A. florea* and *A. mellifera* beeswaxes, and showed each to be extremely homogenous as evidenced by the very small standard deviations in the physical values of the samples measured. Shortly after, Narayana (1970) and Phadke et al. (1971) determined that carbon chain length was, on average, shorter in the three Asian beeswaxes than in *A. mellifera,* which accounts for the lower melting points of the Asian waxes. Progress in wax chemistry advanced with gradually improved analytical techniques of both thin-layer and gas-liquid methods of chromatography in the 1940 and 1950s (Touchstone 1993).

16.2 Chemical Composition

The composition and origin of *A. mellifera* comb beeswax has relatively recently been summarised by Tulloch (1980), and is shown in Table 16.1. The major components are defined as those exceeding more than 5 % of each fraction; those of lesser abundance are regarded as minor constituents. Tulloch regarded, as major

components, those which constituted more than 1 % of each fraction; those of lesser abundance were regarded as minor constituents. Nevertheless, if a particular fraction is itself small, then a given compound may well be 'major' in that fraction, but very minor with respect to the bulk composition of a beeswax sample. Tulloch (1980) regarded the large number of minor hydrocarbons as probably disproportionate, because of the relative ease with which they can be separated, vis-à-vis the seven groups of esters. The residue of some 44 % of beeswax is taken up entirely by minor constituents, to which Tulloch ascribed the relatively low melting point of intact beeswax and its plasticity.

By combining both gasliquid and thin-layer methods of chromatography Tulloch (1973, 1974, 1975, 1980) also studied the composition of waxes from different honeybee species. He found that the waxes from different *A. mellifera* races were very similar as a group, but the unsaturated C_{31} hydrocarbon peak was smaller and the C_{35} hydrocarbon peak larger in the African bee, *A. m. scutellata,* than in the European races of *A. mellifera*. By contrast, he reported that waxes of the Asian bees, *A. cerana, A. dorsata* and *A. florea*, resemble each other more closely than any of them do to *A. mellifera* waxes as previously reported by Narayana (1970) and Phadke et al. (1971). In the Asian waxes there is a smaller pool of free fatty acids (analysed as methyl esters), reduced amounts of C_{31} and C_{33}, but increased C_{25} hydrocarbons compared to *A. mellifera* waxes. The recordings from the gas-liquid chromatography analyses by Tulloch are shown in Fig. 16.1.

Despite the assiduous efforts of numerous chemists who have sought to analyse the composition of beeswax, we have very few observations on the chemistry of newly secreted wax scales. Huber (1814) investigated the solubility properties of wax scales and of fragments of newly fashioned white comb wax. He observed that the wax scales readily dissolved in turpentine (presumably comprising then, as now, a pot-pourri of terpenes, but mainly the monoterpenes $\acute{\alpha}$- and β-pinene), but that comb wax left a white residue. When scale and comb wax samples of equivalent weight were placed in vessels of sulphuric ether (probably diethyl ether), the former became opaque but did not dissolve, while the latter dissolved leaving a white residue in the vessel.

When Huber allowed the ether to evaporate from the vessels, he always obtained a recoverable layer of scale wax residue, which led him to conclude that if the scales were indeed crude wax, then the bees must impregnate them with some additional substance to obtain the whiteness and ductility of newly constructed comb wax. To this we can add the observations of Young (1963), who analysed wax scales for the presence of (2-^{14}C)-acetate that had been injected into wax-producing bees. He found that the label was incorporated in the free acid and ester fractions of wax scales. Finally, Lambremont and Wykle (1979) performed a thin-layer chromatographic separation of scale wax and found the resulting chromatographic pattern similar to that obtained by Tulloch (1970) from cappings wax, with the exception that their chromatograms lacked activity at the diester position.

Subsequently, Davidson and Hepburn (1986) compared the glycerols of scale and comb wax. Their assays showed that the monoacylglycerol and diacylglycerol

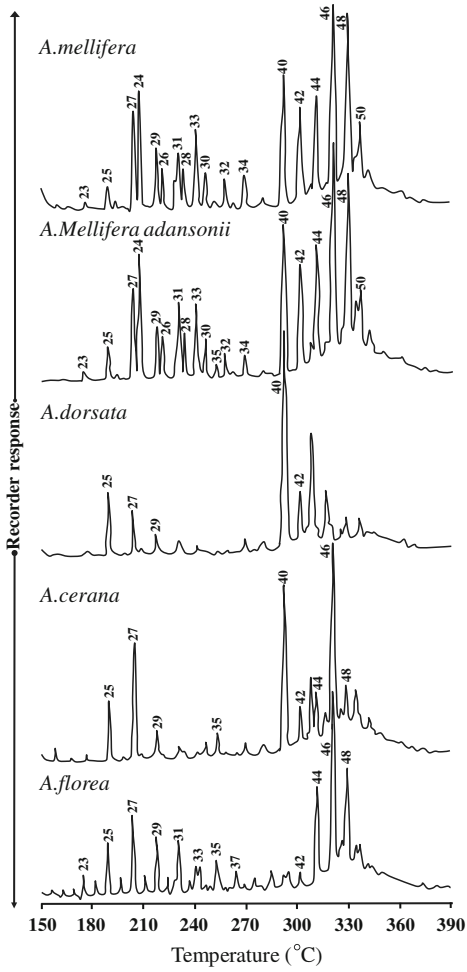

Fig. 16.1 The spectra obtained from gas–liquid chromatographic analyses of *A. mellifera, A. m. scutellata* (= *adansonii*), *A. dorsata, A. cerana* and *A. florea* comb waxes. Hydrocarbons are indicated by odd-numbered peaks (23–35), free acids by even-numbered peaks (24–34) and monoesters by even numbers (40–50) (Tulloch 1980)

fractions comprised about 91 % of the total glycerol in scale and comb wax. While the total level of the acylglycerols were the same in scale and comb wax, the diacylglycerols dominated the scale wax glycerol pool, and the monoacylglycerols the comb wax glycerols. Within the acylglycerol fractions there were substantially more double-bonded fatty acids in scale than in comb wax. Although about 50 % of the fatty acid fractions were the same in the two waxes, there was a significantly greater degree of saturation in the fatty acids of comb wax.

In the absence of hard analytical knowledge as to the total composition of beeswax scales vis-à-vis that of newly built comb, a rather circuitous route must be taken to assess the possible differences among European, African and 'africanized' (*A. m. scutellata*) subspecies. Of equal importance, what exactly is it that a honeybee worker does when she chews scales in the process of comb construction? To this end Eckert (1922, 1927) repeated the basic experiment of Dumas and Edwards

(1843) to assess the effects of cane sugar versus honey on the composition of wax. He compared the fresh, white wax of newly constructed combs built by bees fed sugar, with the yellowish wax produced by a colony given nectar and honey, and found no differences between them. The dimension of age was added to composition studies by Jordan et al. (1940), who compared old comb wax, wax newly secreted by young bees and new wax produced by bees of more than a month old. Replicate and parallel measurements were made on cleaned combs, but no significant differences were found between the waxes of young and old bees. These two waxes did, however, differ from old comb wax in that the latter had an iodine number twice that of the former. This they attributed to a greater contamination of the old wax by carotenoids derived from pollen.

16.3 Chemometrics

Titschack (1969) analysed and tabulated the acid, saponification and ester values for *A. mellifera* African waxes, ranging in origin from Morocco and Ethiopia through the Ivory Coast and south to Mozambique. Because these data were sorted by countries, individual results cannot confidently be ascribed to any particular honeybee subspecies (Hepburn and Radloff 1998). Nonetheless, there were statistically significant differences in composition between several African waxes from different sources, pointing to possible genetic differences among the races. This approach was extended by Tulloch (1980) who showed that the waxes of Asian honeybees were chemically different from those of *A. mellifera*, and that the African and European subspecific profiles of *A. mellifera* waxes also differed.

With the development of high resolution capillary gas chromatography, this work has been extended, particularly by Brand-Garnys and Sprenger (1988). They characterised the waxes of different *A. mellifera* races on the basis of unique hydrocarbon and ester profiles, and recognised 16 subspecific waxes, ten of which are of African geographical origin (Table 16.2). Unfortunately no information is given as to the origin of these waxes, or of variations between the samples, so these data elude chemotaxonomic analysis. Recently, Beverly et al. (1995) showed that the pyrolysis-mass spectral peaks obtained from European and African beeswaxes differed in their relative intensities, but no unique molecules peculiar to any specific wax were obtained. Nonetheless, this approach might be a useful line of further inquiry.

With even more sophisticated gas-chromatographic methods than previously available Aichholz and Lorbeer (1999) and Aichholz et al. (2000) re-examined the comb waxes of the Asian honeybees, *A. andreniformis, A. cerana, A. dorsata, A. florea* and *A. laboriosa* as well as *A. mellifera,* and showed that they are complex mixtures of homologous neutral lipids containing a range of 20–64 carbon length molecules. Aichholz et al. (2000) investigated beeswaxes with high temperature gas chromatography and obtained a characteristic elution pattern for the waxes of each honeybee species, confirming and extending the earlier analyses of Tulloch (1980) and Brand-Garnys and Sprenger (1988).

Table 16.2 Wax characteristics of different *A. mellifera* races (Brand-Garnys and Sprenger 1988)[a]

Races	R1	R2	R3	R4	R5	R6	Type
adansonii	0.181	0.267	0.079	1.314	0.76	1.238	II
anatolica	0.261	0.341	0.019	0.908	0.721	0.905	III
capensis	0.257	0.222	0.055	1.121	1.095	1.54	III
carnica	0.184	0.351	0.017	0.921	0.678	0.937	II
caucasica	0.237	0.274	0.003	1.178	0.725	0.914	III
iberica	0.26	0.155	0.01	1.401	0.706	1.012	II
intermissa	0.213	0.285	0.076	0.958	0.768	1.163	II
jemenitica	0.235	0.328	0.027	0.883	0.893	0.846	I
lamarckii	0.215	0.262	0.168	0.952	0.943	1.329	IV
ligustica	0.264	0.257	0.015	1.124	0.685	0.975	II
litorea	0.261	0.212	0.048	1.324	0.748	1.281	II
mellifera	0.323	0.167	0.009	1.282	0.785	0.981	III
monticola	0.269	0.212	0.052	1.082	1.001	1.438	II
nubica	0.218	0.255	0.087	1.19	0.829	1.256	II
scutellata	0.228	0.247	0.063	1.13	0.891	1.358	II
unicolor	0.211	0.254	0.101	1.191	0.689	1.1	II

[a] R1 is defined as the quotient of the quantity of hydrocarbons and 27 carbon atoms out of the total hydrocarbon pool and so on. Types are defined as the sequence of the absolute quantity of straight chain esters 40, 42 and 44 carbon atoms

In another analysis of beeswaxes Puleo (1991) published gas chromatograms of the comb waxes of African *A. m. scutellata* and European *A. m. ligustica* honeybees, and demonstrated striking differences in both their hydrocarbon and straight chain monoester fractions. In the former, the percentage of C_{33}:1 unsaturated hydrocarbon is greater than the concentrations of C_{29} and C_{31} saturated hydrocarbons, while the converse occurs in the latter subspecies. Also, the percentage of C35:1 unsaturated hydrocarbon is ten times greater in *A. m. scutellata* ($\sim$1.2) than in *A. m. ligustica* ($\sim$0.2). Likewise, there is a lower percentage concentration of C_{48} relative to the C_{46} esters in *A. m. scutellata* than in *A. m. ligustica* (Puleo 1991). He also reported that there are also minor components associated with the hydrocarbon fraction, in that the even-numbered, straight chain hydrocarbons vary in length from C_{22} to C_{34} and may constitute 0.02–0.2 % of the total.

Following Tulloch (1980), Aichholz et al. (2000) defined the major compound families as those exceeding 5 % of the total, so that alkanes, alkenes, free fatty acids, monoesters, diesters and hydroxymonoesters are the major compound families, while fatty alcohols and hydroxydiesters are minor constituents (Table 16.3). There are notable species-specific differences in the waxes among honeybee species (Table 16.3), but all share a complex mixture of homologous neutral lipids: C_{25}–C_{29} alkanes, C_{40}–C_{54} monoesters, C_{42}–C_{52} hydroxymonoesters, and C_{56}–C_{58} diesters (Aichholz and Lorbeer 1999; Aichholz et al. 2000). Presently our knowledge of the composition of the waxes of all honeybee species is nearly equal; however, pathways of synthesis remain available only for *A. mellifera* (Hepburn et al. 1991). Given what is known of species-specific composition

Table 16.3 The major compound families of *A. andreniformis*, *A. florea*, *A. cerana*, *A. mellifera*, *A. dorsata* and *A. laboriosa* comb waxes (Aichholz and Lorbeer 1999)

Compound family	*A. andreniformis*	*A. florea*	*A. cerana*	*A. mellifera*	*A. dorsata*	*A. laboriosa*
Alkanes total	18.5	12.5	11.4	12.8	10.8	10.8
Alkenes total	5.9	7.5	7.4	2.9	0.6	5.3
Diene total	3.4	–	–	–	–	–
Hydrocarbons total	27.8	20	18.8	15.7	11.4	16.1
Fatty acids total	2.6	0.8	3.6	18	4.9	4.3
Fatty alcohols total	–	0.4	1.8	0.6	–	–
Monoesters total	27.5	41.1	33.4	40.8	36.9	37.5
Hydroxymonoesters total	13.6	9.1	18.1	9.2	23.3	23.6
Diesters total	12.9	15.7	12.2	7.4	11.9	8.8
Hydroxydiesters total	3.9	2.3	3	–	1.4	1.1
Esters total	57.9	68.2	66.7	57.4	73.5	71
Total	88.3	89.4	90.9	91.7	89.8	91.4

(Table 16.4), there is considerable opportunity for biochemical studies of beeswaxes in future.

16.3.1 Chemometric Classification of Beeswaxes

For any experimental study into the numerous interactions between pheromones and comb and/or cuticular waxes known to occur (Breed et al. 1995a, b, 1998), it is essential to know the chemical composition of the waxes involved and to be able to classify them. The chemical compositions of comb and cuticular waxes of honeybees have been extensively investigated (Blomquist and Ries 1979; Blomquist et al. 1980; Lockey 1985; Hepburn 1986; Francis et al. 1989), but with different methods. In a seminal paper, Frölich et al. (2000) established objective and quantitative chemometric tools for distinguishing between comb waxes of different ages and the cuticular waxes from different castes and sexes of *A. m. carnica*. Previously there had been no studies on chemical composition of different age classes of comb waxes using quantitative classification tools.

When Frölich et al. (2000) analyzed their fractions by gas chromatography, 56–75 % of the total mass of the wax samples could be identified (Table 16.5). All comb waxes of different age classes were dominated by long-chain aliphatic compounds, with chain lengths ranging in length from C_{21} to C_{54} (Fig. 16.2). The chain lengths exhibited a bimodal distribution, and there were no differences in chain length distributions among wax scales, new, middle-aged, and old comb waxes respectively. The respective medians for the shorter and longer chain length distributions were also fairly close. Chain lengths were in the range of C_{42} to C_{44} for all comb wax classes (Fig. 16.2). These data are consistent with those of other studies on *A. mellifera* (Basson and Reynhardt 1988), as well as waxes of the Asian honeybee species (Narayana 1970; Phadke et al. 1971).

Table 16.4 Comparison of the compound composition of derivatised comb waxes of *A. mellifera, A. cerana*, *A. florea, A. andreniformis, A. dorsata* and *A. laboriosa* by GC–FID analysis on a SOP-50-PFD column (modified from Aichholz and Lorbeer 1999)

Structure	Peak	*Apis mellifera*	*Apis cerana*	*Apis florea*	*Apis andreniformis*	*Apis dorsata*	*Apis laboriosa*
Alkane C23	1	0.4	0	0	1.1	0.4	0.3
Alkane C25	3	1.5	0.9	1.5	7	4.3	3.8
Alkane C27	10	6.2	8.2	6.3	4.9	3.6	3.6
Alkane C29	17	2.6	2.3	3	2.8	1.2	1.7
Alkane C31	22	1.5	0	1.2	1.8	0.9	1
Alkane C33	26	0.3	0	0.5	0.5	0.4	0.4
Alkane C35	30	0.3	0	0	0.4	0	0
Alkene C27	8	0	0	0.6	0.5	0	0
Alkene C29	16	0	0.6	1	1	0	0
Alkene C31	21	0.8	0	2.3	0	0	0.3
Alkene C33	25	2.1	0.4	3	0	0.6	1.9
Alkene C35	29	0	5.4	0.6	1	0	1.7
Alkene C37	34	0	1	0	1.4	0	0.8
Alkene C39	38	0	0	0	1.3	0	0.6
Alkene C41	41	0	0	0	0.7	0	0
Fatty acid C20	13	1.1	0	0	0.8	0.8	0
Fatty acid C22	19	0.7	0	0	0	0.3	0.4
Fatty acid C24	24	6	0	0	0	1.4	0.7
Fatty acid C26	27	2.1	0.5	0	0	0	0
Fatty acid C28	31	2.6	1.2	0.4	0.5	0	0
Fatty acid C30	35	2.1	1.9	0.4	0.4	0	0
Fatty acid C32	39	1.6	0	0	0.2	0.3	0.6
Fatty acid C34	43	1.5	0	0	0.3	1.4	1.8
Fatty acid C36	46	0.3	0	0	0.4	0.7	0.8
Fatty alcohol C33	32	0.3	1.8	0.4	0	0	0
Fatty alcohol C35	36	0.3	0	0	0	0	0
Diene C35	28	0	0	0	0.4	0	0
Diene C37	33	0	0	0	0.9	0	0
Diene C39	37	0	0	0	1.1	0	0
Diene C41	40	0	0	0	1	0	0
Diester C54	67	0	0	0	0	1	0.6
Diester C54	68	1.2	0	0.7	0.7	5.6	4.1
Diester C56	69	0	0	0	0	1	0.9
Diester C56	70	1.2	0.6	1	1	2.4	2
Diester C58	72	0	0	0.8	0.6	0.5	0.3
Diester C58	73	1.4	2.3	5.2	4.2	1	0.9
Diester C60	75	0	1.1	1.1	0.9	0	0
Diester C60	76	2	5.3	4.2	3.4	0.4	0
Diester C62	78	0	0.7	0.7	0	0	0
Diester C62	79	1.2	1.6	1.7	1.6	0	0
Diester C64	81	0.4	0.6	0.3	0.5	0	0

(continued)

Table 16.4 (continued)

Structure	Peak	*Apis mellifera*	*Apis cerana*	*Apis florea*	*Apis andreniformis*	*Apis dorsata*	*Apis laboriosa*
Hydroxydiester C50	71	0	0.7	0	0.4	1	0.7
Hydroxydiester C52	74	0	0	0	0.6	0.4	0.4
Hydroxydiester C54	77	0	1	1.1	1.6	0	0
Hydroxydiester C56	80	0	1	0.6	0.9	0	0
Hydroxydiester C58	82	0	0.3	0.6	0.4	0	0
Hydroxymonoester C40	48	0	0	0	0.4	3.3	2.3
Hydroxymonoester C40	49	0.9	0	0	0.4	9.6	8.4
Hydroxymonoester C42	51	0	0	0	0	4	4.5
Hydroxymonoester C42	52	0.8	0.4	0.4	0.8	2.5	2.6
Hydroxymonoester C44	54	0	2.8	0	0	1.3	1.3
Hydroxymonoester C44	55	1.8	0	3.3	4.3	0.5	0.6
Hydroxymonoester C46	57	0.9	9.2	0	0	0.4	0.4
Hydroxymonoester C46	58	2.3	0	2.9	4.7	0.3	0.4
Hydroxymonoester C48	61	0.6	4.4	0	0	0.3	0.5
Hydroxymonoester C48	62	1.6	0	1.5	1.9	0.5	0.9
Hydroxymonoester C50	64	0	0.5	0	0.8	0.3	0.7
Hydroxymonoester C50	65	0.3	0.8	0.7	0	0.3	0.5
Hydroxymonoester C52	66	0	0	0.3	0.3	0	0.5
Monoester C38	42	0	0	0	0	0.5	0.7
Monoester C40	44	6.6	0.7	1.5	1.3	26.8	24.9
Monoester C42	47	4.6	0.9	3.4	1.5	4.7	4.5
Monoester C44	50	5.7	4.8	9.7	7.7	0.7	1
Monoester C46	53	11.9	23.7	17	10.7	0.9	1.6
Monoester C48	56	9	2.2	7.3	4.7	1.7	2.7
Monoester C50	60	2.6	0.6	1.8	1.3	1.2	1.6
Monoester C54	63	0.4	0.5	0.4	0.3	0.4	0.5

