PHYSICO-CHEMICAL CHARACTERISTICS OF WAXES PRODUCED BY THE AFRICAN HONEYBEE, *Apis mellifera scutellata*

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Errata

p 67: footnote of table, insert "(%)" after "Results"

p 68: figure legend, insert "(%)" after "profiles"

p 75: line 1, insert "($p < 0.05$)" after "increase"
line 7, insert "($p < 0.05$)" after "Figure 3.5"

p 100: line 14, insert "($p < 0.05$)" after "difference"

p 124: footnote of table, add "Tests were performed at 23°C and the data is expressed as the $\bar{x} \pm$ S.D."

p 125: table caption, insert "for data of Table 5.1" after "strength"

p 126: table caption, insert "for data of Table 5.1" after "strain"

p 127: table caption, insert "for data of Table 5.1" after "stiffness"

p 128: figure legend, insert "native" after "of"

p 129: figure legend, insert "native" after "of"

p 130: figure legend, insert "native" after "of"

Abstract

In this dissertation the physical and chemical alterations induced by mastication and manipulation of wax by the worker bee in honeycomb construction, and the subsequent contribution afforded the structural integrity of the nest, are elucidated.

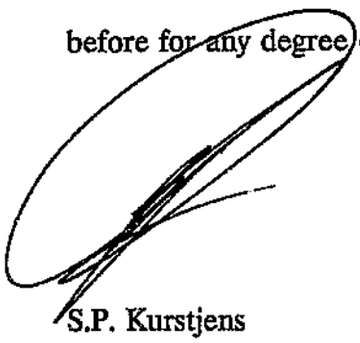
In comb building, the freshly secreted wax scales are mandibulated together with a frothy salivary emulsion, and added piece-meal to form honeycomb. Textural modifications were revealed using X-ray crystallography. While virgin scale wax is highly structured, with the crystallites aligned approximately perpendicular to the planar surface, comb wax has a random crystallographic arrangement. This reflects a disruption of the crystallite structure following the mechanical insult of mastication. Chemical analyses included investigation of both lipid and proteinaceous elements. Lipid composition was evaluated by enzyme-catalyzed as well as thin-layer and gas-liquid chromatographic methods. The results indicate a reduction in scale diacylglycerols with a corresponding increase in comb saturated monoacylglycerols. Such modifications are highly suggestive of lipase activity within the salivary addition. The proteins of comb and scale wax were analyzed electrophoretically, under reduced conditions. Each wax possesses unique

polypeptide fractions, in addition to sharing common protein species. It is speculated that those in common represent integral proteins, such as transport molecules, while the disparities noted may be due to salivary enzymatic degradation, or even glycosylation.

The effects of these textural and chemical alterations on the mechanical behaviour of the waxes was assessed. Tensile tests were performed on a variety of scale and comb wax preparations over the range of temperatures likely to impinge on the honeybee nest. These investigations reveal the specific structural contributions made by each of the physico-chemical alterations described. Further, they demonstrate that while scales are ideal moulding materials due to their high distensibility and low stiffness, the greater resistance to deformation and lower potential for extension makes comb wax a superior structural material. The mechanical advantage for including propolis and cocoon silk within the comb structure was also investigated. Tensile testing indicates that the resultant composite material is structurally superior, largely due to the presence of silk reinforcement.

Declaration

I declare that the work reported in this dissertation was carried out by me in the Department of Physiology, University of the Witwatersrand. Where help was received it has been acknowledged. This work has not been submitted before for any degree or examination in any other University.



S.P. Kurstjens

Signed this 20th day of July 1990, Johannesburg

Dedication

This thesis is dedicated to my parents, and the memory of my grandmother, Mathilda Baglietto, *su alma ke ripose in passe.*

Acknowledgements

I am indebted to the following people whose assistance has been essential in the preparation of this dissertation. My mentor Professor H.R. Hepburn, for his much valued insight, guidance and support; Dr. E. McClain, for her kind acceptance of the responsibility of co-supervisor, a task she excelled at; Professor F.R.L Schoening and Dr. B.C. Davidson for their expert supervision in the execution of Chapters 2 and 3 respectively; Professor D. Mitchell for the facilities made available in the Department of Physiology and Dr. D. Scriven for his assistance with the preparation of the manuscript. The financial support of the C.S.I.R. is gratefully acknowledged.

