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

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)
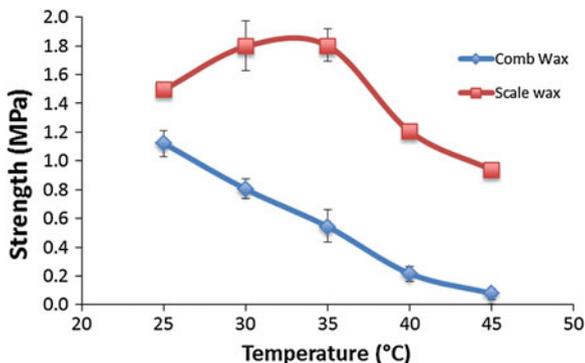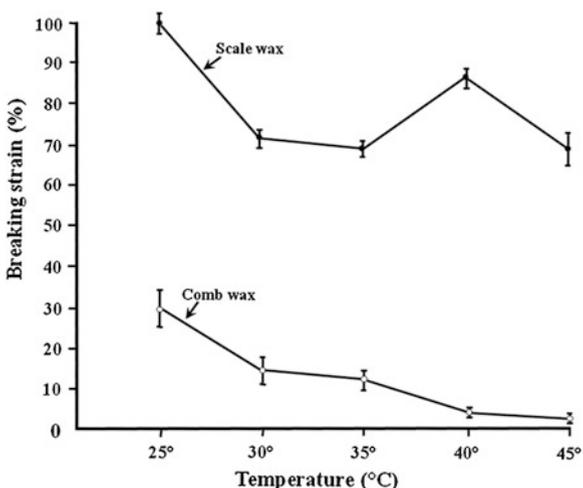

Fig. 14.2 Tensile breaking strain of *A. m. scutellata* scale wax and comb wall wax at different temperatures (Kurstjens et al. 1985)

(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)
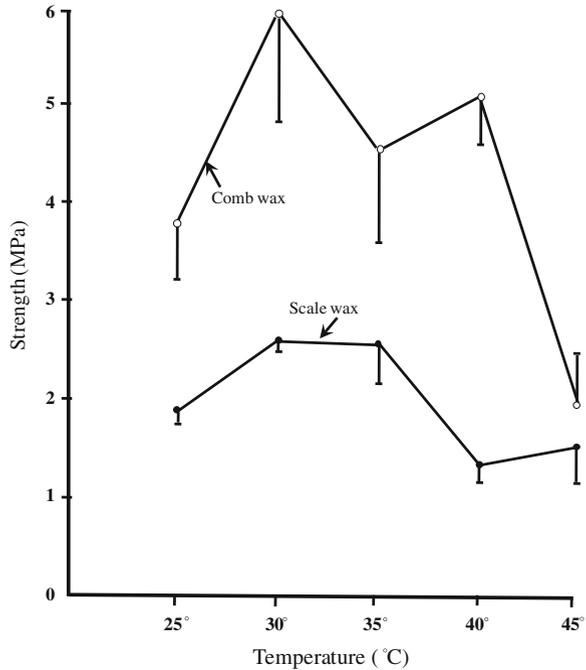
Neither the theory of plastics nor our current knowledge of wax chemistry is yet sufficiently robust to explain the basis of this behaviour. The stiffness (which is a measure of a material's resistance to deformation), of the two beeswaxes is shown in Fig. 14.3.

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)
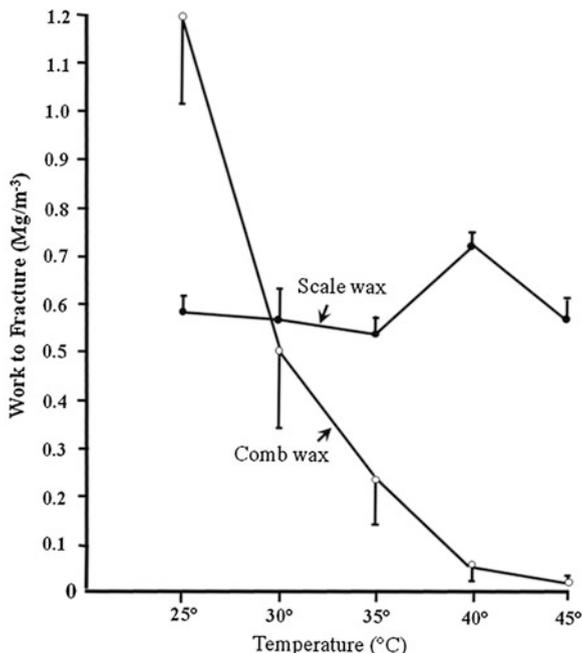
energetically advantageous (Pirk et al. 2011). The results of mechanical tests on scale and cell wall waxes are empirical in nature; they describe phenomena but do not explain how they come about.