Table 16.5 Analytical yields derived from gas chromatographic analyses of *A. m. carnica* scale and comb waxes (Frölich et al. 2000)

Sample type	Relative amounts of masses (%), Means ± 95 % confidence intervals[a]		
	Identified in GC	Unidentified in GC	Polar fraction
Comb waxes			
Wax scales	71 ± 2.2	4.2 ± 2.24	25 ± 1.8
New wax	68 ± 2.1	3.0 ± 2.13	29 ± 6.6
Middle-aged wax	70 ± 1.9	4.6 ± 1.93	26 ± 2.6
Old wax	69 ± 1.5	5.4 ± 1.51	26 ± 4.0
Cuticular wax			
Workers	67 ± 0.7	1.6 ± 0.76	31 ± 5.1
Drones	54 ± 0.7	2.2 ± 0.72	43 ± 6.2
Queens	57 ± 2.8	7.7 ± 2.79	36 ± 9.7

[a] Fractions 1–3 were subjected to gas-chromatographic (GC) analysis. The values given are related to the total mass of fractions 1–4. The limit of detection was 0.01 % and the decimals were set accordingly

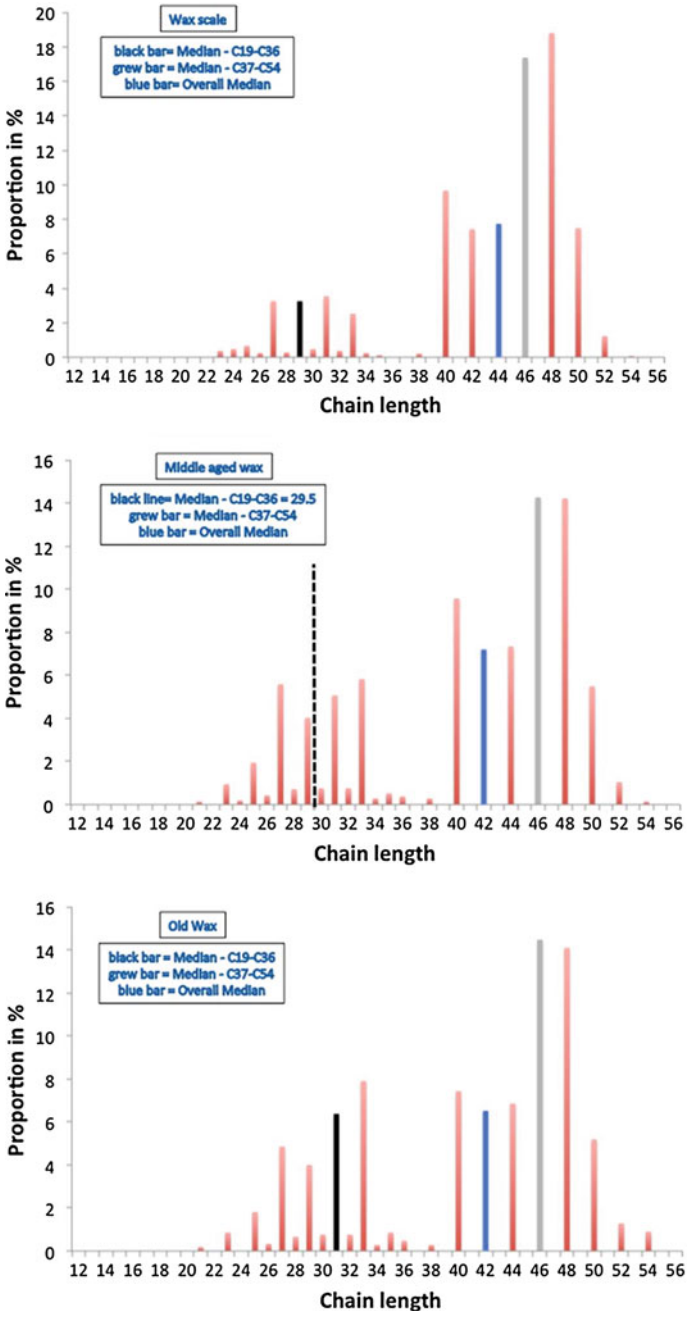

Fig. 16.2 Distribution of chain lengths of *A. m. carnica* comb waxes. $Median_1$ refers to the chains ranging from C_{19} to C_{36}; $Median_2$ refers to the chains ranging from C_{37} to C_{54}; and $Median_{all}$ characterizes the whole range of chain lengths (Frölich et al. 2000)

Table 16.6 Relative chemical composition of *A. m. carnica* comb waxes of different ages (Frölich et al. 2000)

Substance classes	Relative amounts of masses (%), Means ± 95 % confidence intervals[a]			
	Wax scales ($N = 6$)	New wax ($N = 6$)	Middle-age wax ($N = 6$)	Old wax ($N = 6$)
Alkanes	11 ± 4.9	13 ± 1.7	15 ± 1.7	14 ± 1.1
Alkenes	3.4 ± 1.43	6.0 ± 1.04	8.8 ± 0.98	12 ± 1.3
Alkadienes	0.06 ± 0.044	0.24 ± 0.041	0.72 ± 0.077	2 ± 0.21
Branched alkanes	0.00 ± 0.008	0.19 ± 0.117	0.46 ± 0.053	0.95 ± 0.12
Esters	57 ± 6.9	57 ± 3.6	47 ± 4	48 ± 4.3
Unsaturated alkyl esters	13 ± 3.3	11 ± 0.7	12 ± 1.4	9.5 ± 1.54
Hydroxzalkyl esters	8.0 ± 3.08	7.9 ± 5.72	8.1 ± 1.57	6.4 ± 0.98
Acids	1.3 ± 2.00	0.14 ± 0.158	0.51 ± 0.338	0.08 ± 0.10
Alcohols	0.41 ± 0.239	0.53 ± 0.317	0.74 ± 0.128	0.48 ± 0.20
Unidentified	5.6 ± 2.97	4.2 ± 2.99	6.2 ± 2.59	7.3 ± 2.03

[a] The values given related to the total mass of fractions 1–3; limit of detection at 0.01 %, decimals were set accordingly

Table 16.7 Relative chemical composition of *A. m. carnica* hydrocarbon fractions of comb waxes of different ages (Frölich et al. 2000)

Substance classes	Relative amounts of masses (%), Means ± 95 % confidence intervals[a]			
	Wax scales (N = 6)	New Wax (N = 6)	Middle-age wax (N = 6)	Old wax (N = 6)
Alkanes	75 ± 1.2	67 ± 0.9	60 ± 0.3	50 ± 0.6
Alkenes	24 ± 1.1	31 ± 0.7	35 ± 0.2	40 ± 0.5
Alkadienes	0.38 ± 0.071	1.2 ± 0.03	2.9 ± 0.05	7.0 ± 0.09
Branched alkanes	0.05 ± 0.048	1.0 ± 0.31	1.8 ± 0.05	3.3 ± 0.08

[a] The values given related to the total mass of fraction 1; the limit of detection was 0.01 % and the decimals were set accordingly

The chemical compositions of all waxes were dominated by long-chain alkyl esters contributing 47 % ± 4.0 to 57 % ± 6.9 of the total of fractions 1–3 (Table 16.6).

With the increasing age of comb wax, the overall median of the different age classes decreases, but the relative contributions by alkenes, alkadienes and branched alkanes increased from 3.4 % ± 1.43 (alkenes), 0.06 % ± 0.044 (alkadienes) and 0.00 % ± 0.008 (branched alkanes) in wax scales, to 12 % ± 1.3, 2.0 % ± 0.21 and 0.95 % ± 0.129 in old comb wax respectively. These systematic changes of alkene, alkadiene, and branched alkane contents were even more pronounced when the hydrocarbon fraction (fraction 1) alone was analysed. In this case, the contributions of the three substance classes to the total of hydrocarbons increased from 24 % ± 1.1, 0.38 % ± 0.071 and 0.05 % ± 0.048 in wax scales, to 40 % ± 0.5, 7.0 % ± 0.09 and 3.3 % ± 0.08 in old comb wax respectively (Table 16.7).

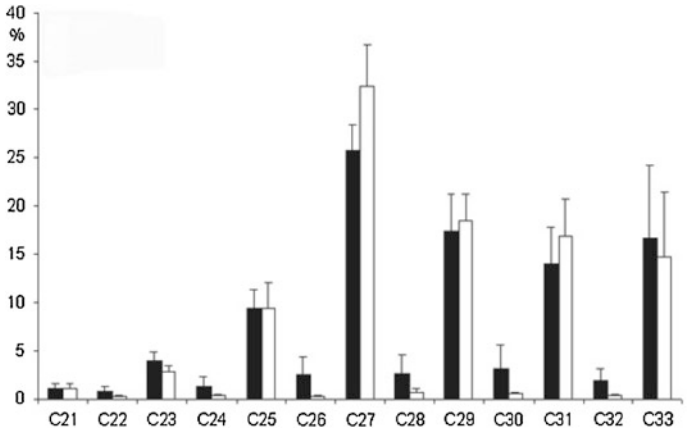

Fig. 16.3 Histogram of the averaged peak areas of the alkanes extracted from *light coloured* (*white columns*) and *dark coloured* (*black columns*) *A. m. ligustica* beeswax samples. The relative peak areas are normalized to the most abundant alkane. Cx refers to *n*-alkane with x carbons in its chain. Y axis = % (from Namdar et al. 2007)

More recently, Namdar et al. (2007) published GC and GC/MS analyses of light and dark coloured *A. m. ligustica* and *A. m. syriaca* combs (Fig. 16.3). They found that, as beeswax ages and darkens, its *n*-alkane composition changes. The amount of even numbered *n*-alkanes (C_{22}–C_{32}), is significantly higher in darker coloured beeswax compared to light beeswax. They attributed these differences, at least in part, to the accumulation of cuticular residues known to contain C_{23} to C_{32} odd and even numbered *n*-alkanes. They determined the presence of odd and even numbered *n*-alkanes, and showed that there was a clear predominance of the C_{27} alkane, with only very small amounts of even numbered *n*-alkanes in the range of C_{22}–C_{32}. Also, darker beeswax contains on average about 3 times more even numbered *n*-alkanes than lighter coloured beeswax.

16.3.2 Discrimination and Classification of Beeswaxes

Before introducing this topic, it is often important to identify and separate pure beeswax from contaminant resins, such as slumgum, which occur in beeswax samples (Grout 1946; Morales-Corts et al. 2010). It was recently reported that waxes and contaminating resins can readily be identified by differential scanning calorimetry (Zhang et al. 2012). Quantitative criteria for the distinction between comb age classes, castes are possible based on chemical features of the respective waxes are both desirable and possible Frölich et al. (2000) subjected their data to a discriminant function analysis which allows the predictive classification of cases (wax samples) by computation of classification functions. These functions are not

to be confused with discriminant functions. Only substance classes that could be positively identified by gas chromatography-mass spectrometry, were included. The results of their analysis functions achieved 99.3 % unambiguous discrimination into the classes: wax scales, new wax, middle aged wax and old wax.

The chemical changes recorded by Frölich et al. (2000) during the ageing process of comb wax, seem to consist of two parallel processes. They proposed that the decrease in chain length with age (process 1), may be due to lipolytic enzymes (Kurstjens et al. 1985; Davidson and Hepburn 1986; Hepburn 1986), which bees add to the wax scales during their conversion into comb wax. These enzymes might be esterases, and this could result in a decrease in long-chain esters and subsequently an increase in shorter chains. The second process (2), may be due to spontaneous physical and chemical processes rather than the direct influence of the bees. The olfactory system of the honeybee is very sensitive to hydrocarbon compounds (Page et al. 1991), the clearly distinguishable wax compositions may be cues for the honeybees to distinguish different regions of the nest for allocating tasks, or to identify nestmate bees they meet in the darkness of the nest (Tautz 2009) (cf. Chap. 5). Phiancharoen et al. (2011) calculated the weighted frequency distributions of the compounds in Table 16.4 to determine the average chain length of each type of wax as shown in Table 16.8. There were no significant differences among the waxes, although there is a trend suggesting that the waxes of the dwarf honeybees have the longest chain lengths. This is surprising because, as a general rule, stiffness, strength, yield stress and other properties increase with increasing carbon chain length in polymers (Salamone 1996), but this relationship does not hold for beeswaxes.

In a further study on wax discrimination Phiancharoen et al. (2011) performed a cluster analysis of beeswax composition, based on the data of Aichholz and Lorbeer (1999) (Table 16.4) to assess their relative affinities, as measured by the Euclidean distances using the unweighted pair-group centroid amalgamation rule. A parsimonious unweighted pair-group analysis based on the distribution of the chemical constituents for 82 elution peaks of the derivatized comb waxes of *A. andreniformis*, *A. cerana*, *A. dorsata, A. florea*, *A. laboriosa* and *A. mellifera* is shown in Fig. 16.4. The giant honeybee group (*A. dorsata* and *A. laboriosa*) is clearly separated from the other species, as are the dwarf species (*A. andreniformis* and *A. florea*), while *A. mellifera* is placed close to its sister-group, *A. cerana*.

The Euclidean distances of beeswaxes presented a very similar picture, which is consistent with the recent analyses of *Apis* species, in which three distinct clusters of sister-groups result from morphometric (Alexander 1991), behavioural (Raffiudin and Crozier 2007) and DNA sequence analyses (Arias and Sheppard 2005): (1) dwarf bees (*A. andreniformis* and *A. florea*); (2) giant honeybees (*A. dorsata* and *A. laboriosa*); and (3) a cluster consisting of the medium-sized bees (*A. cerana*, *A. koschevnikovi*, *A. mellifera*, *A. nigrocincta* and *A. nuluensis*). In any event, the close proximity of the beeswax unweighted pair-groups to those based on DNA and morphometrics, suggests that the wax glands and the products of secretions were highly conserved features during honeybee evolution (Fig. 16.4).

Table 16.8 Weighted frequency distributions for carbon chain length variation in *A. mellifera, A. cerana, A. florea, A. andreniformis*, *A. dorsata* and *A. laboriosa* waxes calculated from the data in Table 16.4 (Phiancharoen et al. 2011)

Structure	*A. mellifera*		*A. cerana*		*A. florea*		*A. andreniformis*		*A. dorsata*		*A. laboriosa*	
	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.
C20	1.1	7.9	0.0	0.0	0.0	0.0	0.8	6.0	0.8	5.9	0.0	0.0
C22	0.7	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.3	2.4	0.4	3.2
C23	0.4	3.3	0.0	0.0	0.0	0.0	1.1	9.5	0.4	3.4	0.3	2.5
C24	6.0	51.8	0.0	0.0	0.0	0.0	0.0	0.0	1.4	12.3	0.7	6.1
C25	1.5	13.5	0.9	8.2	1.5	13.8	7.0	65.4	4.3	39.5	3.8	34.3
C26	2.1	19.6	0.5	4.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C27	6.2	60.2	8.2	80.4	6.9	68.8	5.4	54.5	3.6	35.7	3.6	35.1
C28	2.6	26.2	1.2	12.2	0.4	4.1	0.5	5.2	0.0	0.0	0.0	0.0
C29	2.6	27.1	2.9	30.5	4.0	42.8	3.8	41.2	1.2	12.8	1.7	17.8
C30	2.1	22.7	1.9	20.7	0.4	4.4	0.4	4.5	0.0	0.0	0.0	0.0
C31	2.3	25.7	0.0	0.0	3.5	40.1	1.8	20.9	0.9	10.3	1.3	14.6
C32	1.6	18.4	0.0	0.0	0.0	0.0	0.2	2.4	0.3	3.5	0.6	6.9
C33	2.7	32.1	2.2	26.4	3.9	47.5	0.5	6.2	1.0	12.1	2.3	27.4
C34	1.5	18.4	0.0	0.0	0.0	0.0	0.3	3.8	1.4	17.5	1.8	22.1
C35	0.6	7.6	5.4	68.6	0.6	7.8	1.8	23.5	0.0	0.0	1.7	21.5
C36	0.3	3.9	0.0	0.0	0.0	0.0	0.4	5.4	0.7	9.3	0.8	10.4
C37	0.0	0.0	1.0	13.4	0.0	0.0	2.3	31.8	0.0	0.0	0.8	10.7
C38	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	7.0	0.7	9.6
C39	0.0	0.0	0.0	0.0	0.0	0.0	2.4	35.0	0.0	0.0	0.6	8.4
C40	7.5	108.0	0.7	10.2	1.5	22.1	2.1	31.4	39.7	583.6	35.6	514.1
C41	0.0	0.0	0.0	0.0	0.0	0.0	1.7	26.0	0.0	0.0	0.0	0.0
C42	5.4	81.6	1.3	19.8	3.8	58.9	2.3	36.1	11.2	172.9	11.6	175.9
C44	7.5	118.8	7.6	121.4	13.0	211.1	12.0	197.3	2.5	40.4	2.9	46.1
C46	15.1	250.0	32.9	549.4	19.9	337.9	15.4	264.7	1.6	27.0	2.4	39.9

(continued)

Table 16.8 (continued)

Structure	*A. mellifera*		*A. cerana*		*A. florea*		*A. andreniformis*		*A. dorsata*		*A. laboriosa*	
	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.
C48	11.2	193.5	6.6	115.0	8.8	155.9	6.6	118.4	2.5	44.1	4.1	71.1
C50	2.9	52.2	2.6	47.2	2.5	46.1	2.5	46.7	2.8	51.4	3.5	63.2
C52	0.0	0.0	0.0	0.0	0.3	5.8	0.9	17.5	0.4	7.6	0.9	16.9
C54	1.6	31.1	1.5	29.4	2.2	43.9	2.6	52.5	7.0	138.9	5.2	101.4
C56	1.2	24.2	1.6	32.5	1.6	33.1	1.9	39.8	3.4	70.0	2.9	58.6
C58	1.4	29.2	2.6	54.7	6.6	141.3	5.2	112.7	1.5	32.0	1.2	25.1
C60	2.0	43.2	6.4	139.4	5.3	117.4	4.3	96.4	0.4	8.8	0.0	0.0
C62	1.2	26.8	2.3	51.8	2.4	54.9	1.6	37.1	0.0	0.0	0.0	0.0
C64	0.4	9.2	0.6	13.9	0.3	7.1	0.5	12.0	0.0	0.0	0.0	0.0
Total	91.7		90.9		89.4		88.3		89.8		91.4	
Mean		39.7		43.9		44.4		42.5		40.9		40.7
SD		55.7		98.4		73.9		58.4		104.8		92.4

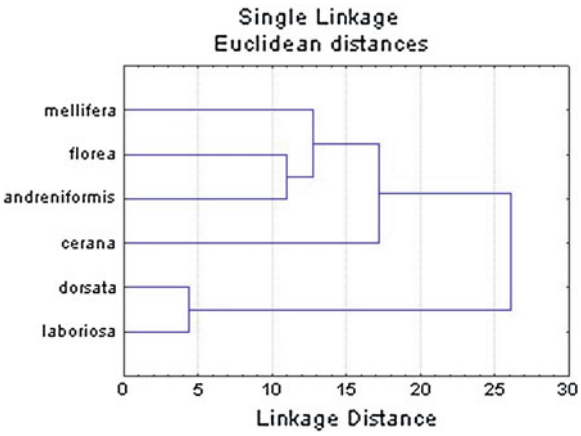

Fig. 16.4 Hierarchical clustering diagram derived from single linkage clustering compound composition of derivatized comb wax (Phiancharoen et al. 2011)

16.4 The Proteins of Beeswax

That beeswax might contain non-lipoidal material has been a very real possibility since Huber (1814) showed that beeswax scales and comb wax have different solubility characteristics. A century later Lineburg (1924) described in detail how worker bees chew and maul wax scales, adding a frothy substance to them. Kurstjens et al. (1985) pursued this probability as a by-product of their studies on the physical changes that occur in the conversion of wax scales into fashioned comb. They found that scale wax did not exhibit a detectable monoglyceride fraction, but had a relatively large pool of diglycerides. In comb wax there was a pronounced monoglyceride fraction, and the diglyceride fraction was considerably less than that in scale wax.