I further wish to declare that the X-ray crystallographic data for untreated comb wax (Chapter 2) was obtained while reading for the degree of Bachelor of Science with Honours in the Faculty Science at the University of the Witwatersrand. Further, the gas-liquid chromatography data for scale and comb wax (Chapter 3) was included with kind permission from Professor H.R. Hepburn and Dr. B.C. Davidson.

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Chapter 1

1. General Introduction

Few animals in nature demonstrate the social organization and evolutionary adaptations of the honeybee, Apis mellifera. These complex individuals reside in matriarchal colonies, and occupy communal dwellings. Here they behave as a truly altruistic functional unit, carefully executing their particular tasks under the tactile and pheromonal command of the queen. As with the most sophisticated of social communities, the daily chores are distributed amongst the workers. One of the most important of these tasks is the construction and maintenance of the principal structural material of the nest - the honeycomb.

The honeybee colony reproduces by swarming. This phenomenon is reputedly initiated by a deficiency in available queen pheromone, either as a consequence of aged queens or from overcrowding with impedance of signal distribution (Free, 1977). As a last stage in the process, a new location for the colony is selected. Motivated by the limited food supply available to the swarm, scout bees endeavour to rapidly secure a suitable nesting site (Lindauer, 1961). Numerous factors influence their choice. Distance from the parent nest, cavity volume as well as orientation of the setting are key considerations (Lindauer, 1955; Seeley and Morse, 1976). Prospective sites are presented to the colony by the scouts, who perform nest dances similar to those used to communicate the location of nectar and pollen. Preference is generally shown for those locations 500 to 600 meters from the parent site with cavity volumes between 20 and 80 liters. These criteria do, however, vary

greatly among bees of different races (Gould, 1982). Once a nest site has been selected, comb construction may begin in earnest.

Prior to the mid-eighteenth century, naturalists held the opinion that comb wax was either retrieved directly from plants and flowers by foraging bees, or was transformed from pollen. The Ancients, including Aristotle and Pliny the Elder, described bees gathering wax from a variety of plants, and returning to the hive with their harvest firmly attached to their hind legs (Fraser, 1931). Based on more detailed observations, De Reaumur (1740) suggested that bees regurgitate the wax crop, thus processing it into a suitable construction material. Dobbs (1750) further endorsed this theory of active alteration, but proposed that the transmuted product one was of defecation. The first accurate report postulating the origin of wax was made by Hornbostel in 1744. Having observed wax scales in situ, he hypothesized that bees secrete wax, rejecting the then current belief based on the physical dissimilarities between beeswax and plant constituents. Hunter (1792) corroborated this by experimentally demonstrating the difference in molten characteristics of pollen and wax scales. He too suggested wax scales to be a secretion of bees, and extended the concept by coupling the synthesis of scales with the construction of comb. In view of these considerations, and with the advent of sophisticated light and subsequent electron microscopic techniques attention understandably focused on defining the presence of a wax gland complex.

Initial studies by Graber (1872) and Holz (1878) suggested the presence of a triad of functionally active cells within the wax mirror cuticle of the honeybee; the epidermal cells, oenocytes and adipocytes. Histologically, the striated epidermal monolayer is subtended by vacoulated fat cells interspersed with oenocytes in a ratio of three to one (Hepburn, 1986). In a sequential series of paraffin sections, Rösch (1930) observed both fat body and oenocyte in close proximity to the wax mirror epidermis. Subsequent sections clearly demonstrated them discharging their contents into the epidermal cytoplasm. The association of these cells as a consolidated wax organ was further corroborated by Boehm (1965). She described the syncluronous cellular development of the wax gland complex of normal wax-secreting bees, and speculated over their intricate interactive processes. Exhaustive cytological data revealed that oenocytes develop at the expense of the adjacent adipocytes, and led Boehm to suggest that the fat cells stimulated wax epidermal cell development either directly or via substances sequestered within the oenocytes. Once contained in the epidermal cells, the wax precursors are transported to the surface of the cuticle via a convoluted system of wax canal tubules, continuous with the epidermal cells and penetrating the inner wax mirror epicuticle (Locke, 1961a).