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 %).
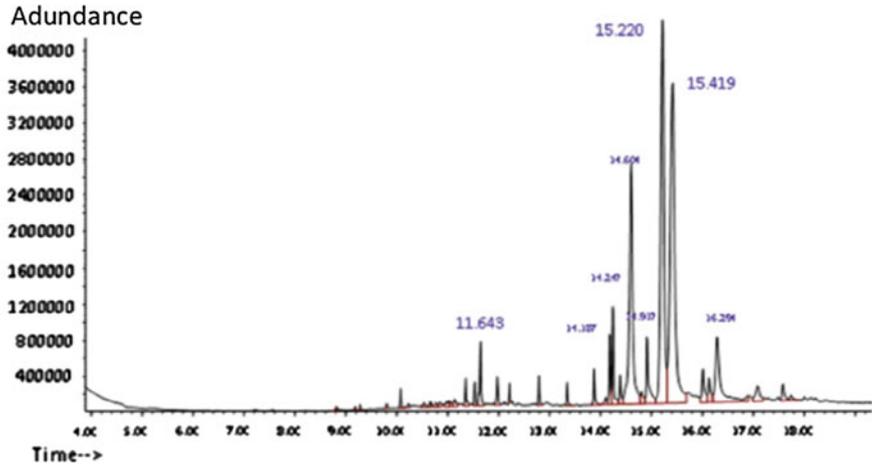
Fig. 14.6 Chromatogram of the sticky band material collected from *A. florea* nests. Preliminary identifications were based on the WILEY 7 N library database. To consider only the main constituents: $R_t = 11.643$, no satisfying library match; $R_t = 14.604$, triterpene (amyrin); $R_t = 14.917$, heptacosane; $R_t = 15.220$, 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)
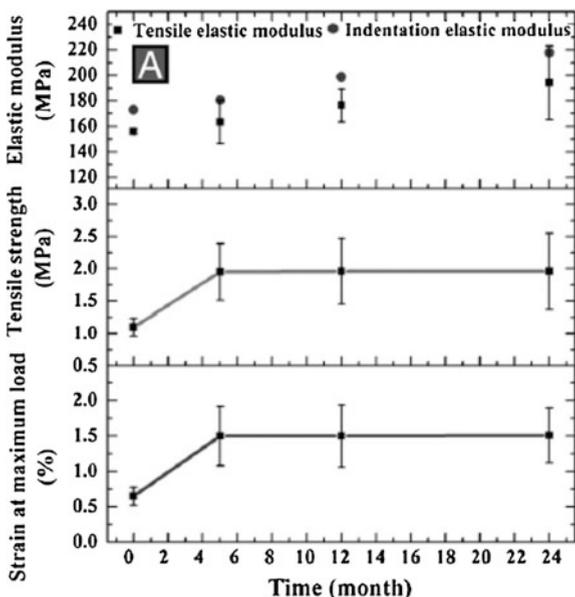
strain fields in new and old combs using a linear elastic finite element model at 25 °C. For newly constructed comb, the maximum normal stress and the corresponding strain along the axis of the cell were found to be 72 kPa and 0.05 % respectively for the combined weight of honey and worker bees, which 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).