These gross chemical differences between wax scales and finished combs led directly to a search for proteinaceous material that could be added to the wax during chewing, and which might have the expected lytic properties, as had been noted decades earlier by Lineburg (1924). In the search for bee-derived proteins in beeswax, it was essential to preclude any contamination of the scale and comb waxes used in the analyses. Such wax was obtained by keeping small colonies of bees made from newly enclosed brood, confined in a laboratory with no opportunity to forage, nor access to pollen or honey. The bees were only fed a syrupy solution of sucrose. Kurstjens et al. (1985) were able to confirm that scale wax obtained under these conditions contained about 2 μg of protein /mg of wax, and that comb wax contained about 6 μg of protein/mg of wax.

Because beeswax is hydrophobic, it was surmised that it is transported through the pore canals to the exterior surface of the wax mirror by lipophorins. This appears to be the major transport mechanism of hydrophobic natural products in insects (Gilbert and Chino 1974; Haruhito and Chino 1982). Because the lipid composition changes in the conversion of scales into comb wax (Kurstjens et al. 1985), it is also likely that some lipolytic protein is introduced into the scale wax when the bees chew it (Lineburg 1924; Kurstjens et al. 1985). In a series of

Table 16.9 Volatile components of beeswax characterized by gas chromatography-mass spectrometry (Ferber and Nursten 1977)

Hydrocarbons	Alcohols	Carbonyls
p-cymene	*cis*-linalol oxide (5-membered)	Octanal
Durene	*trans*-linalol oxide (5-membered)	Nonanal
Isodurene	*cis*-linalol oxide (6-membered)	Decanal
Decane	*trans*-linalol oxide (6-membered)	
Dodecane	Hotrienol	
Tridecane	ά-terpineol	
Tetradecane	Guaiacol	
Pentadecane	Benzyl alcohol	
Hexadecane	2-phenethyl alcohol	
Naphthalene	Phenol	
ά-methylnaphthalene		
β-methylnaphthalene		

electrophoretic studies on the beeswax proteins of *A. m. capensis* and *A. m. scutellata*, Kurstjens et al. (1990) showed that the substructures of the wax scale and comb protein fractions contained 11 and 13 bands respectively. Seven of these bands were common to both scale and comb waxes for both subspecies.

The proteins ranged between 19 and 100 kD. Bands 1, 2, 6 and 17 (about 97, 89, 66, and 19 kD respectively), were unique to scale wax, while bands 3, 4, 10, 11 and 15 (87, 82, 54, 47 and 43 kD respectively), were unique to comb wax. The waxes shared bands 5, 7–9, 12, 14 and 16 (70, 60, 57, 55, 51, 44 and 29 kD respectively). The densitometric scans showed the relative molecular weight distributions of the bands, and that band 17 is dominant in scale wax, while bands 7–12 are collectively dominant in comb wax. Although wax scales and comb wax contain both unique and shared proteins, their functions are unknown. However, two kinds of lipophorins occur in honeybees (Ryan et al. 1984), and it was surmised that apolipophorin II of honeybees at 78 kD is very close to the 82-kD fraction of comb wax, and to the 70-kD fractions shared by both comb and scale waxes. Although workers chew wax during comb-building, sometimes almost intact scales can be seen in cell walls (Casteel 1912; Zhang et al. 2010), this too points to the addition of a salivary secretion because when incorporated in scale wax, the diacylglycerol component of scales is reduced, and the monoacylglycerol fraction of comb wax increases (Davidson and Hepburn 1986).

16.5 Plant-Derived Aromatic Volatiles and Colourants in Beeswax

Although beeswax has long been a very valuable commodity and its aroma one of its particularly favoured qualities, no analyses of these volatiles were undertaken until the work of Ferber and Nursten (1977). They used a combined GC-MS

Table 16.10 Components of propolis recovered from beeswax (Puleo 1991, and references therein)

1	Citronellol
2	Cinnamic acid
3	Cinnamyl alcohol
4	Coumaric acid, *p*-hydroxycinnamic acid
5	Coumaric acid, *p*-methoxycinnamic acid
6	Cinnamyl-*p*-coumate
7	Vanillin, 4-hydroxy-3-methoxybenzaldehyde
8	Isovanillin, 3-hydroxy-3-methoxybenzaldehyde
9	Caffeic acid, 3,4dihydroxycinnamic acid
10	Ferulic acid, 4-hydroxy-3-methoxycinnamic acid
11	Ferulic acid, 2-hydroxy-4-methoxyacetophenone
12	Ferulic acid, 2-hydroxy-4,6-methoxyacetophenone
13	Pterostilbene, 4-hydroxy-2-4-dimethoxystilbene
14	Pterostilbene, 2′-hydroxy-4′,6′-dimethoxychalcone
15	Pterostilbene, 2′-hydroxy-4-acetyl-5-hydroxy-2-methyl-2H-3H-naptho (1,8-b,c)pyran
16	Pterostilbene, 2′-hydroxy-4,4′6′-trimethoxychalcone
17	Pterostilbene, 2′-hydroxy-3,4,4′-trimethoxychalcone
18	Xanthorrhoeol, 4-acetyl-5-hydroxy-2-methyl-2H-3H-naptho(1,8-b,c)pyran
19	Xanthorrhoeol, 3,5-dimthoxybenzyl alcohol
20	Benzoic acid
21	Benzyl alcohol
22	Sorbic acid, hexa-2,4-dienoic acid
23	Eugenol, 4-aliyl-2-methoxyphenol
24	Lanosterol
25	Squalene
26	Cholesterol
27	Chrysin, 5,7-dihydroxyflavone
28	Techochrysin, 5-hydroxy-7-methoxyflavone
29	Acacetin, 5,7-dihydroxy-4′- methoxyflavone
30	Acacetin, 5-hydroxy-4′,7′- dimethoxyflavone
31	Quercetin, 3,3′4′,5,7-pentahydroxyflavone
32	Kaempferide, 3,5,7-trihydroxy-4′-methoxyflavone
33	Rhamnocitrin, 3,4′,5- trihydroxy-4′,7-methoxyflavone
34	Rhamnocitrin, 3,5-dihydroxy-4′,7-methoxyflavone
35	Galangin, 3,5,7-trihydroxyflavone
36	Isalpinin, 3,5-dihydroxy-7-methoxyflavone
37	Pectolinarigenin, 5,7-dihydroxy-4′,6-methoxyflavone
38	Apigenin, 4′5,7-trihydroxyflavone
39	Kaempferide, 3,4′5,7-tetrahydroxyflavone
40	Flavone, 5-hydroxy-4′7-methoxyflavenone
41	Pinostrobin, 5-hydroxy-7-methoxyflavenone
42	Pinocembrin, 5,7-dihydroxyflavenone
43	Sakuranetin, 4′,5-dihydroxy-7-methoxyflavenone
44	Quercetin-3,3′dimethyl ether, 4′,5′7-trihydroxy-33′-dimethoxyflavone
45	Pinobanksin, 3,5,7-trihydroxyflavenone
46	3-Acetylpinobanksin, 5,7-hihydroxy-3-acetylflavenone

approach, and for positive identification, they used retention indices of ±0.10 for unknowns and standards on each of two columns of differing polarity, as well as acceptable mass spectral data (Table 16.9). In view of the now well-established interactions between pheromones and comb and/or cuticular waxes (Breed et al. 1995a, b, 1998), it is essential to know the chemical composition of the waxes involved, and to be able to classify same. The aromatic volatiles detected in *A. mellifera* wax and listed by Ferber and Nursten (1977) could lead to unimagined possibilities for studies on nestmate recognition.

Subsequently Puleo (1991) performed an exhaustive analysis of the minor constituents of beeswax. Table 16.10 demonstrates the extraordinary diversity of plant-derived compounds (collectively, propolis). Among them is a large percentage of chromophoric (C = C, C = O, N = N, C–NO_2) and auxochromic (C–OH, CNH_2, COOH) groups, which contribute to the strong colour of beeswax. This results from the fact that the auxochromes enhance the colouring capacity of the chromophores (Puleo 1991).

References

Aichholz R, Lorbeer E (1999) Investigation of comb wax of honeybees with high-temperature gas chromatography and high-temperature gas chromatography-chemical ionization mass spectrometry I. High-temperature gas chromatography. J Chromatogr A 855:601–615

Aichholz R, Lorbeer E, Pechhacker H, Hüttinger E (2000) The chemical compounds of beeswax from *Apis* species. In: Proceedings of 4th Asian apicultural association international conference, Kathmandu, pp 152–154

Alexander BA (1991) A cladistic analysis of the genus *Apis*. In: Smith DR (ed) Diversity in the genus *Apis*. Westview Press, Boulder, pp 1–28

Arias MC, Sheppard WS (2005) Phylogenetic relationships of honeybees (Hymenoptera: Apinae: Apini) inferred from nuclear and mitochondrial DNA sequence data. Mol Phylogenet Evol 37:25–35

Basson I, Reynhardt EC (1988) An investigation of the structures and molecular dynamics of natural waxes. I. Beeswax. J Phys D: Appl Phys 21:1421–1428

Bellier J (1906) Beeswax from Annam. Ann Chim Anal 11:366–368

Beverly MB, Kay PT, Voorhees KJ (1995) Principal component analysis of the pyrolysis-mass spectra from African, africanized, and European beeswax. J Anal Appl Pyrol 34:251–263

Blomquist GJ, Ries M (1979) The enzymatic synthesis of wax esters by a microsomal preparation from the honeybee *Apis mellifera* L. Insect Biochem 9:183–188

Blomquist GJ, Chu AJ, Remaley S (1980) Biosynthesis of wax in the honeybee, *Apis mellifera* L. Insect Biochem 10:313–321

Brand-Garnys EE, Sprenger J (1988) Beeswax: new aspects of a classic raw material for cosmetics. Seifen, Öle, Fette 114:547–552. (In German)

Breed MD, Garry MF, Pearce AN, Hibbard BE, Bjostad L, Page RE (1995a) The role of wax comb in honey bee nestmate recognition. Anim Behav 50:489–496

Breed MD, Page RE, Hibbard BE, Bjostad L (1995b) Interfamily variation in comb wax hydrocarbons produced by honey bees. J Chem Ecol 21:1329–1338

Breed MD, Leger EA, Pearce AN, Wang YJ (1998) Comb wax effects on the ontogeny of honey bee nestmate recognition. Anim Behav 55:13–20

Büchner G (1906) On Indian Ghedda-wax. Chem-Ztg 43:528–529. (In German)

Büll R (1977) Das grosse Buch vom Wachs: Geschichte, Kultur, Technik. Callwey, Munchen

Casteel DB (1912) The manipulation of the wax scales of the honey bee. Circ US Bur Entomol No 161:l–13
Coggshall WL, Morse RA (1995) Beeswax: production, harvesting, processing and products. Wicwac Press, Cheshire
Cowan TW (1908) Wax craft. Sampson Low, London
Davidson BC, Hepburn HR (1986) Transformations of the acylglycerols in comb construction by honeybees. Naturwissenschaften 73:159–160
Dumas JB, Edwards HM (1843) Note sur la production de la cire des abeilles. Ann Sci Nat Paris 20:l–8
Eckert A (1922) Über Bienenwachs. Ein Beitrag zur Frage der Herkunft des Bienenwachses. Lotos Naturwiss Z 70:293–299
Eckert A (1927) Das Werden des Wachses nach der Biologischen Seite. Bienenpflege Ludwigsburg 49:193–198
Ferber CEM, Nursten HE (1977) The aroma of beeswax. J Sci Food Agric 28:511–518
Francis BR, Blanton WE, Littlefield J, Nunamaker RA (1989) Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera: Apidae). Ann Ent Soc Am 82:486–494
Frölich B, Tautz J, Riederer M (2000) Chemometric classification of comb and cuticular waxes of the honeybee *Apis mellifera carnica*. J Chem Ecol 26:123–137
Gilbert LI, Chino H (1974) Transport of lipids in insects. J Lipid Res 15:439–456
Grout RA (1946) Production and uses of beeswax. In: Grout RA (ed) The hive and the honeybee. Dadant and Sons, Hamilton
Grün AD, Halden W (1929) Analyse der Fette und Wachse. Hirschwaldsche Buchhan'dlung, Berlin
Haruhito K, Chino H (1982) Transport of hydrocarbons by the lipophorin of insect hemolymph. Biochim Biophys Acta 710:341–348
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR, Radloff SE (1998) Honeybees of Africa. Springer, Berlin
Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax in honeybees. Apidologie 22:21–36
Hooper D (1904) Indian bees'-wax. Agric Ledg 7:201–238
Hooper D, Buchner G (1906) On Indian beeswaxes. Anal Bioanal Chem 45:657–658 (In German)
Huber F (1814) Nouvelles observations sur les Abeilles. English translation, 1926. Dadant, Hamilton
Ikuta H (1931) The investigation of Japanese beeswax. Anal Chem 3:430–437
Ikuta H (1934) The investigation of Japanese beeswax (III): composition of hydroxy fatty acid. Analyst 59:353–357
Jordan R, Tischer J, lllner E (1940) Vergleich des von Altbienen erzeugten wachses mit 'Jungfernwachs' und gewohnlichem bienenwachs. Z Vergl Physiol 28:353–357
Kurstjens SP, Hepburn HR, Schoening FRL, Davidson BC (1985) The conversion of wax scales into comb wax by African honeybees. J Comp Physiol B 156:95–102
Kurstjens SP, McClain E, Hepburn HR (1990) The proteins of beeswax. Naturwissenschaften 77:34–35
Lambremont EN, Wykle RL (1979) Wax synthesis by an enzyme system from the honey bee. Comp Biochem Physiol B63:131–135
Lineburg B (1924) Comb-building. Am Bee J 64:271–272
Lockey KH (1985) Insect cuticular lipids. Comp Biochem Physiol B81:263–273
Morales-Corts MA, Gómez-Sánchez R, Pérez-Sánchez J, Prieto-Calvo C (2010) Characterization of bee keeping wastes for use in seedling production. Spanish J Agric Res 8:493–500
Namdar D, Neumann R, Sladezski Y, Haddad N, Weiner S (2007) Alkane composition variations between darker and lighter colored comb beeswax. Apidologie 38:453–461
Narayana N (1970) Studies in Indian honeys and bees waxes. Central Bee Research Institute, Pune
Page RE, Metcalf RA, Metcalf RL, Erickson EH, Lampman RL (1991) Extractable hydrocarbons and kin recognition in honeybees (*Apis mellifera* L.). J Chem Ecol 17:745–756

Phadke RP (1961) Some physico-chemical constants of Indian beeswaxes. Bee Wld 42:149–153
Phadke RP, Nair KS, Nandekar KU (1971) Indian beeswaxes. II. The nature of their chemical constituents. Indian Bee J 33:3–5
Phiancharoen M, Duangphakdee O, Hepburn HR (2011) The biology of nesting. In: Hepburn HR, Radloff SE (eds) Honeybees of Asia. Springer, Berlin, pp 109–132
Puleo S (1991) Beeswax minor components: a new approach. Cosmet Toil 106:83–89
Raffiudin R, Crozier RH (2007) Phylogenetic analysis of honeybee behavioural evolution. Mol Phyl Evol 43:543–552
Roberts OD, Islip HT (1922) The constants of Indian beeswax. Analyst 47:246–251
Ryan RO, Law JH, Schmidt JO (1984) Chemical and immunological properties of lipophorins from seven insect orders. Arch Insect Biochem Physiol 1:375–383
Salamone JC (1996) Polymeric materials encyclopedia. CRC Press, Boca Raton
Tautz J (2009) The buzz about bees: biology of a superorganism. Springer, Berlin
Touchstone C (1993) History of Chromatography. J Liquid Chromatogr 16:1647–1665
Titschack G (1969) Bienenwachs—Analytik und Kennzahlen. Fette, Seifen, Anstrichtmittel 71:369–379
Tulloch AP (1970) The comparison of beeswax and other waxes secreted by insects. Lipids 5:247–258
Tulloch AP (1973) Comparison of some commercial waxes by gas liquid chromatography. J Am Oil Chem Soc 50:269–272
Tulloch AP (1974) Composition of some natural waxes. Cosmet Perfum 89:53–54
Tulloch AP (1975) Chromatographic analysis of natural waxes. J Chromatogr Sci 13:403–407
Tulloch AP (1980) Beeswax—composition and analysis. Bee Wld 61:47–62
Ueno S (1915) Corean beeswax. Analyst 40:343–344
Young RG (1963) The biosynthesis of beeswax. Life Sci 2:676–679
Zhang K, Duan H, Karihaloo BL, Wang J (2010) Hierarchical, multilayered cell walls reinforced by recycled silk cocoons enhance the structural integrity of honeybee combs. Proc Nat Acad Sci 107:9502–9506
Zhang RG, Zhang H, Zheng Z, Zheng H, Feng Y, Wen W (2012) Characterization of five natural resins and waxes by differential scanning calorimetry (DSC). Adv Mat Res 418–420:643–650

Chapter 17
Synthesis of Beeswax

Abstract The notion that honeybees secrete wax and not gather it from blossoms was first shown in the mid-18th century (Hornbostel 1744). Later, Huber (1814) observed that newly settled swarms do not gather pollen but construct combs, and he concluded that beeswax was the secretory product of the glands of the wax mirrors and fuelled by honey. However, the actual amount of fatty material present in bees, before and after their incarceration in experimental cages and in combs constructed in the interim, had to be determined. This Dumas and Edwards (1843) did, and they concluded that the amount of fatty material present at the onset of the experiment could not account for the wax produced by the end of the experiment; hence bees both synthesise and secrete wax. A century later, Piek (1961, 1964) fed captive bees (1-^{14}C)-acetate, (UL-^{14}C)-glucose and deuterated water and recovered the labels both from bees and newly constructed combs. Lambremont and Wykle (1979) incubated homogenates of the wax glands with (1-^{3}H)-tetracosanol and recovered the label only in the wax ester fraction and the ^{3}H wax ester fraction, which yielded a ^{3}H-fatty alcohol with the same R_f value as authentic tetracosanol. Blomquist and Ries (1979) showed that long-chain primary alcohols, fatty acids and the acyl group of acyl-CoA were incorporated in wax monoesters, and that (1-^{14}C)-palmitate entry into the monoester pool was enhanced by ATP, CoA and $MgCl_2$, while the addition of palmitoyl-CoA resulted in a fivefold yield increase. Subsequently, the specific cellular sites for the origin of hydrocarbons and fatty acids within the wax gland complex and the necessary ultrastructural correlates of this activity and of their transport, were determined Hepburn et al. (1991).

17.1 Introduction: Proof of Beeswax Synthesis

At the outset of this chapter, it must be pointed out that all published work on the synthesis of beeswax by honeybees has been restricted to *A. mellifera*; none of the Asian species have been examined in this regard as yet. More than two centuries

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_17,

Fig. 17.1 François Huber (2 July 1750–22 December 1831) was a blind Swiss naturalist who, with the assistance of François Burnens, was able to carry out investigations that laid the foundations of scientific knowledge of the life history of the honeybee. His *Nouvelles Observations sur les abeilles* was published in Geneva in 1792, and was revised and published in 1814 (this was the edition that was used by CP Dadant for an English translation in 1926). It is in this volume that Huber described, in considerable detail, the construction of comb and experiments on the respiration of bees. It remains a modern honeybee text and is still cited today

ago, careful observations and shrewd inferences led Hornbostel (1744), and then Hunter (1792), to conclude that honeybees secrete wax, a view that ran counter to the 2000 year-old belief that bees gather wax from blossoms, as was promulgated by Aristotle (Fraser 1931). Having reached the same conclusion, quite independently, Huber (1814) (Fig. 17.1) attempted an experiment to show that bees actually synthesise wax rather than merely secrete it as a chyme-treated, transmuted form of pollen. Added to this was the contentious problem of the route of secretion: was wax secreted from the proboscis (de Réaumur 1740), the anus (Dobbs 1750), or from the wax mirrors, as Hornbostel, Hunter and Huber believed?