From emergence, the numerous infertile female worker bees are busily engaged in various tasks. The initial concept of polyethism suggested a rather rigid age-related division of labour within the honeybee colony, with young

workers confined to in-nest duties and older bees involved in foraging and other free-ranging tasks (Butler, 1609; Huber, 1814). Within this framework, the process of wax secretion and manipulation is predominant in 12 to 18 day old bees as one of the final in-house responsibilities (Rösch, 1927). This temporal apportionment is, however, subject to variation. In a detailed series of experiments, Rösch (1927) described several conditions in which the wax gland epithelium of worker bees may again assume functional status. Renewed wax production was reported in isolated foragers, newly nesting swarms and the survivors of starvation. Wax production and secretion would thus appear to be subject to multifactorial control.

Of the potential mediators, queenrightness is the most extensively researched. Dreischer (1956) demonstrated a fourfold increase in wax prod·ction by queenright bees when compared to an equal sized queenless colony. Darchen (1957) subsequently proposed that volatile pheromones emanating from the head of the queen were the source of chemical control. Wax production is further effected by the state of the residing queen. Colonies headed by mated queens produce significantly more comb than those of virgin queens (Rajashekharappa and Channabasavanna, 1979), possibly as a result of differences in the quality of pheromonal signals produced (Crewe, 1982). Predictably, the presence of the mediating pheromones influence wax production by enhancing wax gland development (Hepburn et al., 1984). The availability of nectar and pollen also modulates comb construction. Koch

(1961) reported an association between the onset of spring, the appearance of blossoms and comb construction. Freudenstein (1960), on the other hand, showed increased wax production in pollen fed bees compared to deprived groups. Concomitant histological analyses revealed an increase in wax gland epithelial height and oenocyte diameter, indicating a key role for protein in wax synthesis and secretion.

Comb construction incorporates both major and minor building operations (Meyer and Ulrich, 1952). In the minor proceedings, three to nine day old nurse bees engage in cell capping, edge thickening and cell shaving of both honey and brood cells (Meyer and Ulrich, 1952). These manipulations are characterized by the reworking of available nest wax (Darchen, 1980). The principal process involves the genesis of the honeycomb. Worker bees commence construction on the side walls or roof of the nest cavity, with up to three primary sites per comb. Here thick layers of wax are positioned, and gently drawn out into cells by elongating and thinning. The first row of cells generally comprise irregular pentagons, while those contiguous are classically hexagonal (Huber, 1814). This shape facilitates maximal utilization of the available comb-face, allowing for the very minimum of dead-space. Initially, the growth of the comb is faster in the downward than lateral direction (Hepburn, 1986), but with eventual confluence of the various building sites, a precise, unified geometrical structure results. This mathematical precision is further extended to included equal and parallel spaces between adjacent

combs (Darchen, 1954). The honeycomb cells are of three basic types : small cells for the rearing of worker bees, larger, deeper cells for drone brood and the more specialized queen cells. Drone cells occur toward the outer edges of the comb, grouped amongst the predominant worker cells (Seeley and Morse, 1976). Queen cells are more predominant at the extreme inferior regions of the comb-face, and are constructed in response to the loss or removal of the queen from the colony (Huber, 1792).

Two major difficulties are unique to the wax builders. Firstly the comb cells are horizontal, or inclined at 77 degrees to the vertical foundation. The construction process is thus highly dependent upon an astute spatial awareness. Further, the exact geometrical structure of the honeycomb demands precise manipulation and alignment of the building material. Anatomically the honeybee worker is well adapted to overcome these challenges. Martin and Lindauer (1966) ascertained that sensory hair plates, located at the base of the neck, were responsible for bees perceiving the direction of the force of gravity. Following obliteration of these plates, bees failed to construct proper combs. Surprisingly enough, bees do appear capable of comb construction in the weightless environment of outer space (Vanderberg et al., 1985). Sensory organs located in the tips of the antennae may alone, or in combination with the additional tactile circuit of mandible and neck organ, detect the thickness and quality of cell walls (Martin and Lindauer, 1966). Structures responsible for controlling the diameter and

orientation of the cell walls remain unclear, although sensory input from the forelegs of the queen appears essential in distinguishing drone from worker cells, possibly by detecting the difference in cell diameter (Koeniger, 1970).