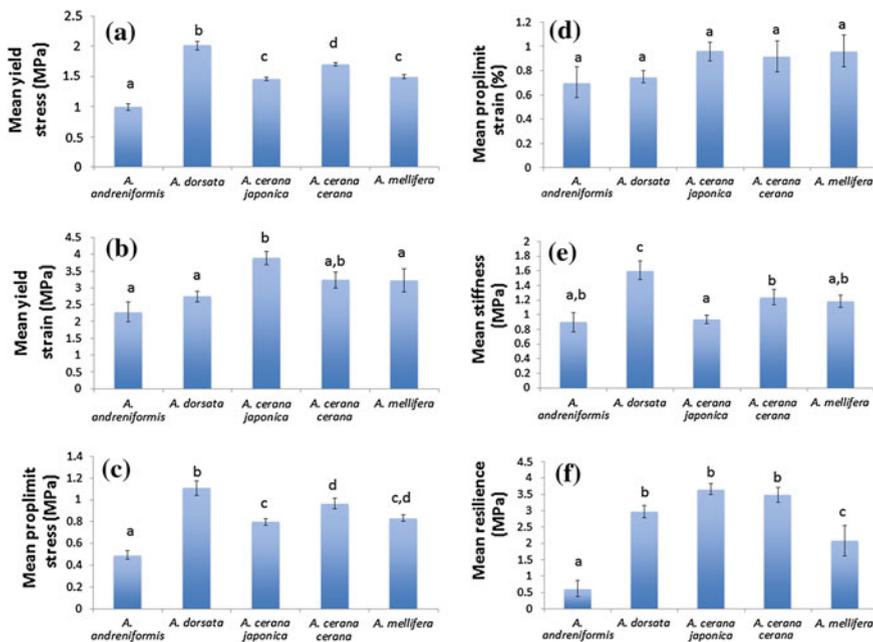
Fig. 14.8 Comparisons among the waxes of five honeybee species (*A. andreniformis, A. dorsata, A. cerana japonica, A. cerana cerana* and *A. mellifera*) for six mechanical measures: **a** yield stress, **b** yield strain, **c** stress at proportional limit, **d** strain at the proportional limit, **e** 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
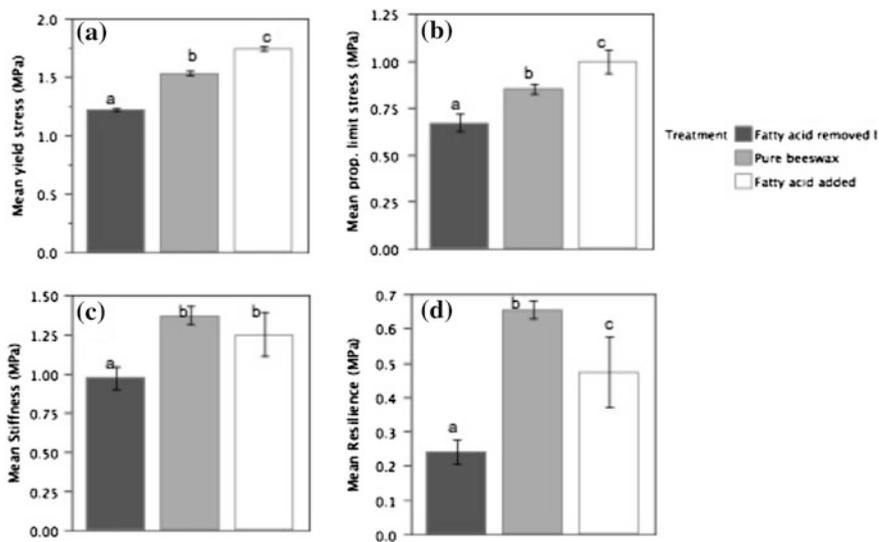
Fig. 14.9 The effects of fatty acids on the mechanical properties of *A. mellifera* beeswax. Values represent means and standard errors for beeswax with the free fatty acids removed, unmodified beeswax, and beeswax with added stearic acid. Four mechanical properties were examined: **a** yield stress; **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   ⍺-Helical Silk