17.1.1 François Huber (1814)

A key piece of evidence that led to Huber's experiments were observations, by both de Réaumur and Hunter, that newly settled swarms do not gather pollen, but avidly construct combs, whereas old established colonies readily gather pollen.

Huber converted these observations into a series of experiments. He fully appreciated that the time element must be such as to preclude the elaboration of wax from pollen that might have been ingested before the experiment. Huber hived a swarm in a wax-free skep, and placed it in a room where the bees were given water and honey, but no pollen. Five days later, the bees had eaten the honey and produced new combs. He repeated this experiment for about a month, and always obtained the same result: a continuous supply of honey was sufficient for comb construction to proceed. He then mounted the reciprocal experiment and fed pollen to the bees but not honey. After 8 days he found neither any combs in the skeps nor wax scales on any of the bees. He concluded correctly, if prematurely (cf. below), that beeswax was the secretory product of the glands of the wax mirrors and that the fuel for synthesis was honey, wax was not made from pollen.

Huber anticipated any objections that may arise (the honey he fed his bees might be contaminated with wax), and performed a complementary experiment. Oddly enough he did not mention pollen contamination of honey. He incarcerated three colonies and fed one of them syrup made from white sugar, another syrup from brown sugar, and the third honey. Eight replicated feeding trials always resulted in the production of wax combs in the apparent absence of pollen. Unfortunately, in attempting to discredit pollen as the source of wax, Huber missed the significance of pollen as an essential source of protein. His experiments certainly showed that sugar drives wax secretion, even if his conclusions about pollen were equivocal. Berzelius and Thénard, distinguished academic chemists of the day, regarded Huber's conclusions with reserve, and rightly stated that it had not been conclusively shown that bees have the faculty to produce wax (Holmes 1985); nor had any other animal been shown to be capable of synthesising lipids (Dumas and Edwards 1843).

17.1.2 The Chemists: Dumas and Edwards (1843)

To legitimise the claims of Huber, it was clearly necessary to determine the actual amount of fatty material present in bees before their incarceration in any experimental cage and again at the end of the experiment, as well as that contained in any combs which may have been constructed in the interim. After a few false starts, Dumas and Edwards (1843) hived a small swarm of bees, having removed 5 % to sample for fat analysis. They determined the mean fat content and mass of the standing population of the colony, and of samples obtained from three other such colonies. They proceeded to measure: (1) the wax content of the honey which they fed to their other colonies of confined bees; (2) collected wax scales dropped on the floor of the hive; (3) the amount of comb produced over the 11 days of the experiment; (4) the amount of fatty material contained in larvae and eggs present in the comb, and finally; (5) they measured the amount of fatty material present in the bodies of the bees at the end of the experiment. The results from the experiment are shown in Table 17.1.

Table 17.1 Results obtained by Dumas and Edwards (1843) in their experiment to prove that honeybee workers can synthesize wax

	Input (mg)	Output (mg)
1. Mean fatty material per bee at the beginning of the experiment	1.80	
2. Wax particles in honey fed to bees did not exceed	0.38	
3. Mean quantity of fatty substance available per bee (1 + 2)	2.20	
4. Mass of wax produced per bee during the experiment (2 + 3)		6.40
5. Mean fat content of bees at the end of the experiment (5)		4.30
6. Balance (4 + 5 − 3)		8.50

They concluded that the amount of fatty material present at the onset of the experiment, both as body fat and wax present in honey, was insufficient to account for the amount of wax produced by the end of the experiment; hence bees both synthesise and secrete wax.

17.2 Routes of Synthesis

Of far greater importance than the proof that honeybees synthesise wax, was the demonstration for the first time by Dumas and Edwards (1843) that *an animal* could synthesise fats. This was a burning issue among chemists of the 1840 period, championed on theoretical grounds by the German chemist, Justus von Liebig (Fig. 17.2), and actually opposed by the French under the leadership of Jean-Baptiste Dumas (Fig. 17.3). Thus it is only fitting that Dumas and Edwards should have answered this question in their 'balance sheet' studies of bees. Oddly enough, Dumas and Edwards did not consider their results from bees to hold any significance to the questions as to whether animal are able to synthesize fats. They, and other academicians of their day, were only willing to accord this trait to animals after it had been shown to be so shortly after with Persoz's experiments with geese, and Boussingault's work on pigs (Florkin 1977; McCosh 1984). The confirmation by Dumas and Edwards that sugar or honey was sufficient for the secretion of wax and building of comb, gradually seeped into the general apicultural literature as a method for producing wax (Langstroth 1853; Dzierzon 1861), and soon after, the first attempts to quantify the energetics of the process appeared.

17.3 Biochemical Investigations on Beeswax Synthesis

17.3.1 Hypothetical Scheme for Beeswax Synthesis

A thorough analysis of the synthesis of beeswax is clearly predicated on knowledge of its composition; and, while studies on the latter had been in progress for a good 150 years (Grün and Halden 1929), real headway was only made

Fig. 17.2 Justus Freiherr von Liebig (12 May 1803–18 April 1873) was a German chemist who made major contributions to agricultural and biological chemistry. On theoretical grounds he championed the idea that animals could synthesize lipids, and was proven right with the works of Dumas and Edwards (1843) on honeybees, Persoz (1843) on geese, and Boussingault (1843) on pigs, as cited by McCosh (1984)

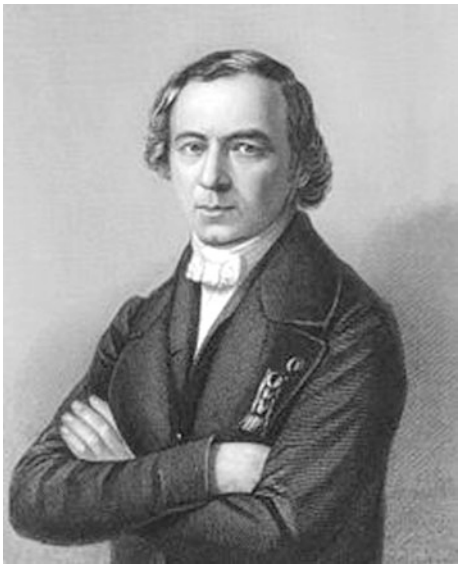

Fig. 17.3 Jean Baptiste André Dumas (14 July 1800–10 April 1884) was a French chemist, renowned for his experiments on organic analysis and synthesis and the determination of relative atomic masses and molecular weights. Although opposed to the idea that animals might synthesize lipids on theoretical grounds, his experiments with honeybees, published in 1843, provided the first proof that an animal could synthesize lipids

intermittently over the past 75 years. Following an extensive series of wax analyses, Chibnall and Piper (1934) and Chibnall et al. (1934) postulated that the primary alcohols of beeswax are formed as reduction products of the corresponding acids. They also suggested that the hydrocarbons arise through decarboxylation of the corresponding acids, hypotheses that remained untested. In an early series of experiments, Piek (1961, 1964) investigated wax synthesis in or

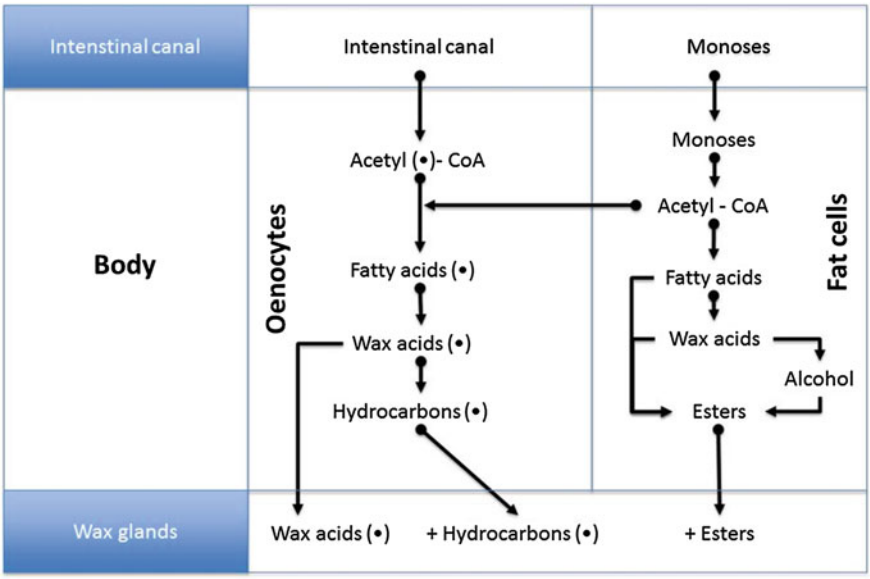

Fig. 17.4 Hypothetical scheme for the synthesis of beeswax based on feeding *A. mellifera* bees with labelled acetate. Asterisks indicate the fate of labelled ^{14}C-labelled material (Piek 1964)

which he fed captive bees (1-^{14}C)-acetate, (UL-^{14}C)-glucose and deuterated water for two weeks, and then recovered the labels from both the bees and the newly constructed combs.

Piek found that both the hydrocarbon and free acid fractions were labelled, but was unable to measure any activity in either the fatty alcohols or wax esters. Coupling his results with the prevailing histological picture of the wax gland complex and the views of Chibnall and Piper (1934), Piek proposed a hypothetical scheme for the synthesis of wax. He concluded that esters are produced by the fat cells (they did not take up acetate, but sequestered monoses from the haemolymph), the hydrocarbons and free wax acids by the oenocytes; the products of both tissues being delivered to the wax glands on the surface of the cuticle as shown schematically in Fig. 17.4.

That Piek did not recover any labelled material in either the ester or alcohol fractions is, in retrospect, possibly attributable to the low specific activity of his starting material and/or to the loss of label through other metabolic pathways. Young (1963) had touched on this problem by injecting (2-^{14}C)-acetate into the body cavities of bees rather than feeding them. Twelve hours after injection he recovered labelled material in both the wax ester and free acid fractions of beeswax scales. Young's results are obviously inconsistent with Piek's conclusion that the fat body cannot metabolise acetate; but, the possibility of partitioning wax synthesis in different tissues prior to secretion, remained an untested and viable possibility.

Fig. 17.5 Proposed route for monoester synthesis in *A. mellifera* (Lambremont and Wykle 1979)

17.3.2 Monoester Synthesis

Two refinements in the study of wax synthesis appeared somewhat later, when Lambremont and Wykle (1979) prepared homogenates of worker bees' wax glands and incubated their cell-free preparations with (1-^{3}H)-tetracosanol. The metabolism of this primary alcohol is such that the label is only known to be recoverable as tritiated water, and the unmetabolised alcohol in wax esters derived from the alcohol. After incubation, they recovered the label only in the wax ester fraction, and showed that the ^{3}H wax ester fraction yielded a ^{3}H-fatty alcohol having the same chromatographic R_f value as authentic tetracosanol. The labelled ester also had the same chromatographic mobility as those of other wax monoesters, which had previously been shown by Tulloch (1970) to consist mainly of palmitates of C_{24}–C_{34} alcohols.

Lambremont and Wykle (1979) concluded that their labelled ester was most probably the monoester, tetracosyl hexadecanoate. They established that the enzyme activity of their preparation was functional between a pH of 6.5–7.4, with maximum activity at a pH of 7.1 at 37 °C, and that Coenzyme A and Mg^{2+} are cofactors in a monoester synthesis driven by ATP. The temperature value of 37 °C for maximum enzyme activity is within 5 % of brood nest temperature and of wax producing bees (Hepburn and Muller 1988). Lambremont and Wykle regarded the enzymes that synthesise the wax esters as not specific for long chain alcohols, because both hexadecanol and tetracosanol were readily incorporated in ester synthesis. Lambremont and Wykle (1979) claimed that tetracosanol and short-chain alcohols are absent from wax monoesters, possibly because the wax gland does not form shorter chain alcohols as opposed to the specificity characteristics of the relevant enzymes. This is open to debate in view of Tulloch's report (1971) on the presence of tetracosanol in commercial samples of beeswax. Finally, Lambremont and Wykle (1979) suggested a route for monoester synthesis that had been previously shown to occur in other animals and in plants (Fig. 17.5).

In a parallel study of ester synthesis, Blomquist and Ries (1979) also used a microsomal preparation of workers' wax glands to investigate the incorporation of long-chain primary alcohols, fatty acids and the acyl group of acyl-CoA into wax monoesters. They showed that (1-^{14}C)-palmitate entry into the monoester pool was enhanced by ATP, CoA and $MgCl_2$, while the addition of palmitoyl-CoA resulted in a fivefold increase in monoester synthesis when labelled tetracosanol was used as the substrate. Accordingly, they concluded that the acyl group of acyl-CoA is transferred to the primary alcohol during the synthesis of monoesters. The works of

both Lambremont and Wykle (1979) and Blomquist and Ries (1979) were based entirely on the recovery of reaction products from microsomal preparations, and the methods and the conclusions they reached were the same. However, neither study had precluded the possible synthesis of epicuticular lipids as distinct from those of wax scales, until Blomquist et al. (1980) specifically addressed this problem.

17.3.3 Cuticular and Comb Waxes

The composition of the wax of the epicuticle of worker honeybees differs quantitatively from that of comb wax (Lockey 1991). While the major component of the epicuticular lipids is hydrocarbon (~58 %), the hydrocarbon content of comb wax is relatively low (~13–17 %) as monoesters comprise the largest component (Tulloch 1971). Blomquist et al. (1980) analysed the major fractions of the epicuticular waxes by gas–liquid chromatography, and found that they were qualitatively similar to those of comb wax. When Blomquist et al. (1980) injected radio-labeled acetate into worker honeybees that were not actively producing comb, they recovered much of the radioactivity in the hydrocarbon fraction. In bees actively producing comb wax, a higher percentage of radioactive products were recovered in the monoester fraction.

Blomquist et al. (1980) also recorded the dramatic effect of age on the distribution of radioactivity from acetate into the various wax fractions from honeybees studied during the summer months. The major wax component synthesized by the wax-secreting age group was monoester, while in both younger and older bees hydrocarbon was the major wax component formed (Fig. 17.6). Both in vivo and in vitro experiments, using insects actively producing comb wax, showed that the abdomen produced significant amounts of monoester, hydrocarbon and other esters, whereas the thorax synthesized mostly hydrocarbon. These data show that the epidermal cells and wax glands each produce a wax with a distinct composition, and that the age and seasonal differences observed in wax synthesis are due to the presence or absence of active wax glands (Blomquist et al. 1980).

Using winter bees, which are not actively engaged in any significant comb-building (although some wax scales are still secreted—Cassier and Lensky 1995), Blomquist et al. (1980) demonstrated that the cuticular waxes, while qualitatively similar in composition to comb wax, differ quantitatively as shown in Table 17.2.

Comb wax is considerably poorer in hydrocarbons but moderately richer in monoesters than the epicuticular waxes of worker honeybees. Turning to the synthesis of wax, Blomquist et al. (1980) proceeded to show that more label could be recovered from the hydrocarbon fraction of bees not actively secreting wax and, conversely, that more label could be recovered from the monoester products of bees actively secreting wax (Table 17.3). Similarly, an analysis of summer bees, (workers raised during spring and the beginning of summer), showed that the greatest amount of labelled acetate was recovered from 11- to 18-day-old bees, those at the peak of wax production (Rösch 1927; King 1928), while labelled

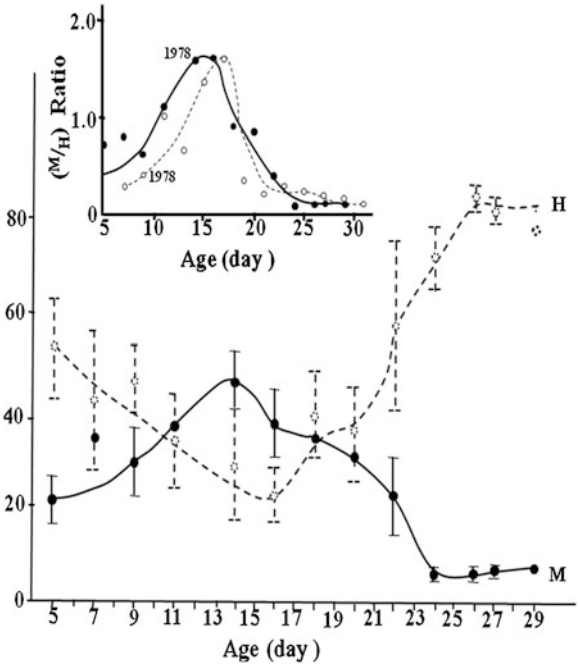

Fig. 17.6 Distribution of (1-^{14}C)-acetate in the hydrocarbon and monoester fractions of *A. mellifera* worker bees of wax secreting age. *H* hydrocarbon, *M* monoester (Blomquist et al. 1980)

Table 17.2 Chemical composition of *A. mellifera* cuticular and comb waxes (Blomquist et al. 1980)

Class	Percent by weight	
	Cuticular wax	Comb wax
Hydrocarbon	58	13–17
Monoester	23	31–35
Diester	9	10–14
Triester	2	
Free fatty acids		3
More polar material	9	34

hydrocarbons dominated the products of both younger and older bees. Against this, they also found that the relative composition of the hydrocarbon pool varied with age (Table 17.4), and independently of whether the bees were actively secreting wax. The hydrocarbon pool also varied seasonally. Finally, on the evidence that the hydrocarbons are derived from acetate, Blomquist et al. (1980) suggested that the (Z)-C_{23}–C_{29} alkenes are derived from fatty acids desaturated at the 9 position, while those desaturated at the 8 and 10 positions serve as intermediates in the formation of longer chain alkenes.