Casteel (1912) provides the most detailed observations on the manipulation of wax scales in the process of honeycomb construction. He closely monitored the building activities of bees in an observation hive through a binocular microscope. The wax produced by the honeybee occurs as eight well-formed scales upon the surface of the ventral wax mirrors. These wax plates are easily defined by examining the sternal aspect of a stretched abdomen, and occur as four pairs of smooth oval areas lying on either side of the midventral line, usually covered by the next preceding plate. Unless accidentally dislodged, the wax scales are always removed and manipulated by the parent bee. Workers never assist each other in the process of removal, although free scales are, as a rule, recovered and built into comb by other workers.

Bees involved in scale removal assume a particular attitude. All other activities cease and the insect remains motionless. The forelegs and mandibles are raised from the comb-face and the neck is flexed. One hind-leg is lifted and the bulk of the body weight is thus supported upon three legs. The first tarsal segment of the elevated limb is passed along the ventral surface of the extended abdomen, encountering the protruding wax scales of the corresponding side. With the application of pressure, the scale is engaged by

the tarsus, and slowly withdrawn from the wax pocket. The hind leg then rapidly transfers the scale anteriorly where it is manipulated by the mandible usually in association with the forelegs. Casteel (1912) identified the medial tarsus, specifically the transverse row of spines on the planta, the pollen combs, as instrumental in scale removal. He thus rejected the current concept that the wax shears acted as the scale-removal organ (Huber, 1814).

Once transferred to the mouth, the wax is masticated and mixed with saliva. Scales of varying size are treated differently. Thin, small scales are usually masticated to completion before the resultant flaky emulsion is added to the comb. Here, little assistance is required from the forelegs in manipulation. Larger, thicker scales invariably need to be secured by the legs, and are processed piecemeal, a small portion being applied to the comb at a time. Not all scales are fully mandibulated and occasionally unchewed portions of wax scales are incorporated into the comb (Casteel, 1912). In mastication the wax scales are subject to repeated attrition by the wax nippers, or sharpened mandibular edges, and the chewed fragments are expelled via the median keel (Lineburg, 1924).

In addition to these precise details on the mechanics of comb construction, Casteel (1912) also reported upon the innate alterations he noted in the wax following chewing. In the transformation of scale wax into comb, the wax was observed to become translucent rather than transparent, change in colour, and

become more pliable. These modifications Casteel ascribed to alterations in the chemical composition of scale wax accomplished through mastication and blending with saliva. In effect, these observations served only to confirm that already experimentally established by Huber in 1814. In a series of solubility studies, Huber showed the properties of fresh wax scales to differ from those of newly built comb. In the first experiment, wax scales dropped into spirits of turpentine dissolved completely and disappeared before reaching the bottom of the vessel without rendering the fluid turbid. Similar sized fragments of newly constructed white comb, however, were not totally dissolved, many particles remaining suspended in the liquid. In the second experiment, comb fragments immersed in sulphuric ether disintegrated and fell in powder to the bottom, whereas similarly treated wax scales preserved their shape and lost only their transparency, becoming a dull white colour. From these results Huber concluded that scale wax was "less compounded" than that of comb. He therefore surmised that wax scales were in some way prepared in the construction process, and speculated that bees impregnate the scales with a frothy substance thus imparting the ductility and whiteness of comb.

Once the waxen nest is fully established, the structural integrity of the comb is preserved by constant maintenance. Besides minor reparative building operations, with the passage of time adjuvant constructional materials in the form of silk and propolis are incorporated. Propolis, or bee glue, is a hard

resinous material derived by bees from plants (Hornbostel, 1744). It fulfils a range of functions, from blocking holes and cracks in the nest to strengthening comb bases as a composite with wax (Huber, 1814). Very recently, propolis has been demonstrated to exhibit antibacterial activity against a variety of commonly encountered cocci and Gram-positive rods, including the human tubercle bacillus (Grange and Davey, 1990). The authors attribute this property to the high flavonoid content. Brood is generally reared in the central, lower areas of the comb. Just prior to pupation the honeybee larvae cover the walls of their cells with silk (Arnhart, 1919). The silk is impacted in the wall with the aid of anal secretions (Jay, 1964). Consequently, with successive generations of brood the silk to wax ratio increases (Chauvin, 1962), possibly augmenting the aged, failing primary waxen structure.