Honeybee silk is a ⍺-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)
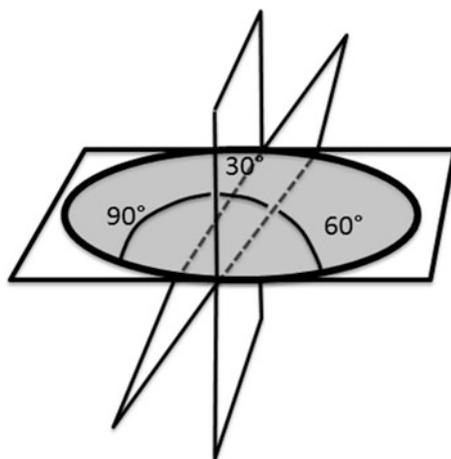
done in the manufacture of sheets of beeswax foundation), showed enhanced
texture at higher temperatures (38 °C) than at lower ones (17 and 29 °C), and the
same was true of ribbons annealed at higher temperatures. New comb wax kept at
38 °C for 5 months was more crystalline than that stored at 15 °C. 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 observa-
tions 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 άradiation 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 άradiation. 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 ($a \neq b \neq c$, $ά = \gamma = 90°$, $\beta \neq 90°$) and

orthorhombic ($a = b = c$, $\acute{\alpha} = \beta = \gamma = 90°$). 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 $a \sin \beta$ in the monoclinic unit cell. The definitive crystallographic parameters of $a \sin \beta$ and b were obtained from a linear plot of $d^2 = h^2$ versus d^2k^2 using the equation: $d^2h^2 = -a^2 \sin2 \beta/b^2$. $d^2k^2 + a^2 \sin^2 \beta$ (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 $a \sin \beta$ 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
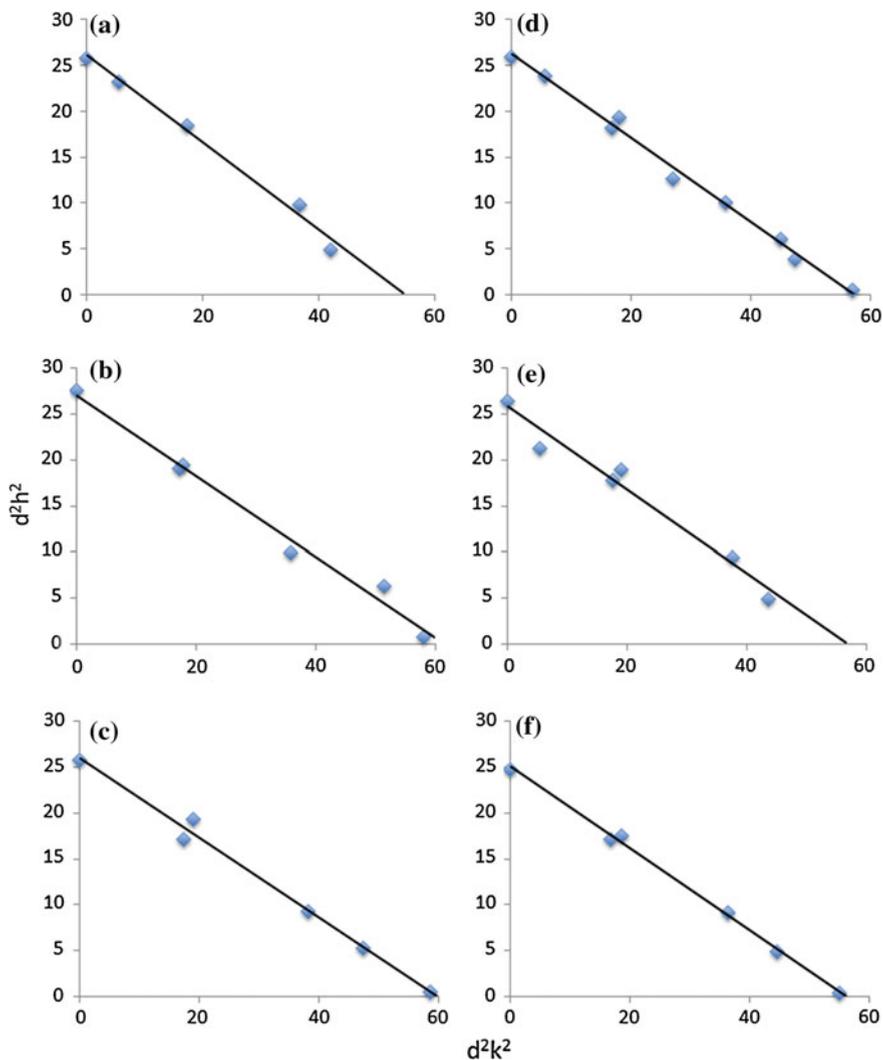
Fig. 14.12 d^2h^2/d^2k^2 plots for all six preparations of *A. m. scutellata* beeswax: **a** natural comb wax for which $\acute{a}$ sin $\beta = 0.506$ nm, $b = 0.756$ nm; **b** sheeted comb wax for which $\acute{a}$ sin $\beta = 0.503$ nm, $b = 0.772$ nm; **c** soxhlet-extracted and sheeted comb wax for which $\acute{a}$ sin $\beta = 0.513$ nm, $b = 0.776$ nm; **d** beeswax scales for which $\acute{a}$ sin $\beta = 0.502$ nm, b = 0.750; **e** sheeted scale wax for which $\acute{a}$ sin $\beta = 0.519$ nm, $b = 0.781$ nm; **f** soxhlet-extracted and sheeted scale wax for which $\acute{a}$ 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)
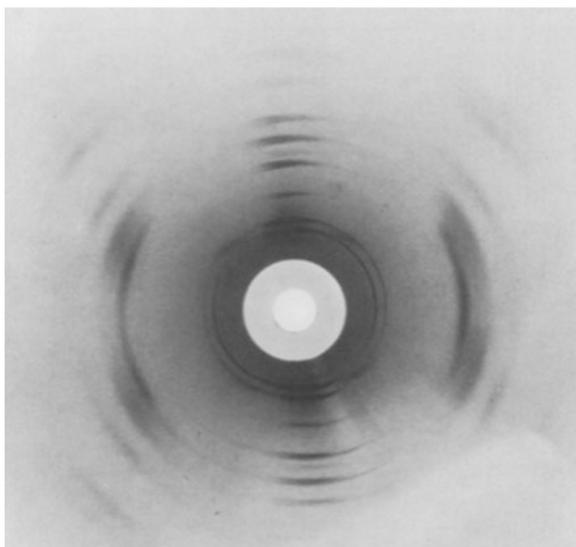
Table 14.3 A comparison of the long-spacing reflections of *A. m. scutellata* wax scales and comb wax (Kurstjens et al. 1985)

	Strong reflection	
	d (nm)	*d* (nm)
Comb wax	7.06 ± 0.05	6.40 ± 0.05
	(n = 9)	(n = 6)
Virgin wax scales	7.07 ± 0.04	6.22 ± 0.04
	(n = 15)	(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 ἀ-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 re-crystallisation, 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.

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