A most interesting piece of natural history traced by Blomquist et al. (1980) involved monitoring the distribution of 1-(1-^{14}C)-acetate in both the monoester and hydrocarbon fractions of adult worker bees (Fig. 17.7). They noted that the rise and fall in wax secretion is closely related to that of labelled monoester

Table 17.3 Incorporation of (1-^{14}C)-acetate into *A. mellifera* comb wax (Blomquist et al. 1980)

Class	Percent distribution	
	Insects not producing Comb wax[a]	Insects actively producing Comb wax[b]
Hydrocarbon	47 ± 3[c]	32 ± 6
Monoester	18 ± 2	35 ± 7
Diester	7 ± 1	9 ± 2
Triester	12 ± 1	3 ± 1
Free fatty acids		
More polar lipid	16 ± 3	21 ± 4

[a] Insects used in April 1978 when they were not actively producing comb wax
[b] Insects were actively producing comb wax as evidenced by wax scales on the ventral abdomen. Insects were used in June 1978
[c] Values are the mean ± S.D. Five groups of five insects per group were used in each experiment

Table 17.4 Changes in the composition of the major hydrocarbons in *A. mellifera* with worker age (Blomquist et al. 1980)

Hydrocarbon component	Percentage composition					
	7 days	9 days	16 days	18 days	22 days	26 days
nC_{23}	2.8[a]	2.5	2.3	4.1	3.8	25.8
$nC_{23:1}$	0.7	0.4	0.3	0.2	1.1	4.6
nC_{25}	3.0	3.0	2.5	5.7	5.7	27.2
$nC_{25:1}$	1.3	0.9	1.1	1.3	2.3	7.6
nC_{27}	7.8	9.4	7.8	11.9	14.5	12.4
$nC_{27:1}$	0.7	0.3	0.2	3.2	1.1	1.8
nC_{29}	7.1	10.4	9.9	10.0	9.0	6.8
$nC_{29:1}$	1.8	1.6	2.2	1.1	2.6	0.8
nC_{31}	6.9	8.5	8.3	7.5	5.7	3.6
$nC_{31:1}$	19.4	17.7	22.8	19.6	17.2	3.2
nC_{33}	1.4	1.5	1.4	1.0	1.0	0.4
$nC_{33:1}$	43.6	41.2	40.1	32.4	35.2	5.6
$nC_{35:1}$	3.5	2.6	1.1	0.7	0.8	0.2
% Saturated	29.0	35.3	32.2	40.2	39.7	76.2
% Unsaturated	71.0	64.7	67.8	59.8	60.3	23.8
% C_{23}–C_{29} Components	25.2	28.5	26.3	37.5	40.1	87.0
% C_{31}–C_{35} Components	74.8	71.5	73.7	62.5	59.9	13.0

[a] Values are the mean of two groups of fifteen insects per group. The range for each value was less than 15 % of the mean

synthesis, and, conversely, to that of hydrocarbon synthesis. This observation is in accordance with the fact that the increase in monoester synthesis, observed in bees during the northern summer which are actively producing wax, is absent from autumnal bees, which normally do not produce wax, nor show any increase in monoester synthesis. These few and hard-won battles towards unravelling wax synthesis in honeybees might well gain impetus from the striking advances made

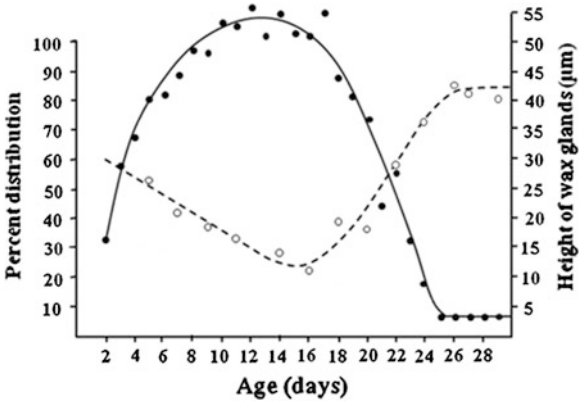

Fig. 17.7 Distribution of (1-^{14}C)-acetate in the hydrocarbon fraction (*open circles*) of *A. mellifera* worker honeybees and the corresponding rise and fall of the epithelium (*closed circles*) of the wax gland in relation to worker age (Hydrocarbon data from Blomquist et al. 1980; wax gland data from Rösch 1927)

by plant chemists in recent years. That many of the components of wax are synthesised from acetate is now accepted as a general principle.

The older view of Chibnall et al. (1934) has been supplanted by the discovery by Kolattukudy (1967a): hydrocarbons and their derivatives are produced by an 'elongation-decarboxylation' mechanism, by which fatty acid synthetase (a multi-enzyme protein that catalyzes fatty acid synthesis) produces palmitic acid. This end-product is elongated through the addition of C_2 units until the growing chain is eventually decarboxylated with the release of hydrocarbons, a pathway which precludes both ketones and secondary alcohols from being sources of wax hydrocarbons. Kolattukudy (1968) further suggested that there are chain-elongating enzymes with different specificities, but this idea awaits confirmation. The origin of fatty alcohols from exogenous fatty acids has also been confirmed with the discovery of fatty acyl-CoA reductase (Kolattukudy 1969). Finally, Kolattukudy (1967b) demonstrated the existence of a protein which catalyses an acyl-CoA-dependent esterification of fatty alcohols giving rise to wax esters. The biosynthesis of long-chain fatty acids requires fatty acid synthetase; however, exogenous units ranging in length from C_2 to C_{24} can be incorporated in syntheses by plants, for which malonyl-CoA is the elongating agent and NADPH the reductant (Kolattukudy et al. 1976) (Fig. 17.8).

17.4 Cellular Basis of Synthesis

Since the work of Clements (1959) on the fat body of locusts, the adipocytes have emerged as the major seat of intermediary metabolism in insects (Candy 1985; Keeley 1985). The peripheral adipocytes, such as those associated with the wax

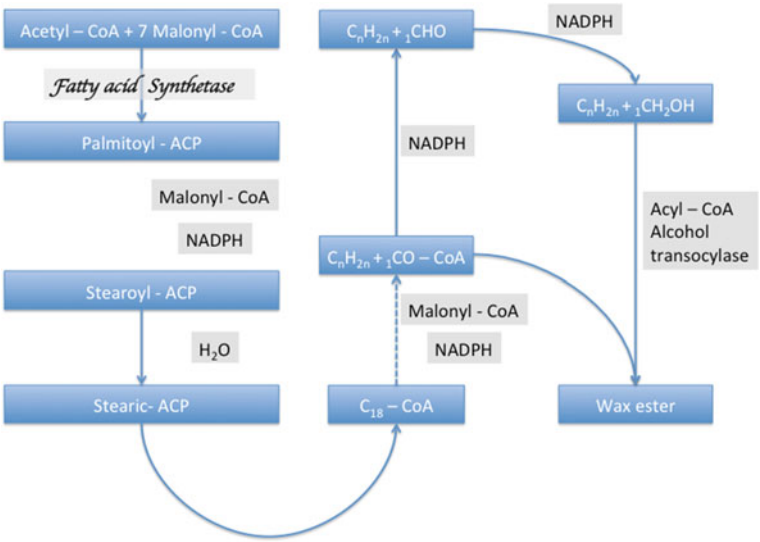

Fig. 17.8 Proposed route for the synthesis of wax esters in plants (Kolattukudy 1980)

mirror epidermis, are regarded as the primary site of lipid synthesis and storage (Dean et al. 1985). However, production of specific classes of compounds is not understood. In studies of hydrocarbon synthesis, Diehl (1973, 1975) demonstrated that the oenocytes of locusts produce hydrocarbons from acetate. Chino (1985) showed that lipophorins transport both hydrocarbons and diacylglycerol from the oenocytes to the cuticle in cockroaches. Similarly, the elongation-decarboxlyation of long chain fatty acids, proposed by Kolattukudy (1967a), has found support in the synthesis of alkanes (Major and Blomquist 1978) and alkenes (Dwyer et al. 1981) in cockroaches.

Because cellular synthesis ultimately depends on mitochondrial respiration, the possibility of neuroendocrine control in relation to wax synthesis must be briefly considered. Altmann (1959) showed that extracts of the corpora allata from laying workers increased the respiratory rate of normal queenright workers, a result clouded by the stimulation of ovarial activation in the recipients. This problem was ultimately clarified in other insects by Slama (1964) and Wiens and Gilbert (1965), who showed that respiration at the cellular level is controlled by the corpora cardiaca. There is also evidence that lipogenesis in the insect fat body is stimulated by the corpora cardiaca (Downer and Steele 1972), and possibly governed by juvenile hormone, since allatectomy results in high levels of lipid production (Gilbert 1967; Steele 1985).

While lipogenesis proceeds from carbohydrate precursors in the fat body (Chino and Gilbert 1965), the rate-limiting reaction in the conversion of glucose to lipid has not been identified. Still, extracts of the corpora allata accelerate glycolysis (Steele 1985). It is also of interest that 20-hydroxyecdysone stimulates

Table 17.5 Changes in hydrocarbons in the wax gland epidermis of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Hydrocarbon	Bee age (days)							
	0	3	6	9	12	15	18	21
25:0	20.6	22.3	21.9	21.3	21.6	24.0	23.2	22.5
27:0	39.7	37.0	33.3	35.0	33.6	37.4	37.8	43.7
29:0	6.9	7.4	8.3	9.7	8.4	10.4	12.2	9.9
31:0	4.4	2.5	3.1	3.9	3.7	2.1	3.7	4.2
33:0	10.3	11.1	12.5	14.6	15.9	12.5	13.4	12.7
35:0	2.9	3.7	2.1	1.9	3.7	3.1	2.4	2.8
Total	83.8	84.0	81.2	86.4	86.9	89.5	92.7	95.8
33:1	4.4	3.7	6.3	5.8	5.6	4.2	2.4	1.4
35:1	11.8	12.3	12.5	7.8	7.5	6.3	4.9	2.8
Total	16.2	16.0	18.8	3.6	13.1	10.5	7.3	4.2

hydrocarbon synthesis in flies (Arnold and Regnier 1975), and that the oenocytes of a beetle can synthesise ecdysteroids (Romer et al. 1974). Finally, Gast (1967), and then Robinson (1985), showed that the implantation of active corpora allata into young bees resulted in the hypotrophy of hypopharyngeal glands and the movement of bees away from brood care, while allatectomy extended the life of these glands (Imboden and Luscher 1975). However, somewhat later, Muller and Hepburn (1994) experimentally established, by allatectomy and corpora allata implants, that neither Juvenile Hormone III nor the corpora allata play a role in regulating either the onset of wax secretion nor the amount of wax secreted.

17.4.1 Chemical Composition and the Ages of Worker Bees

Hepburn et al. (1991) conducted studies on wax synthesis and secretion in honeybees to identify specific cellular sites for the origin of hydrocarbons and fatty acids within the wax gland complex, and to establish the necessary ultrastructural correlates of this activity and of their transport. Of equal importance, they measured the actual rate of wax secretion in bees of different ages, to assess how well chemical composition of the tissues and cycles of ultrastructural change corresponded with the cycles of wax production within the division of labour. They developed a technique to isolate the epidermis, oenocytes and adipocytes, and were able to study each tissue separately. The hydrocarbons and fatty acids of the epidermis and oenocytes were analyzed in bees of the same age as those used in the ultrastructural studies (Tables 17.5 and 17.6). There was an increase in the saturated hydrocarbons dominated by the 2 C_5 and 2 C_7 groups, and a decrease in the 3 C_3 fractions of the oenocytes in relation to age (Table 17.5). The saturated groups increased at the expense of the unsaturated groups, particularly in the case of 33:1.

Table 17.6 Changes in hydrocarbons in the wax gland oenocytes of the Cape honeybee, *A. m. capensis,* workers with age (Hepburn et al. 1991)

Hydrocarbon	Bee age (days)							
	0	3	6	9	12	15	18	21
25:0	23.8	23.0	21.9	26.9	26.6	30.0	29.9	31.0
27:0	38.8	37.2	35.8	36.1	40.7	44.0	47.8	51.7
29:0	4.5	6.4	7.5	12.0	9.7	8.0	6.3	2.0
31:0	3.0	2.6	2.8	2.8	3.5	2.0	3.0	3.4
33:0	6.0	6.4	4.7	4.6	5.3	4.0	4.5	3.4
35:0	1.5	1.3	0.9	0.9	1.8	2.0	3.0	1.7
Total	77.6	76.9	73.6	83.3	87.6	90.0	92.5	93.2
31:1	4.5	6.4	8.5	6.5	5.3	4.0	4.5	3.4
33:1	17.9	16.7	17.9	10.2	7.1	6.0	3.0	3.4
Total	22.4	23.1	26.4	16.7	12.4	10.0	7.5	6.8

The trends for the epidermal cells are similar, but on a smaller scale (Tables 17.5 and 17.6). Notable differences include an increase in the 2 C_9 pool, while 2 C_5 and 2 C_7 remained about the same.

Among the unsaturated groups, there was a marked reduction of 35:1 in the epidermis in relation to age (Table 17.5). The fatty acid profiles of the oenocytes and epidermal cells in relation to age are given in Tables 17.7 and 17.8 respectively.

While the total pool of saturated fatty acids in the oenocytes remained much the same, there were notable increases in 12:0, and decreases in 16:0 and 24:0, in relation to age (Table 17.7). Only minor changes occurred in the unsaturated fatty acids pool. The epidermal cells showed even fewer changes in fatty acid composition in relationship to age (Table 17.8).

Values for scale wax were based on samples harvested over several years in other age-related experiments. Thus, these values represented the already averaged content of thousands of individual wax scales taken from as many bees between the ages of 3- and 21- days-old (an internal control run established no differences between wax scale samples of that were freshly secreted or 2 years old.) Because of the necessity to pool wax scale samples, the data of Tables 17.5, 17.6 and 17.7 were re-expressed as total averages for direct comparison with scale wax in Tables 17.8 and 17.9. The former provided insight into the metabolic activities of the wax gland tissues, on an age-related basis, the latter allowed comparisons of average product content. The average content of scale wax hydrocarbons showed a 50 % reduction in the saturated 2 C_5 groups, compared with the two wax gland tissues (Tables 17.9 and 17.10). Also, the C_{33} hydrocarbons of the oenocytes were far less than those of either the epidermis or scale wax. The other hydrocarbons were much the same for tissue and scale wax (Table 17.7). In the case of the fatty acids, there were large differences between the short chain (C_{12} and C_{14}) and the long chain (C_{24} to C_{28}) groups in both the tissues and the scale wax. There were also notable differences between the tissues and scale wax among the unsaturated fatty acids (Table 17.10).

Table 17.7 Changes in fatty acids in the wax gland oenocytes of the Cape honeybee, *A. m. capensis,* workers with age (Hepburn et al. 1991)

Fatty acid	Bee age (days)							
	0	3	6	9	12	15	18	21
12:0	0.0	0.0	0.0	0.0	3.1	7.0	14.1	28.8
14:0	4.4	4.6	4.8	3.9	4.2	3.5	2.5	2.5
16:0	19.3	17.6	15.0	14.7	14.5	12.0	10.9	8.4
18:0	4.1	3.9	4.1	4.4	4.7	4.8	5.2	4.4
20:0	2.2	1.9	1.7	1.9	1.8	1.9	1.8	1.7
22:0	1.2	1.1	1.2	1.6	1.8	2.0	2.2	1.9
24:0	48.5	48.9	50.8	50.9	54.3	49.2	45.2	34.8
26:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	79.7	77.0	77.6	77.4	79.4	80.4	81.9	82.5
16:1n9	2.3	3.7	4.7	4.6	3.9	3.2	3.0	2.8
18:1n9	4.5	5.4	5.3	5.5	6.0	4.9	3.6	3.9
20:1n9	1.5	1.9	1.4	1.5	1.1	1.0	1.0	0.9
Total	8.3	11.0	11.5	11.6	11.0	9.1	7.6	7.6
18:2n6	7.4	6.7	6.6	6.6	6.8	6.7	6.6	6.3
18:3n6	2.0	1.5	1.3	1.3	1.4	1.3	1.4	1.4
20:4n6	1.2	1.3	1.2	1.1	1.2	1.1	1.1	0.9
Total	10.6	9.5	9.1	9.0	9.4	9.1	9.1	8.6
18:3n3	1.0	1.3	1.6	1.7	1.1	1.0	1.2	1.0
20:5n3	0.4	0.4	0.4	0.5	0.4	0.3	0.4	0.4
Total	1.4	1.7	2.0	2.2	1.5	1.3	1.6	1.4

17.5 Secretion of Beeswax

The amounts of wax borne on average by worker bees of different ages are shown in Fig. 17.9. Paired comparisons of different age groups showed that not all age groups differed significantly. It is nonetheless worth commenting on the magnitude of the standard deviations. It requires between 24 and 48 h for any particular honeybee worker to produce a moderate-sized wax scale (Hepburn and Muller 1988). Moreover, on harvest, one cannot tell whether an individual honeybee, with no or only a little wax on it, is because it either did not secrete any wax, or had recently removed its scales and added them to the comb-building in progress. Consequently, one cannot relate any specific amount of wax back to a defined zero time. However, the general trend of the data is highly significant, and fully supported by the one-way analysis of variance. Thus, the amount of wax borne per bee is significantly affected by the age class of the bee.

When an adult worker bee emerges from its cell, the cuticle of the wax mirror is about 3 μm thick and, unlike other regions of the exoskeleton which increase in thickness with age (Menzel et al. 1969), it remains the same. Its basic ultrastructure has already been described (Locke 1961; Hepburn 1986; Hepburn et al.

Table 17.8 Changes in fatty acids of the wax gland epidermis of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Fatty acid	Bee age (days)							
	0	3	6	9	12	15	18	21
12:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14:0	2.5	2.5	2.9	3.1	2.8	2.9	2.8	2.7
16:0	26.5	23.8	25.9	24.7	26.2	25.6	25.9	25.3
18:0	10.0	7.0	5.5	4.6	3.3	3.9	3.7	7.6
20:0	0.3	0.3	0.2	0.3	0.3	0.4	0.3	0.2
22:0	0.6	0.6	0.6	0.6	0.4	0.3	0.4	0.2
24:0	21.5	25.8	24.4	27.2	25.0	20.4	23.5	22.5
26:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	61.4	59.9	59.6	60.4	58.1	53.5	56.7	58.5
16:1n9	2.4	2.4	2.1	2.2	2.3	2.1	1.9	2.0
18:1n9	8.0	7.7	7.7	8.4	9.5	9.0	8.5	8.1
20:1n9	2.4	2.4	2.6	2.5	2.6	2.9	2.7	2.6
Total	12.8	12.6	12.4	13.1	14.3	14.0	13.1	12.8
18:2n6	18.1	16.7	17.7	16.2	19.3	22.9	21.0	20.7
18:3n6	0.6	0.6	0.3	0.3	0.3	0.4	0.4	0.4
20:4n6	6.8	9.9	9.9	8.1	7.6	8.8	8.4	7.4
Total	25.4	27.2	27.8	26.6	27.2	32.1	29.8	28.5
18:3n3	0.3	0.3	0.2	0.4	0.4	0.4	0.5	0.3
20:5n3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	0.3	0.3	0.2	0.4	0.4	0.4	0.5	0.3

Table 17.9 Composition (percentage) of hydrocarbons in the wax gland tissues and waxes of the Cape honeybee, *A. m. capensis*, workers (Hepburn et al. 1991)

Hydrocarbon	Oenocytes	Epidermis	Scale wax	Comb wax
25:0	26.6	22.2	11.4	6.6
27:0	41.5	37.2	39.8	33.3
29:0	7.0	9.0	8.1	13.8
31:0	2.8	3.5	4.1	8.9
33:0	4.6	12.9	14.6	15.4
35:0	1.6	2.8	3.3	3.3
Total	84.3	87.6	81.3	81.3
31:1	5.4	–	–	–
33:1	10.3	4.2	4.9	6.5
35:1	0.0	8.2	13.8	12.2
Total	15.7	12.4	18.7	18.7

1991; Cassier and Lensky, 1995). The epidermis was earlier reported to lack both dermal glands and, more importantly, smooth endoplasmic reticulum (SER) during peak wax secretion (Sanford and Dietz 1976; Hepburn et al. 1991). This oversight was later amended by Cassier and Lensky (1995) who provided electron

Table 17.10 Average composition (percentage) of the fatty acids in wax gland tissues and waxes of the Cape honeybee, *A. m. capensis*, workers (Hepburn et al. 1991)

Fatty acids	Oenocytes	Epidermis	Scale wax	Comb wax
12:0	6.6	–	–	–
14:0	3.8	2.7	8.3	4.7
16:0	14.1	25.4	15.2	20.2
18:0	4.5	5.7	4.9	1.4
20:0	1.9	0.3	1.8	1.8
22:0	1.6	0.5	5.0	3.3
24:0	47.8	23.7	28.3	35.8
26:0	–	–	3.0	2.9
28:0	–	–	1.9	2.1
Total	80.3	58.4	68.4	72.1
16:1n9	3.5	2.8	0.9	1.9
18:1n9	4.9	8.4	18.2	15.0
20:1n9	1.3	2.6	–	–
Total	9.7	13.1	19.1	16.9
18:2n6	6.7	19.1	7.2	6.9
18:3n6	1.5	0.4	3.5	2.0
20:4n6	1.1	8.4	–	–
Total	9.3	28.1	10.7	8.9
18:3n3	1.2	0.4	1.8	2.1
20:5n3	0.4	–	–	–
Total	1.6	0.4	1.8	2.1

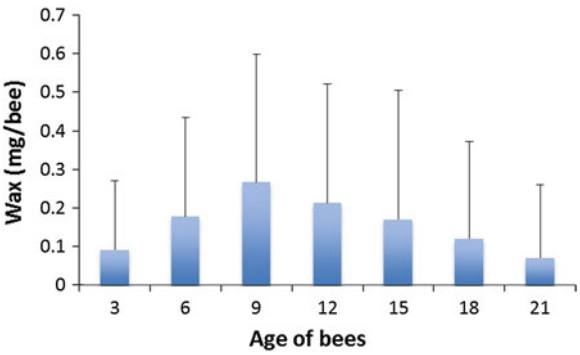

Fig. 17.9 Wax secretion (mg) by individual *A. m. capensis* worker honeybees of different ages (Hepburn et al. 1991)

micrographs of SER in epidermal cells. The idea that the epidermis has no role in the actual synthesis of beeswax is not new (Holz 1878). The major role of the epidermis in the production of wax appears to be the development of an elaborate system of small transport tubules (Reimann 1952; Locke 1961; Hepburn 1986; Cassier and Lensky 1995). The most notable and dynamic feature of the oenocytes is the abundant SER, whose rise and fall are synchronized with measured periods of secretion (Hepburn and Muller 1988), and which is considered to be indispensable for lipogenesis.