Few studies have attempted to characterize either the extent of the chemical and mechanical alteration imparted during comb construction, or the possible significance thereof. Preliminary diffraction studies and crystallographic analyses have suggested that scale wax possess a crystalline nature, as does comb cell wall (Dujardin, 1850; Schmidt, 1941). Further, alterations in lipid chemical composition following mastication have been alluded to by Lambremont and Wykle (1979) as an aside to their investigation of wax synthesis in honeybees. In this thesis then, the textural and biochemical alterations manifest through mandibulation and salivary action are examined in detail. These differences further serve to explain the recorded significant

alteration in mechanical and structural behaviour following mastication. In addition, the maturation of the honeycomb nest is investigated, and the mechanical advantage obtained from adjuvant materials quantified.

Chapter 2

2. The Crystalline Nature of Beeswax

2.1 Introduction

Beeswax is secreted by the worker bee in the form of tiny scales from the four pairs of wax glands located on the undersurface of the abdomen. In comb construction, the scale is removed from the wax pocket by the hind tarsus, becoming impaled on several of the pollen spines. It is transferred to the mouth, where it is manipulated by the mandibles, often assisted by the fore-legs if the scale is large. The edges of the scale are masticated and the fragments impregnated with a frothy liquid. The jaws cut the resultant narrow, opaque, white ribbon which is then applied at the comb-face (Lineburg, 1924).

Huber (1814) first suggested that the properties of fresh wax scales differed from those of newly built comb. He observed that virgin scales dropped into turpentine dissolved quickly and completely, while similar sized fragments of newly constructed, white comb were not completely dissolved, many pieces remaining suspended in the liquid. When placed in ether, however, comb wax disintegrated and fell in powder to the bottom. Virgin wax scales retained their size and shape, and lost only their transparency. This demonstration of the difference in solubility characteristics suggests a significant disparity in the basic chemical composition of comb and scale wax. Further, as vigorous mandibulation is integral to the construction process, this difference may be

extended to include an alteration in physical characteristics. Central to this is the variation in texture or crystallite arrangement which occurs in the conversion of virgin scales to comb wax.

The crystalline nature of scale wax was initially demonstrated by Dujardin (1850). He observed that the individual layers of the scale wax strongly depolarised light at an angle inclined to the depolarised plane. He thus surmised that scales were textured, with the crystals aligned obliquely to the surface plane of the scale. This notion was later validated by Ambronn (1892). The inclination of crystallites in virgin scale wax was further corroborated in the X-ray diffraction studies of Woog and Yannaquis (1935).

Brewster (1815) showed that comb wax, made transparent with Canada balsam, depolarised light in all directions and exhibited no neutral axis. Based on his initial characterization of materials by their reflective and refractive effect on plane-polarised light, he thus assigned comb wax to his third species of crystals formed by the successive deposition and induration of thin layers. This crystalline nature of comb wax was independently demonstrated by Ehrenberg (1849), and has subsequently been confirmed in other studies (Gaubert, 1910a,b; Schmidt, 1941).

Beeswax consists of a complex mixture of various classes of lipid components. Comb wax produced by Apis mellifera has been shown to comprise the

following constituents: hydrocarbons (14%), monoesters (35%), diesters (14%), triesters (3%), hydroxy monoesters (4%), hydroxy polyesters (8%), acid esters (1%), acid polyesters (2%), free acids (12%), free alcohols (1%) and unidentified (6%) (Tulloch, 1980). Each of these fractions further contain a number of both major and minor components. Despite these elements forming a composite of widely divergent chain lengths, Schoening (1980) was able to observe the X-ray diffraction pattern of commercial beeswax. He demonstrated that, in addition to an amorphous component, beeswax consists of two crystalline fractions. Beeswax shows a diffractogram pattern with paraffin-like side spacings which may be attributable to monoesters, while the long-spacings recorded are indicative of diesters, including free acids and hydrocarbons.

In this chapter, the crystallite arrangement of freshly secreted virgin scales and native comb wax are investigated and compared, and the textural rearrangement evoked during mastication elucidated.