On the other hand, adipocytes are the primary site of intermediary metabolism in insects (Downer 1985; Keeley 1985), and the large quantities of lipid, protein and glycogen in the adipocytes associated with the wax gland support this generalization. The early mobilization of lipid from the adipocytes (Table 17.11) suggests that it might produce beeswax precursors. However, the absence of communicating junctions between adipocytes and oenocytes, and the fact that adipocyte lipid reserves are depleted prior to both the maximal development of the oenocyte SER and wax production, mitigates against this possibility. Likewise, at maximal wax production, the lipid content of the adipocyte is more or less constant. Finally, paraffins are synthesized by oenocytes and triglycerides by the adipocytes of locusts (Diehl 1973), and in beetles, lipid oxidation proceeds in oenocytes after they have taken up lipid droplets through the plasma membrane reduction–oxidation system of the adipocytes (Romer et al. 1974). Collectively these observations do not support an adipocyte origin for beeswax lipids.

Although the fine structure of the wax mirror cuticle and its wax transport tubules have now been visualized (cf. Hepburn 1986; Cassier and Lensky 1995), there remains the problem of the physical transport of beeswax precursors. Kurstjens et al. (1990) reported a partial characterisation of the proteins of wax scales and comb wax, in which some 17 fractions were separated. Two of these fractions have been implicated in wax precursor transport, on the grounds that their molecular weight distributions closely approximate those of known honeybee apolipophorins. Thus, it is highly probable that hydrocarbons and fatty acid precursors of beeswax may be synthesized in the oenocytes, an interpretation strongly supported by the data of Hepburn et al. (1991), and then transported through the haemolymph to the surface of the insect in the form of primary or modified apolipophorins (Kurstjens et al. 1990; Hepburn et al. 1991), probably derived from the epidermis (Cassier and Lensky 1995).

Before comparing the chemical content of the wax gland tissues with that of scale wax, it is important to note that the hydrocarbon and fatty acid contents of *A. m. capensis* comb wax (Tables 17.9 and 17.10) are virtually identical to those of its sister-race, *A. m. scutellata*, as reported by Tulloch (1980); results which lend confidence to the analyses presented here. The nature and origins of the large differences between scale and comb wax (Tables 17.9 and 17.10) are post-secretory phenomena, and have been dealt with in detail elsewhere (Kurstjens et al. 1985; Davidson and Hepburn 1986; Hepburn and Kurstjens 1988; and cf. Chaps. 13 and 14). The general trends in the hydrocarbon profiles of the oenocytes include an age-related increase in the saturated components (Table 17.6), reflecting considerable synthetic activity. By comparison, the epidermal hydrocarbons showed more modest changes in relation to the ages of the bees (Table 17.5); these are pronounced among the more minor groups, the unsaturated compounds. The hydrocarbons of the epidermis probably reflect oenocyte-derived material in transit because its age-related changes in hydrocarbons are not synchronized with the cycle of secretion.

There is an apparent discrepancy between the high C_{25} and low C_{35} content of the oenocytes vis-à-vis wax scales; but it could be that C_{33} is formed from C_{25} and

Table 17.11 Volume changes in the wax gland adipocytes of the Cape honeybee, *A. m. capensis,* workers with age (Hepburn et al. 1991)*

Feature	Bee age (days)								
	0	4	6	9	12	14	16	18	21
Whole cell[a]	65 ± 26	110 ± 52	130 ± 100	117 ± 48	123 ± 52	120 ± 87	143 ± 65	95 ± 37	97 ± 32
RER[b]	2	8	5	4	4	3	3	2	2
Mitochondria[b]	5	10	8	8	10	9	11	12	13
Lipid droplets[b]	61	24	22	21	17	25	18	17	21
Protein granules[b]	–	10	19	22	7	1	6	–	–
Glycogen granules[b]	5	3	14	6	10	5	7	10	7

*Adipocyte volume ($\times$ 1000 μm^3). Bees of age 0 days were significantly different ($P < 0.05$) from those of 4–16-day-old groups but not each other, but both were significantly different ($P < 0.05$) from the 4–16-day-old groups

[a] whole cell volume

[b] organelle volume

Table 17.12 Volume changes in the wax gland oenocytes of the Cape honeybee, *A. m. capensis*, workers with age (Hepburn et al. 1991)

Feature	Bee age (days)								
	0	4	6	9	12	14	16	18	21
Whole cell[a]	8 ± 4	36 ± 13	54 ± 16	39 ± 10	45 ± 13	40 ± 14	52 ± 22	32 ± 10	30 ± 12
SER[b]	65 ± 10	83 ± 14	82 ± 6	83 ± 24	81 ± 9	82 ± 10	8 ± 12	85 ± 12	74 ± 15
Mitochondria[b]	10	9	8	8	8	9	7	5	7
Glycogen granules[b]	23	7	8	9	9	7	6	7	15

[a] Oenocyte volume ($\times$ 1000 μm^3). Bees of age 0, 4 and 6 days were significantly different ($P < 0.05$) from each other and from the groups 9–18-day-olds. But there were no significant differences within the 9–18-day-old group. Similarly, bees of 18- and 21-day-olds were not significantly different from each other but were different ($P < 0.05$) from the 9–18-day-old groups

[b] Organelle volume density expressed as a percentage of mean oenocyte cytoplasm occupied by organelle. For SER, bees of age 0 and 21 days were significantly different ($P < 0.05$) from ages 4–18-day-olds. There were no significant differences between any of the 4–18-day-old groups

Table 17.13 Volume changes in the wax gland adipocytes of Cape worker, *A. m. capensis,* honeybees, with age (Hepburn et al. 1991)

Feature	Bee age (days)								
	0	4	6	9	12	14	16	18	21
Whole cell[a]	65 ± 26	110 ± 52	130 ± 100	117 ± 48	123 ± 52	120 ± 87	143 ± 65	95 ± 37	97 ± 32
RER[b]	2	8	5	4	4	3	3	2	2
Mitochondria[b]	5	10	8	8	10	9	11	12	13
Lipid droplets[b]	61	24	22	21	17	25	18	17	21
Protein granules[b]	–	10	19	22	7	1	6	–	–
Glycogen[b] granules	5	3	14	6	10	5	7	10	7

[a] Adipocyte vol (x 1000 μm^3). Bees of age 0 were significantly different ($P < 0.05$) from those of 4–6-day groups but there were no difference within the 4–6-day old groups. Similarly, bees of 18 and 21-days-old were not different from each other, but both were significantly different ($P < 0.05$) from the 4–6-day old groups

[a] whole cell volume

[b] organelle volume

C_{27} outside the oenocytes. The fatty acid profiles of the epidermal cells lacked evident patterns of change consistent with the ageing of the bees or with the cycle of wax synthesis and secretion (Table 17.8)—excepting C_{18}. By contrast, large differences among the fatty acid pools of the oenocytes related both to the ages of the bees and to the cycle of synthesis (Table 17.7) and secretion (Fig. 17.6). Likewise, the average composition for fatty acids in oenocytes more closely mirrors those of scale wax than that of the epidermis (Table 17.8). The presence and increase of C_{12} in the oenocytes coupled to its absence from epidermis and scale wax (Tables 17.7, 17.8 and 17.10), further suggests that the oenocytes perform chain elongation reactions. The decrease in C_{16} and C_{24} in the oenocytes over time is also consistent with synthesis and subsequent export. The oenocytes are the only cells of the wax gland complex whose developmental fate closely matches periods of wax synthesis (Tables 17.11, 17.12 and 17.13).

Unlike those of the epidermis, the hydrocarbon and fatty acid profiles of isolated oenocytes shared much in common with newly secreted wax scales. That the oenocytes are the probable source of beeswax hydrocarbons is supported by the close cyclical changes in ultrastructure that coincide with age-related cycles of secretion of beeswax by worker honeybees. These interpretations are consistent with the histochemical data of Reimann (1952), the autoradiographic studies of Piek (1964), studies of hydrocarbon synthesis in other insects by Diehl (1973, 1975), and with the electron microscopical study results of Cassier and Lensky (1995). Finally, it must be remembered that comb wax also mediates the acquisition of nestmate recognition cues in honeybees. Indeed comb wax in the colony, and the hydrocarbon layer of the epicuticle most probably serve as continuous media for hydrocarbon-soluble substances used by honeybees in nestmate recognition (Breed et al. 1988, et seq.) This aspect of the hydrocarbon story is further developed in Chap. 13.

References

Altmann G (1959) Hormon-physiologische Untersuchungen des Stoffwechsels der Honigbiene. Dtsch Bienenwirtsch 2:74–76

Arnold MT, Regnier FE (1975) Stimulation of hydrocarbon biosynthesis by ecdysterone in the flesh fly, *Sarcophaga bullata*. J Insect Physiol 21:1827–1833

Blomquist GJ, Ries MK (1979) The enzymatic synthesis of wax esters by a microsomal preparation from the honeybee, *Apis mellifera* L. Insect Biochem 9:183–188

Blomquist GJ, Chu AJ, Remaley S (1980) Biosynthesis of wax in the honeybee, *Apis mellifera* L. Insect Biochem 10:313–321

Breed MD, Williams KR, Fewell JH (1988) Comb wax mediates the acquisition of nest-mate recognition cues in honey bees. Proc Nat Acad Sci 85:8766–8769

Candy DJ (1985) Intermediary metabolism. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology biochemistry and pharmacology, vol 10. Permagon, Oxford, pp 1–41

Cassier P, Lensky Y (1995) Ultrastructure of the wax gland complex and secretion of beeswax in the worker honey bee, *Apis mellifera* L. Apidologie 26:17–26

Chibnall AC, Piper SH (1934) The metabolism of plant and insect waxes. Biochem J 34:2209–2219
Chibnall AC, Piper SH, Pollard A, Williams EF, Sahai PN (1934) The constitution of the primary alcohols, fatty acids and paraffins present in plant and insect waxes. Biochem J 28:2189–2208
Chino H (1985) Lipid transport: biochemistry of hemolymph lipophorin. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology biochemistry and pharmacology, vol 10. Pergamon, Oxford, pp 115–135
Chino H, Gilbert LI (1965) Lipid release and transport in insects. Biochim Biophys Acta 98:94–110
Clements AN (1959) Studies on the metabolism of locust fat body. J Exp Biol 36:665–675
Davidson BC, Hepburn HR (1986) Transformations of the acylglycerols in comb construction by honeybees. Naturwissenschaften 73:159–160
Dean RL, Locke M, Collins JV (1985) Structure of the fat body. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology biochemistry and pharmacology, vol 3. Pergamon, Oxford, pp 155–210
de Réaumur RAF (1740) Memoires pour servir à l'histoire des insects. Vol 5. L'imprimerie Royale, Paris
Diehl PA (1973) Paraffin synthesis in the oenocytes of the desert locust. Nature 243:468–470
Diehl PA (1975) Synthesis and release of hydrocarbons by the oenocytes of the desert locust, *Schistocerca gregaria*. J Insect Physiol 21:1237–1246
Dobbs A (1750) A letter from Arthur Dobbs Esq; to Charles Stanhope Esq; F.R.S. concerning bees, and their method of gathering wax and honey. Phil Trans R Soc 46:536–549
Downer RGH (1985) Lipid metabolism. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology biochemistry and pharmacology, vol 10. Pergamon, Oxford, pp 77–113
Downer RGH, Steele JE (1972) Hormonal stimulation of lipid transport in the American cockroach, *Periplaneta americana*. Gen Comp Endocrinol 19:259–265
Dumas JB, Edwards HM (1843) Note sur la production de la cire des abeilles. Ann Sci Nat Paris 20:1–8
Dwyer LA, De Renobales M, Blomquist GJ (1981) Biosynthesis of (Z, Z)-6, 9-heptacosadiene in the American cockroach. Biochim Biophys Acta 663:536–544
Dzierzon J (1861) Rationelle Bienenzucht oder Theorie und Praxis. Falch'sche Buchruckerei, Brieg
Florkin M (1977) A history of biochemistry. In: Florkin M, Stotz EH (eds) Comprehensive biochemistry. Elsevier, Amsterdam
Fraser HM (1931) Beekeeping in antiquity. University of London Press, London
Gast R (1967) Untersuchungen über den Einfluss der Könnigensubstanz auf die Entwicklung der endokrinen Drüsen bei der Arbeiterin der Honigbiene (*Apis mellifera*). Insectes Soc 14:1–12
Gilbert LI (1967) Changes in lipid content during the reproductive cycle of *Leucophaea maderae* and effects of the juvenile hormone on lipid metabolism in vitro. Comp Biochem Physiol 21B:237–257
Grün AD, Halden W (1929) Analyse der Fette und Wachse. Hirschwaldsche Buchhandlung, Berlin
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR, Kurstjens SP (1988) The combs of honeybees as composite materials. Apidologie 19:25–36
Hepburn HR, Muller WJ (1988) Wax secretion in honeybees. Naturwissenschaften 75:628–629
Hepburn HR, Bernard RTF, Davidson BC, Muller WJ, Lloyd P, Kurstjens SP, Vincent SL (1991) Synthesis and secretion of beeswax. Apidologie 22:21–36
Holmes FL (1985) Lavoisier and the chemistry of life. University of Wisconsin Press, Madison
Holz H (1878) Das Organ der Wachsbildung. Bienen-Ztg 34:183–184
Hornbostel HC (1744) Neue Entdeckung, wie das Wachs von den Bienen Komt. Hamburg Vermis Bibliothek 2:45–62 (orig. Mellitophilus Theosebastus)
Huber F (1814) Nouvelles observations sur les abeilles. [English translation, 1926] Dadant, Hamilton

Hunter J (1792) Observations on bees. Phil Trans R Soc 82:128–196
Imboden H, Luscher M (1975) Allatektomie bei adulten Bienen-Arbeiterinnen (*Apis mellifera*). Rev Suisse Zool 82:694–698
Keeley LL (1985) Physiology and biochemistry of the fat body. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology biochemistry and pharmacology, vol 3. Pergamon, Oxford, pp 211–248
King GE (1928) The larger glands in the worker honey-bee. A correlation of activity with age and with physiological functioning. Thesis, University of Illinois, Urbana
Kolattukudy PE (1967a) Mechanisms of synthesis of waxy esters in brocolli (*Brassica olearacea*). Biochem 6:2705–2715
Kolattukudy PE (1967b) Biosynthesis of paraffins in *Brassica oleracea*: fatty acid elongation - decarboxylation as a plausible pathway. Phytochem 6:963–975
Kolattukudy PE (1968) Biosynthesis of surface lipids. Science 159:498–505
Kolattukudy PE (1969) Oxidation of paraffins by plant tissues. Plant Physiol 44:315–317
Kolattukudy PE (1980) Cutin, ruberin and waxes. In: Stumpf PK (ed) The biochemistry of plants, vol 4. Academic, London
Kolattukudy PE, Croteau R, Buckner JS (1976) Biochemistry of plant waxes. In: Kolattukudy PE (ed) Chemistry and biochemistry of natural waxes. Elsevier, Amsterdam
Kurstjens SP, Hepburn HR, Schoening FRL, Davidson BC (1985) The conversion of wax scales into comb wax by African honeybees. J Comp Physiol B156:95–102
Kurstjens SP, McClain E, Hepburn HR (1990) The proteins of beeswax. Naturwissenschaften 77:34–35
Lambremont EN, Wykle RL (1979) Wax synthesis by an enzyme system from the honeybee. Comp Biochem Physiol 63B:131–135
Langstroth LL (1853) Langstroth on the hive and the honeybee. Root, Medina (Facsimile, 1977)
Locke M (1961) The cuticle and wax secretion in *Calpodes ethlius* (Lepidoptera: Hesperidae). Q J Microsc Sci 101:333–338
Lockey KH (1991) Insect hydrocarbon classes: implications for chemotaxonomy. Insect Biochem 21:91–97
Major MA, Blomquist GJ (1978) Biosynthesis of hydrocarbon in insects: decarboxyiation of long chain acids to n-alkenes in *Periplaneta*. Lipids 13:323–328
McCosh FWJ (1984) Boussingault, chemist and agriculturist. Reidel, Dordrecht
Menzel R, Wladarz G, Lindauer M (1969) Tagesperiodische Ablagerungen in der Endokutikula der Honigbiene. Biol Zentralbl 88:61–67
Muller WJ, Hepburn HR (1994) Juvenile hormone III and wax secretion in honeybees (*Apis mellifera capensis*). J Insect Physiol 40:873–881
Piek T (1961) Synthesis of wax in the honey bee (*Apis mellifera* L.). Proc K Ned Akad Wet Ser C Biol Med Sci 64:648–654
Piek T (1964) Synthesis of wax in the honeybee (*Apis mellifera* L.). J lnsect Physiol 10:563–572
Reimann K (1952) Neue Untersuchungen über die Wachsdrüse der Honigbiene. Zoo1 Jahrb Abt Anat Ont 72:147-188
Robinson GE (1985) Effects of juvenile hormone analogue on honey bee foraging behaviour and alarm pheromone production. J Insect Physiol 31:277–282
Romer F, Emmerish H, Nowock J (1974) Biosynthesis of ecdysones in isolated prothoracic glands and oenocytes of *Tenebrio molitor* in vitro. J Insect Physiol 20:1975–1987
Rösch GA (1927) Über die Bautatigkeit im Bienenvolk und das Alter der Baubienen. Weiterer Beitrag zur Frage nach der Arbeitsteilung im Bienenstaat. Z Vergl Physiol 6:265–298
Sanford MT, Dietz A (1976) The fine structure of the wax gland of the honeybee (*Apis mellifera*, L). Apidologie 7:197–207
Slama K (1964) Hormonal control of respiratory metabolism during growth, reproduction and diapause in female adults of *Pyrrhocoris apterus* L (Hemiptera). J lnsect Physiol 10:283–303
Steele JE (1985) Control of metabolic processes. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology biochemistry and pharmacology, vol 8. Pergamon, Oxford, pp 99–145

Tulloch AP (1970) The comparison of beeswax and other waxes secreted by insects. Lipids 5:247–258
Tulloch AP (1971) Beeswax: structure of the esters and their component hydroxy acids and diols. Chem Phys Lipids 6:235–265
Tulloch AP (1980) Beeswax - composition and analysis. Bee Wld 61:47–62
Wiens AW, Gilbert LI (1965) Regulation of cockroach fat body metabolism by the corpus cardiacum in vitro. Science 150:614–616
Young RG (1963) The biosynthesis of beeswax. Life Sci 2:676–679

Chapter 18
Material Properties of Honeybee Silk

Abstract Colourless honeybee silk, ~3 μm diameter, is produced through a spinneret at the tip of the labium-hypopharynx. Successive generations of brood apply silk to the cell walls, making the cells smaller, as silk is deposited in the old brood combs. X-ray diffraction data show that honeybee silk contains ά-helical proteins ordered into coiled-coil structures, with an axial periodicity of about 28 nm, and form a four-stranded array parallel to the fibre axis. Honeybee fibroin is crystalline, but, when hydrated, is only half as stiff as when dry, although they are equal in strength. The fibroin is hygroscopic and highly distensible when solvated because of its molecular conformation. The mechanical properties of silk are independent of temperature. Lithium thiocyanate and urea virtually eliminate the yield point of honeybee silk tested both dry and in distilled water, and values for stress in the slope of the solvent-related curves is reduced. The solvents act directly on hydrogen bonds and then the silks behave as unconnected bends during tensile deformation. The components, hierarchical structure and the conditions of their production all affect the mechanical properties of natural silks. The amino acid sequence in honeybee silk protein provides an explanation of why the coiled-coil packing is atypically tight, and the most abundant core residue is the small amino acid, alanine. An atomistic simulation for the unfolding behaviour of ά-helical protein shows that two discrete transition states correspond to two fracture mechanisms. Six honeybee silk genes have now been identified, using a combination of genomic and proteomic techniques.

18.1 Introduction

The honeybee nest contains areas for the storage of nectar and pollen and the rearing of brood. While wax is the basic building material for the nest, with continued use the combs become modified by the addition of silk and propolis (Hepburn and Kurstjens 1988). Thus, much of the honeybee nest gradually changes from a single phase (wax) to a two-phase or composite (wax/silk)

H. R. Hepburn et al., *Honeybee Nests*, DOI: 10.1007/978-3-642-54328-9_18,

material. Some of the material properties of the individual phases of the honeybee nest have now been characterized (Hepburn et al. 1979; Hepburn 1986; Hepburn and Kurstjens 1988; Kurstjens et al. 1985, 1990); but, particularly important recent studies on the molecular structure of honeybee silk (Sutherland et al. 2006, et seq.) necessitate a review of the composition and properties of honeybee α-helical silk (Fig. 18.1), the elastic element in all honeybee combs. This Chapter is largely based on a recent review of honeybee silk (Hepburn et al. 2013).

"Silk" is a functional term used to describe protein fibres spun by honeybees, many different kinds of insects and other invertebrate animals (Fig. 18.1). The spinning of silk by honeybees does not involve either rotating or twisting fibres, as is done in commercial fibre production, but refers to the process of making an insoluble filament from an aqueous protein solution (Sutherland 2010a). In the case of honeybees, just before pupation, the larvae cover the waxen walls of their cells with silk (Huber 1814; Arnhart 1906), paying out the fibres randomly so that, by the end of spinning, the walls are covered by thin sheets of silk in which the individual fibrils are readily discernible (Jay 1964; Zhang et al. 2010a).

Jay (1964) observed that fibres were formed when the honeybee spinneret was drawn away from the cell wall. In contrast, films were formed when the spinneret was dragged over the cell wall, presumably because the substrate stabilized the thin film. Jay (1964) reported that silk is generated from the labial gland as the larvae perform random head movements in all directions, within the cell. Inasmuch as this behaviour may last up to 48 h, it ensures that in the final product (the cocoon), the fibres form a randomised and mechanically, planar isotropic structure. The colourless silk, about 3 μm in diameter (Zhang et al. 2010a), is produced through a slit-like spinneret located at the tip of the combined labium-hypopharynx.

The inference that the silk proteins are highly organized in the gland lumen before the larvae actually begin spinning (Flower and Kenchington 1967), has recently been supported by Silva-Zacarin et al. (2003). These authors showed that silk formation begins during the middle of the 5th instar and finishes at the end of this developmental stage. This process begins in the distal secretory portion of the gland, going towards the proximal secretory portion, and from the periphery to the center of the gland lumen. The silk proteins are released from the secretory cells as a homogeneous substance that polymerizes in the lumen to form compact birefringent tactoids. Secondly, water absorption from the lumen secretion, carried out by secretory and duct cells, promotes the aggregation of the tactoids that form a spiral-shaped filament with a zigzag pattern. This pattern is also the result of silk compression in the gland lumen, and represents a high concentration of macromolecularly, well-oriented silk proteins.

After spinning, the larvae smear a small amount of material from the Malpighian tubules onto the hardened silk layers, and faeces are also excreted between silk layers (Jay 1964). Subsequently, the larvae produce a colourless pollen-free substance and then a yellow pollen-bearing one (from the anus), both of which are applied in turn to the silk base (Verlich 1930; Jay 1964). Nothing further is known

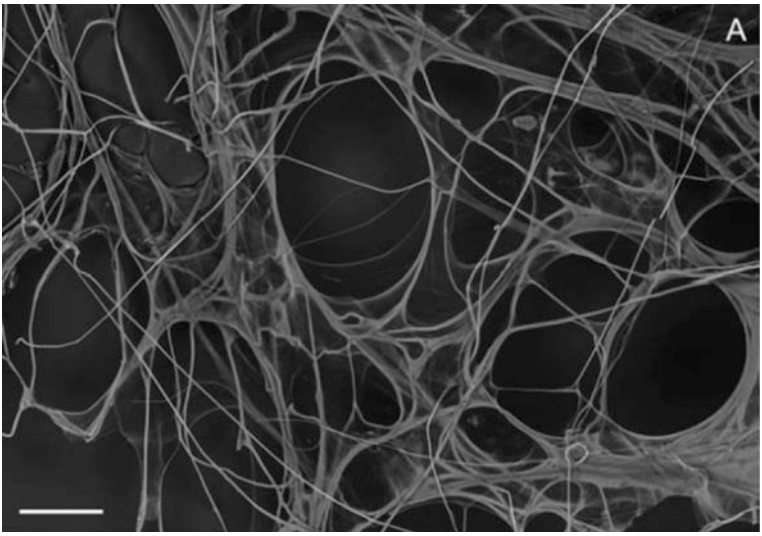

Fig. 18.1 Scanning electron photomicrograph of ά-helical silk fibres produced by *A. mellifera* larvae. Scale bar is 100 μm. Late final instar honeybee larvae were induced to spin silk within plastic tubes, and the clean silk removed before the larvae added any further material (with kind permission of the publishers, from Sutherland et al. 2011a, b)

of these four substances, but they invite the analogy of sizing in paper manufacture.

Successive generations of brood apply more silk to the cell walls so they become smaller, and the mass ratio of silk to wax greater (Chauvin 1962). Thus, old brood combs are heavily impregnated with silk (Fig. 18.2) which is inseparable from the wax except by fairly drastic chemical and/or heat treatments. The development and maturation of brood comb proceeds from a single-phase material (pure white wax), to a coloured, fibre-reinforced, two-phase composite (wax and silk) (Hepburn and Kurstjens 1988; Zhang et al. 2010a). The physical significance of these observations can be illustrated by comparing the properties of the native fibroin, wax-free sheets of silk, silk-free wax, propolis and the final wax-silk composite (Kurstjens et al. 1985; cf. Chap. 4).

18.2 Honeybee Silk: An ά-Helical Protein

Fifty years ago, the crystallographer, KM Rudall (Fig. 18.3), demonstrated in his X-ray fibre diffraction data that silk threads, drawn from honeybee silk glands contain ά-helical proteins assembled into ordered coiled-coil structures, and that their meridional reflections suggested an axial periodicity of about 28 nm (Rudall 1962, 1965). The patterns from honeybee silk fibres were considered most

Fig. 18.2 Longitudinal section of an old, dewaxed comb from *A. m. capensis* showing the layers of silk inside the base and the walls of cells (Hepburn et al. 2007)

Fig. 18.3 KM Rudall, a New Zealander, worked for many years at the then Astbury Department of Biophysics, University of Leeds. He was one of the pioneering crystallographers and molecular biologists who made special and important contributions to the study of the molecular conformations of fibrous proteins fibres, including honeybee silk

consistent with a four-strand coiled-coil structure and a tighter than expected super-helix radius of about 0.52 nm (Atkins 1967). In contrast, the dominant molecular structure in silk of other hymenopteran species is the extended ß-sheet configuration (Warwicker 1960; Sutherland et al. 2007).

So, honeybee silk is an ά-helical fibroin (Rudall 1962), the micelles or crystallites of which form a four-stranded array of coiled-coils parallel to the fibre axis (Atkins 1967). Honeybee fibroin is crystalline, relative to other insect silks (Lucas and Rudall 1968); but hydrated fibres are only half as stiff as dry ones, although they are equal in strength (Hepburn et al. 1979). The fibroin is hygroscopic, and when solvated, is highly distensible, largely owing to its molecular conformation (Lucas and Rudall 1968). These properties of the fibroin are largely suppressed by the cocoon-spinning larvae because the silk is pressed into the wax of the cell wall, possibly aided by the anal secretions, and this immediately water-proofs and checks the silk fibroin against solvation. Thus, it is also likely that inter-micellar friction is enhanced (Warwicker 1960), and the possibility of conformational change restricted (Rudall 1962), effects which are consistent with good stiffness and reduced distensibility (Hepburn et al. 1979). That the silk fibres are spun and randomly arranged in the cell wall overcomes the basic anisotropy of the material, because dewaxed sheets of cocoon silk are planar isotropic on tensile deformation.

18.3 Behaviour of Silk at Different Temperatures

Natural variations in the temperature of honeybee nests invite a consideration of silk behaviour at varied thermal regimes. The independence of the mechanical properties of *A. m. scutellata* silk sheets, when deformed in tension at a fixed rate at different temperatures, is illustrated in Table 18.1.

Sheets of silk maintain the same relative strength and distensibility between 25 and 45 °C, and staunch the plastic flow, and ultimate collapse of wax, at higher temperatures. Consequently changes in stiffness or the energy to fracture the sheet, an index of its relative workability, were not observed. The tensile properties of silk sheets over this range of temperatures are in sharp contrast to those of pure wax (Hepburn et al. 1983), propolis (Hepburn and Kurstjens 1984) and the wax-silk composite of brood combs (cf. Chap. 4). In addition to crystal structure, white comb wax is also affected by the presence of a protein fraction (Kurstjens et al. 1985, 1990). This material is present, quite apart from silk, in both wax scales and in newly constructed combs. In both cases, this partially characterized protein (Kurstjens et al. 1990) is positively associated with enhanced stiffness in both scales and combs. Nothing is known of the molecular behaviour of this protein or how it might contribute to the stiffness of wax. To have assigned this protein to the elastic fraction is somewhat gratuitous.

Table 18.1 Tensile mechanical properties of dewaxed *A. m. scutellata* worker honeybee cocoon silk (Hepburn and Kurstjens 1988)

Temperature °C	Relative tensile strength (Nmm-1)	Breaking strain Percentage (%)	Relative stiffness Nmm-1	Work MJm-3
25	32 ± 16	98	33 ± 14	29 ± 20
30	32 ± 18	81	40 ± 13	28 ± 23
35	26 ± 10	85	31 ± 8	22 ± 14
40	39 ± 17	105	37 ± 14	38 ± 22
45	43 ± 20	106	41 ± 14	48 ± 30

For each value, n = 10

18.4 Relative Crystallinity

Lucas et al. (1960) estimated the relative crystallinity of moth fibroins by calculating short side chain—long side chain ratios. When Hepburn et al. (1979) did the same for honeybee silk, the result suggested that this silk was anomalous because crystalline fibroins generally have a high glycine content and honeybee silk has a very low one, but is nevertheless, relatively crystalline (Atkins 1967). These authors subsequently turned to cellulose, because one feature of cellulose is that the degree of crystallinity is reflected in the sensitivity of its fibres to solution effects. Water can penetrate amorphous regions in a capillary manner thus diminishing the interactions between crystallites; or, alternatively, compete for potential hydrogen-bonding sites within the fibre (Wainwright et al. 1976).

In the work on cellulose it was assumed that hydration loosened the interaction between neighbouring crystalline regions, so reducing stiffness. It was further assumed that the elastic modulus of the dry cellulose approached that of crystalline cellulose. If this were indeed so, then the ratio of modulus wet to modulus dry provides an approximate index of the degree of crystallinity; a ratio of 1 indicating complete and lesser values of progressively less crystallinity. When honeybee silk was examined for hydration sensitivity, expressed as the ratio of the elastic modulus of wet to that of dry fibre, a value of 0.53 showed that this fibre is rather crystalline, a result consistent with other forms of measurement. Tensile stress-strain curves for wet and dry $\acute{\alpha}$-helical honeybee silk are shown in Fig. 18.4. Both wet and dry honeybee silks are characterized initially by linear regions, which terminate in marked yield points at about 0.1 and 0.3 strain respectively. A yield point is defined as a marked decrease in the slope of the stress-strain curve, which occurs over a very small region of strain and, for an $\acute{\alpha}$-helical structure, is associated with the onset of a transconformational change from the $\acute{\alpha}$ to the parallel-β state (Rudall 1962, 1965).

More recently, Zhang et al. (2010b) reported on the microstructures and mechanical properties of honeybee, *A. m. ligustica*, and silkworm, *Bombyx mori*, silks which were examined by environment scanning electron microscopy (ESEM), scanning probe microscopy (SPM), tensile tests, and nanoindentation.

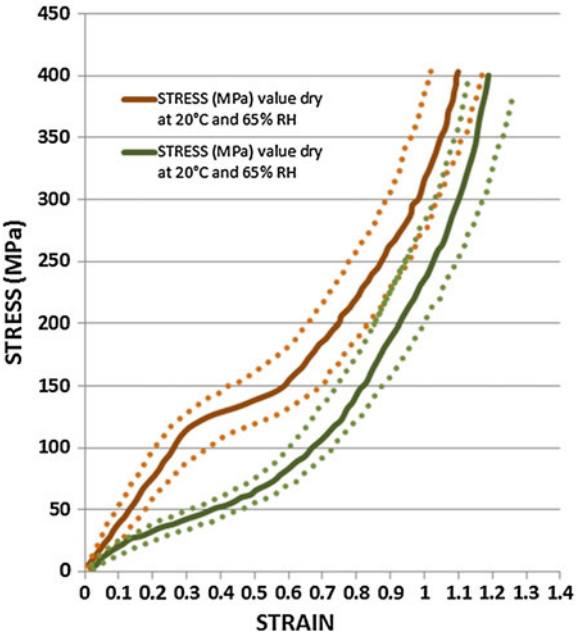

Fig. 18.4 Generalized tensile stress-strain curve for ά-helical silk of *A. m. scutellata* tested dry and then wet (Hepburn et al. 1979)

They concluded that honeybee silk, unlike silkworm silk, is a single fibre with a circular cross-section, which has a much finer, smoother texture than silkworm silk. Honeybee silk exhibits a distinctly linear and brittle elastic mechanical behaviour. Moreover, nanoindentation measurements showed that honeybee silk is much less anisotropic than silkworm silk (Zhang et al. 2010b). The ratio of the longitudinal modulus to the transverse modulus of honeybee silk is 2.0, whereas that of silkworm silk is 18.9. It is probable that the different structural and mechanical properties of honeybee and silkworm silks are likely the result of their specific biological functions (Zhang et al. 2010b).

18.5 Solvent Effects on Silk

A large amount of empirical information on the effects of solvents has accumulated over the past 100 years from the wool, leather and silk industries. A few of these solvents have been studied in considerable detail, and their effects well documented in the general chemical literature. Of these solvents, Hepburn et al. (1979) selected lithium thiocyanate, urea and formamide as high affinity hydrogen bond competitors. Specimens of honeybee silk were tested in these solutions to assess the possible role of distilled water having more than capillary sorptive effects on the general tensile behaviour of the fibres. In the case of honeybee silk, lithium thiocyanate and urea virtually eliminate the marked yield point

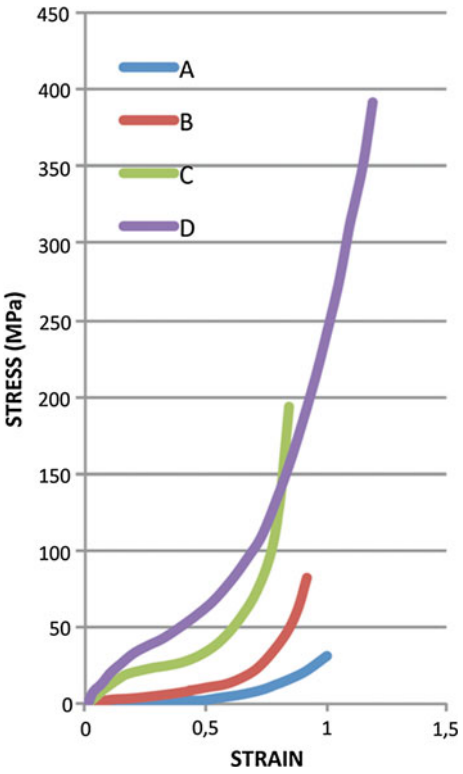

Fig. 18.5 Stress-strain curves to failure of *A. m. scutellata* silk at 20 °C in various hydrogen bond-disruptive solutions: $A = 7$ M urea; $B = 4$ M lithium thiocyanate; $C = 7$ M formamide; $D =$ distilled water (Hepburn et al. 1979)

characteristic of honeybee silk tested both dry and in distilled water. Secondly, the entire slope of the solvent-related curves is markedly reduced, as are the associated values of stress, point for point, along the curves (cf. Figs. 18.4 and 18.5).

These differences can be explained in the following way. An aqueous environment facilitates microfibrillar lubrication, as evidenced by decreasing values of the elastic modulus, and in increasing total extensibility in honeybee silk. On the other hand, organic solvents drastically reduced modulus and stress in honeybee silk, and virtually eliminated the transition from linearity to non-linearity in these curves. We suggest that, in these cases, the solvents are in fact directly acting on hydrogen bonds, so that during tensile deformation, the silks essentially behave as loose collections of unconnected bends (like a bowl of cooked spaghetti or noodles), which require only very small loads to unfold them.

Loose fibres of honeybee silk placed in a 7 M solution of formamide or urea and in a 4 M solution of lithium thiocyanate, showed no change in length, but were remarkably rubbery to the touch and very easily distended. This distensibility was reversible over the ranges examined, 100–200 % (ε_1 = 0.69 − 1.1), and the silk highly reminiscent of solvated resilin (Andersen and Weis-Fogh 1964) and other rubber networks with moderate cross-linking. However, there are basic differences between solvated fibroins and rubber networks; the integrity of the former lies in

Table 18.2 Properties of the proteins of *A. mellifera* ά-helical silk compared with other insects silks (with kind permission of the publishers, from Sutherland et al. 2006)

Species	Protein name	Number of amino acids	Percent of cDNA library clones
Bumblebee	BBF1	327	4
	BBF2	313	14
	BBF3	332	20
	BBF4	357	32
Bulldog ant	BAF1	422	16
	BAF2	411	30
	BAF3	394	26
	BAF4	441	24
Weaver ant	WAF1	391	35
	WAF2	400	22
	WAF3	395	13
	WAF4	443	17
Honeybee	AmelF1	333	6d
	AmelF2	309	7d
	AmelF3	335	11d
	AmelF4	342	7d
Bumblebee	BBSA1	>501	3
Honeybee	AmelSAl	578	13d

the secondary hydrogen bonding topology of the structure, while in the latter, bonding is usually of the sulphydryl covalent type. Thus, we conclude that solvation of honeybee silk in lithium thiocyanate, urea and formamide, and even distilled water, disrupts, the crystalline organization of the fibroin by directly reducing hydrogen bonding in the structure. Properties of the proteins of the ά-helical honeybee silk are shown in Table 18.2.

18.6 Honeybee Silk: An ά-Helical Silk and a Coiled-Coil Protein

It appears to be a general property of natural silks that the components, hierarchical structure and the conditions of their production all affect their mechanical properties (Vollrath and Knight 2001; Shao and Vollrath 2002). It is therefore not surprising that the discovery of the amino acid sequence in honeybee silk protein provided an explanation of why the coiled-coil packing was atypically tight: while the core of coiled-coils usually contains large hydrophobic residues such as leucine and isoleucine, in coiled-coil silk the most abundant core residue is the small amino acid, alanine (Sutherland 2007).

Lucas and Rudall (1968) suggested that the pattern of coiled-coil proteins that occur in the silk gland could be to prevent agglutination of the proteins within the silk gland. Another, not incompatible, reason put forward by Sutherland et al. (2007),

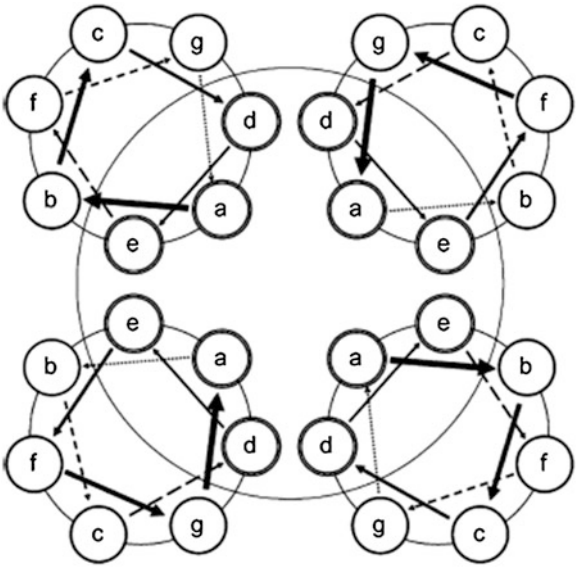

Fig. 18.6 A structural model for a coiled-coil silk as produced by *A. mellifera* honeybees. The ά-helical strands corresponding to each of the fibroins are arranged in an antiparallel tetrameric configuration (direction indicated by arrows). Three residues (**a**, **d**, **e**) from each heptad repeat are buried in the core (with kind permission of Sutherland et al. 2007)

is that it could provide a mechanism to reduce the flow viscosity of the protein solution, in order to allow the concentrated silk dope to pass through the spinneret. Obviously, the behaviour of silk must be based on its chemical composition. Sutherland et al. (2006) were able to identify the coiled-coil silk sequences from silk gland cDNA libraries of European *A. mellifera,* and determine the amino acid sequence of the coiled-coils.

Sutherland et al. (2007) confirmed that honeybee silk is formed from four coiled-coil proteins (fibroins), as originally proposed by Rudall (1962, 1965) on the basis of his X-ray diffraction data. The fibroin proteins contained extensive coiled-coil regions of conserved length, flanked by largely unstructured termini. Sutherland et al. (2007) proposed a structural model for coiled-coil silks (Fig. 18.6). The ά-helical strands corresponding to each of the fibroins are arranged in an antiparallel tetrameric configuration (direction indicated by arrows). Each fibroin contains a continuous predicted coiled-coil region of around 210 residues, flanked by 23–160 residue length N- and C-termini. The cores of the coiled-coils were unusually rich in alanine, a hydrophobic amino acid, in the '**a**' and '**d**' core positions (Fig. 18.6). Sutherland et al. (2011a, b) further provided a schematic top-down view of one strand of a coiled-coil generated from coiled-coil silk proteins such as those that occur in honeybees (Fig. 18.7).

Three residues (**a**, **d**, and **e**) from each heptad repeat are buried in the core. Most known coiled-coils contain predominantly large hydrophobic residues at these positions to maximize the hydrophobic forces stabilizing the core (Woolfson 2005). Sutherland et al. (2007) ascribed the atypical composition of the coiled-coils in bee silks as possibly due to the metabolic constraints of having to produce a continuous and copious secretion of silk during the many hours of larval spinning.

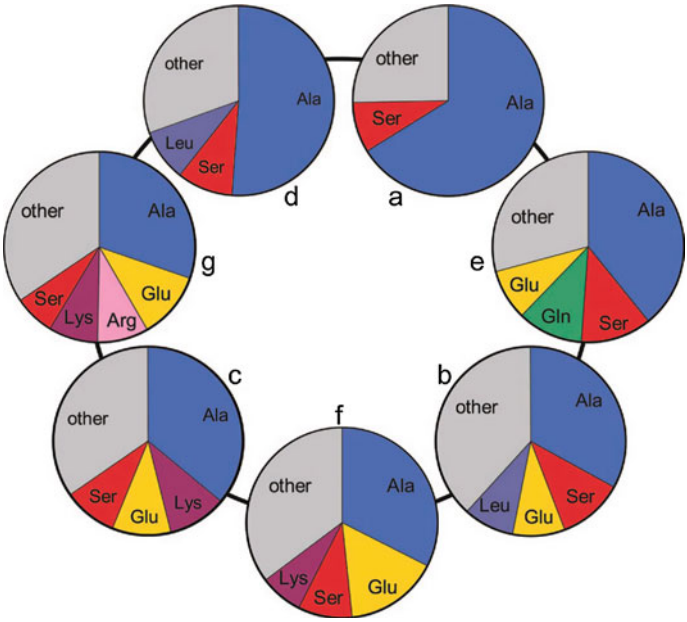

Fig. 18.7 Schematic top-down view of one strand of a coiled-coil generated from coiled-coil silk proteins. Formation of coiled-coils occurs when two strands of protein containing repeats of amino acids in the pattern HPPHPPP (where H are generally hydrophobic residues and P are generally polar residues), come together to shield the hydrophobic residues from the solvent. The heptad repeat is commonly denoted as '**a**–**g**' with '**a**' and '**d**' positions corresponding to the core residues. The relative abundance of different amino acids in each position, averaged over-all silk proteins for seven species, is shown in pie chart form (with kind permission of the publishers, from Sutherland et al. 2011a, b)

Amino acid sequence comparisons indicate that different regions of silk proteins have different levels of sequence constraint. A pairwise alignment of the closely related silk proteins from European *A. mellifera* (Sutherland et al. 2007) and *A. cerana* (Shi et al. 2008) show, on average, 3 % amino acid changes in predicted coiled-coil core positions, 8 % amino acid changes in predicted coiled-coil non-core positions, and 14 % amino acid changes in the N- and C-termini regions (Sutherland et al. 2011a, b). Thus, composition, molecular topology and amino acid content and sequence appear to be highly conserved features in the evolution of *Apis*.

18.7 Molecular Dynamics of ά-Helical Proteins

Over the past few years the molecular dynamics of ά-helical protein behaviour has gained enormous momentum, particularly with the works of Ackbarow et al. (2007, et seq.), who published highly significant work on how hierarchies, multiple

energy barriers and robustness govern the fracture mechanics of ά-helical and β-sheet protein domains. The authors point out that the fundamental fracture mechanisms of protein materials remain largely unknown, in part because of a lack of understanding of how individual protein building blocks respond to mechanical loads. As an example, they report that there is uncertainty as to whether the unfolding behaviour of ά-helical proteins consists of multiple transition state changes continuously with the pulling velocity. Ackbarow et al. (2007) reported on a direct atomistic simulation over four orders of magnitude in time scales of the unfolding behaviour of ά-helical protein, in which they found that two discrete transition states corresponded to two fracture mechanisms.

Whereas the unfolding mechanism at fast fibre extensions involves the sequential rupture of individual hydrogen bonds, unfolding at slower rates involves the simultaneous rupture of several hydrogen bonds. Ackbarow et al. (2007) derived a theory that explicitly considers the hierarchical architecture of proteins, providing a rigorous structure-property relationship. Their results provide evidence that the molecular structure of ά-helical proteins maximizes their robustness with minimal use of building materials (Ackbarow et al. 2007; Buehler and Ackbarow 2007).

Although not directly germane to the present discussion, it is of considerable interest to learn of the existence of both reconstituted honeybee and other fibres produced by recombinant techniques (Wesiman et al. 2010). The coiled-coil silk proteins of honeybees are small compared to the fibrous silk proteins of spiders and silkworms, and therefore can be produced as full length proteins by fermentation in the bacterium *Escherichia coli*. The native coiled-coil silk self-assembles within the silk gland before spinning (Flower and Kenchington 1967), and key elements of this self-assembly are replicated in reconstituted or recombinant silk, potentially allowing straightforward capture of native silk functionality in a biomaterial (Sutherland et al. 2007, 2011a, b, 2012).

Most recently, Sutherland's group described controlled micellar refolding of coiled-coil honeybee silk proteins using the detergent sodium dodecyl sulphate (SDS) (Walker et al. 2013). Their circular dichroism and dynamic light scattering experiments demonstrated that micellar SDS promotes folding of randomly coiled honeybee silk proteins into isolated α-helices, and that removal of detergent micelles, or addition of salt, leads to a coiled-coil formation. They further proposed a mechanism of protein folding:

"In the presence of micellar detergent, hydrophobic residues are associated with the detergent tail groups within the micelles, whereas hydrophilic residues are paired with the detergent head-groups on the micelle's surface. These detergent–protein interactions prevent residue–residue interactions and allow the protein to fold, according to the natural tendency of individual residues. From this condition, when hydrophobic residue–micellar interactions are reduced by lowering detergent levels to below the critical micelle concentration, or by using salt to increase detergent packing in micelles and thereby excluding the protein from the interior, the proteins fold into coiled-coils. We propose that under low SDS conditions,

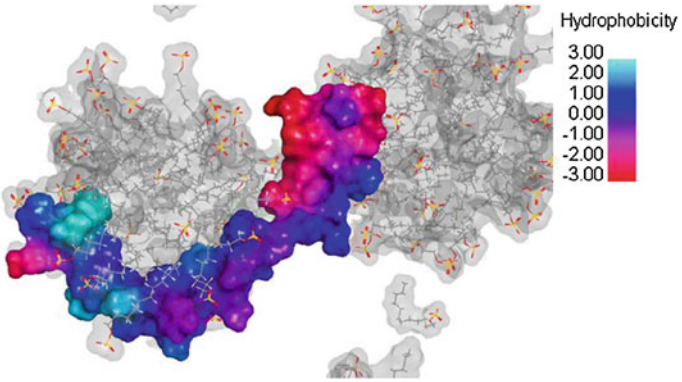

Fig. 18.8 Honeybee silk protein after 8 ns simulation. The hydrophobic residues (*blue*) are situated within the micelle, while the hydrophilic residues (*red*) form a solvent-accessible surface (unpublished, courtesy of T. Sutherland)

hydrophobic–monomeric SDS tail-group and hydrophilic–monomeric head-group interactions (low SDS conditions) or hydrophilic–micellar SDS head-group interactions (high salt conditions), stabilize a transient α-helix intermediate in coiled-coil folding. The folding pathway constitutes a new kind of micellar refolding, which may be profitably employed to refold other proteins rich in coiled-coils." Moreover, in future, this work will likely come within the gambit and purview of patents offices around the world (Sutherland et al. 2010b; Sutherland et al. 2013) (Fig. 18.8).

18.8 Genetic Basis of Honeybee ά-Helical Fibroin

Sutherland et al. (2007) published the results of some pioneering work that described a highly divergent gene cluster in honeybees that actually encodes a novel silk family. Using a combination of genomic and proteomic techniques, they identified four honeybee fibre genes; (*AmelFibroin*1-4) and two silk-associated genes (*AmelSA*1 and 2). The four fibre genes are small, each consisting of a single exon, and are clustered on a short genomic region where the open reading frames are GC-rich amid low GC intergenic regions. The genes encode similar proteins that are highly helical and are predicted to form unusually tight coiled-coils. Despite the similarity in size, structure, and composition of the encoded proteins, the genes have low primary sequence identity. Sutherland et al. (2007) proposed that the four fibre genes have arisen from gene duplication events, but have subsequently diverged significantly. The silk-associated genes encode proteins likely to act as glue (*AmelSA*1), and are involved in silk processing (*AmelSA*2). Although the silks of honeybees and silkworms both originate in larval labial glands, the silk

proteins are completely different in their primary, secondary and tertiary structures, as well as the genomic arrangement of the genes encoding them.

This implies independent evolutionary origins for these functionally related proteins. Six honeybee silk genes have been confidently identified by a combination of genomic and proteomic techniques. Five of these genes, encoding the four proteins and the *AmelSA1* glue protein, are completely novel, with no sequence similarity found to any known gene. The four *AmelFibroin* genes are physically clustered in the genome, and are each composed of a single short exon. Although they encode proteins with similar amino acid composition, helical conformation, and heptad substructure, they share little primary sequence homology. The four related, but diverged genes, may have slightly different roles in coiled-coil formation. All four proteins might be required at fixed ratios for proper silk formation, or expression of the different genes, at varying levels, might allow honeybee silk to adapt rapidly to environmental changes. Alternatively, the four proteins might be functionally equivalent with gene duplication required to support a very high level of expression.

The important and burgeoning field of genomics is concerned with the study of genes and their effects on macroscopic functions, and has led to considerable advances. However, as Ackbarow et al. (2009) noted, genomics does not illuminate material properties, nor the mechanistic relation of hierarchical multi-scale structures and their resulting properties. Elucidating the relation between structure and material properties and multi-scale behavior of protein assemblies, such as the honeybee α-helical silk, represents a grand challenge at the interface of materials science and biology (Ackbarow et al. 2009). This gap in understanding can be closed by systematically studying the material properties of hierarchical protein structures and their effects on the macroscopic properties; an approach, part of a larger effort, to study the role of materials in biology, referred to by Buehler and Keten (2008) as materiomics.

References

Ackbarow T, Chen X, Keten S, Buehler MJ (2007) Hierarchies, multiple energy barriers and robustness govern the fracture mechanics of α-helical and β-sheet protein domains. Proc Nat Acad Sci 104:16410–16415

Ackbarow T, Sen D, Thaulow C, Buehler MJ (2009) Alpha-helical protein networks are self protective and flaw-tolerant. PLoS ONE 4(6):e6015. doi:10.1371/journal.pone.0006015

Andersen SO, Weis-Fogh T (1964) Resilin. A rubberlike protein in arthropod cuticle. Adv Insect Physiol 2:1–65

Arnhart L (1906) Die Zwischenräume zwischen den Wachsdrussenzellen der Honigbiene. Zool Anz 30:719–721

Atkins EDT (1967) A four-strand coiled-coil model for some insect fibrous proteins. J Mol Biol 24:139–141

Buehler MJ, Ackbarow T (2007) Fracture mechanics of protein materials. Mater Today 10:48–58

Buehler MJ, Keten S (2008) Elasticity, strength and resilience: a comparative study on mechanical signatures of α-helix, β-sheet and tropocollagen domains. Nano Res 1:63–71

Chauvin R (1962) Sur le noircissement des vieilles cires. Ann Abeille 5:59–63
Flower NE, Kenchington W (1967) Studies on insect fibrous proteins: the larval silk of *Apis, Bombus* and *Vespa* (Hymenoptera: Aculeata). J R Microsc Soc 86:297–310
Hepburn HR (1986) Honeybees and wax: an experimental natural history. Springer, Berlin
Hepburn HR, Kurstjens SP (1984) On the strength of propolis (bee glue). Naturwissenschaften 71:591–592
Hepburn HR, Kurstjens SP (1988) The combs of honeybees as composite materials. Apidologie 19:25–36
Hepburn HR, Chandler HD, Davidoff MR (1979) Extensometric properties of insect fibroins: the green lacewing cross-β, honeybee $\acute{\alpha}$-helical and greater waxmoth parallel-β conformations. Insect Biochem 9:69–77
Hepburn HR, Armstrong E, Kurstjens SP (1983) The ductility of native beeswax is optimally related to honeybee colony temperature. S Afr J Sci 79:416–417
Hepburn HR, Muerrle T, Radloff SE (2007) The cell bases of honeybee combs. Apidologie 38:268–271
Hepburn HR, Pirk CWW, Duangphakdee O (2013) Physical properties of honeybee silk: a review. Apidologie 44:600–610
Huber F (1814) Nouvelles observations sur les abeilles. [English translation 1926]. Dadant, Hamilton
Jay SC (1964) The cocoon of the honeybee, *Apis mellifera* L. Canad Ent 96:784–792
Kurstjens SP, Hepburn HR, Schoening FRL, Davidson BC (1985) The conversion of wax scales into comb wax by African honeybees. J Comp Physiol B156:95–102
Kurstjens SP, McClain E, Hepburn HR (1990) The proteins of beeswax. Naturwissenschaften 77:34–35
Lucas F, Rudall KM (1968) Extracellular fibrous proteins: the silks. In: Florkin M, Stotz EH (eds) Comprehensive biochemistry, vol 26. Elsevier, Amsterdam, pp 475–558
Lucas F, Shaw JTB, Smith SG (1960) Comparative studies of fibroins: I. the amino acid composition of various fibroins and its significance in relation to their crystal structure and taxonomy. J Mol Biol 2:339–349
Rudall KM (1962) Silk and other cocoon proteins. In: Florkin M, Mason HS (eds) Comparative biochemistry, vol IV. Academic Press, New York, pp 397–433
Rudall KM (1965) Aspects of insect biochemistry. Academic Press, London
Shao ZZ, Vollrath F (2002) Materials: surprising strength of silkworm silk. Nature 418:741
Shi J, Lua S, Du N, Liu X, Song J (2008) Identification, recombinant production and structural characterization of four silk proteins from the Asiatic honeybee *Apis cerana*. Biomaterials 29:2820–2828
Silva-Zacarin ECM, De Moraes RLMS, Taboga SR (2003) Silk formation mechanisms in the larval salivary glands of *Apis mellifera* (Hymenoptera: Apidae). J Biosci Bangalore 28:753–764
Sutherland TD, Campbell PM, Weisman S, Trueman HE, Sriskantha A, Wanjura WJ, Haritos VS (2006) A highly divergent gene cluster in honeybees encodes a novel silk family. Genome Res 16:1414–1421
Sutherland TD, Weisman S, Trueman HE, Sriskantha A, Trueman JWH, Haritos VS (2007) Conservation of essential design features in coiled-coil silks. Mol Biol Evol 24:2424–2432
Sutherland TD, Young J, Weisman S, Hayashi CY, Merrit D (2010a) Insect silk: one name, many materials. Ann Rev Entomol 55:171–188
Sutherland TD, Haritos VS, Trueman HE, Sriskantha A, Weisman S, Campbell PM (2010b) US Patent Application Publication. US2010/0100975 A1, April 22 2010
Sutherland TD, Church JS, Hu X, Huson MG, Kaplan DL, Weisman S (2011a) Single honeybee silk protein mimics properties of multi-protein silk. PLoS ONE 6(2):16489. doi:10.1371/journal.pone.0016489
Sutherland TD, Weisman S, Walker AA, Mudie ST (2011b) The coiled-coil silk of bees, ants, and hornets. Biopolymers 97:446–454

Sutherland TD, Haritos VS, Trueman H, Sriskantha A, Weisman S, Campbell PM (2013) European Patent Specification EP 1 931 702 B1

Verlich AV (1930) Entwicklungsmechanische Studien an Bienenlarven. Z Wiss Zool 136:210–222

Vollrath F, Knight DP (2001) Liquid crystalline spinning of spider silk. Nature 410:541–548

Wainwright SA, Biggs WD, Currey JD, Gosline JM (1976) Mechanical design in organisms. Edward Arnold, London

Walker AA, Warden AC, Trueman HE, Weisman S, Sutherland TD (2013) Micellar refolding of coiled-coil honeybee silk proteins. J Mater Chem B. doi: 10.1039/C3TB20611D

Warwicker JO (1960) Comparative studies of fibroins: II. the crystal structures of various fibroins. J Mol Biol 2:350–362

Weisman S, Haritos VS, Church JS, Huson MG, Mudie ST, Rodgers AJW, Dumsday GJ, Sutherland TD (2010) Honeybee silk: recombinant protein production, assembly and fiber spinning. Biomaterials 31:2695–2700

Woolfson DN (2005) The design of coiled-coil structures and assemblies. Adv Protein Chem 70:79–112

Zhang K, Si FW, Duan HL, Karihaloo BL, Wang J (2010a) Hierarchical, multilayered cell walls reinforced by recycled silk cocoons enhance the structural integrity of honeybee combs. Proc Nat Acad Sci 107:9502–9506

Zhang K, Si FW, Duan HL, Wang J (2010b) Microstructures and mechanical properties of silks of silkworm and honeybee. Acta Biomater 6:2165–2171

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