2.2 Materials and methods

2.2.1 The waxes

The waxes used in this study were produced by the African honeybee Apis mellifera scutellata. Native comb wax was retrieved from apiaries kept in the Transvaal, while freshly secreted virgin scale wax was collected directly from the paired, ventral wax mirrors of numerous bees.

Two additional preparations of both scale and comb wax were also investigated. These included (1) chloroform Soxhlet-extracted and sheeted as well as (2) untreated, sheeted samples. The methodology involved in Soxhlet extraction and wax sheeting is described in Chapter 5, Appendix 5.A.1 and 5.A.2 respectively.

2.2.2 Crystallographic studies

X-ray analysis was employed to characterize the crystallographic texture of all six wax preparations.

The wavelengths of X-rays are of the order of interatomic distances, and a regular array of atoms or molecules may thus serve as a diffraction grating for this radiation. The X-ray diffraction pattern therefore depends directly upon the arrangement of the atoms within the crystal.

The long spacings of long-chain n-aliphatic products reflect the spacing of the crystal lattice planes as determined by the ends of the chain molecules. They thus provide a measure of chain length. The sideways packing of the chains are usually constant, and result in a relatively uniform set of side-spacing reflections. Conversely, long-spacing reflections are present in several higher orders and vary in diameter and intensity. This occurs as a result of the crystal polymorphism in which the substance may occur. Long spacing reflections are thus dependent upon the chain length, the tilt of the chains with respect to the basal planes, and on the nature and position of the characteristic group of the chains (Kreger, 1951).

2.2.3 Long spacings

It is generally accepted that for long spacings to be observed, molecules of a given length must predominate in the sample. If the crystallites are composed of a mixture of chains of widely divergent lengths, no equally spaced planes will be formed and thus no long spacings recorded. If, on the other hand, the

differences in chain lengths are either minimal or vastly different, the long spacings for a composite crystal and a mixture of crystals will be recorded respectively (Kreger, 1951). Beeswax, a complex mixture of different components, will thus tend to exhibit more than one set of long spacing reflections, of which some may be extremely faint. Further, the reflections recorded on the X-ray camera may be difficult to separate because of the relatively small radius of the camera.

In the light of these considerations, long spacings were measured on a diffractometer and special procedures were employed in sample preparation. Reflections from long spacings could be observed reliably only from wax first melted and then solidified between glass slides under slight finger pressure. Long spacings were obtained using manganese filtered K α radiation. During the X-ray measurements the samples were rotated around an axis normal to the specimen plane. To minimize systematic errors at low diffraction angles, diffraction peaks at both positive and negative diffractometer angles were obtained and the angular difference between them calculated. These measurements showed the presence of a strong reflection which was accompanied on its high angle shoulder by a weaker reflection.

2.2.4 Side spacings

Lines due to side spacings were detected in transmission and reflection. Specimens were mounted on a goniometer and oscillated 10° about an axis perpendicular to the beam. Such rotation provides every set of planes an opportunity to reflect the incident beam. Nickel-filtered copper K α 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. A simplified explanation of X-ray photograph interpretation is provided in the Appendix (2.A).

2.3 Results

2.3.1 Side spacings

Side spacings for all specimens were easily observed in transmission and by reflection. They arose as a result of the ordered lateral packing of the long chains.

The relevant crystal structures were monoclinic ($a \neq b \neq c$, $\alpha = \gamma = 90°$, $\beta \neq 90°$) and orthorhombic ($a \neq b \neq c$, $\alpha = \beta = \gamma = 90°$). If the c-axis is taken to be the long-axis of the molecule, then the side spacings, d, are given by the hk0 reflections with the quadratic form

$$\frac{1}{d^2} = \frac{h^2}{a'^2} + \frac{k^2}{b^2}$$

where a' is a in the orthorhombic crystal structure, and a' is a sinß in the monoclinic cell unit. The definitive crystallographic parameters of a sinß and b were obtained from a linear plot of d^2h^2 versus d^2k^2 in the expression

$$d^2h^2 = -\frac{a^2\sin^2\beta}{b^2} d^2k^2 + a^2\sin^2\beta$$

The d values, Miller indices and intensities for the three preparations of virgin scale and comb wax are presented in Tables 2.1 and 2.2 respectively. The relevant plots of d^2h^2 versus d^2k^2 for each wax are given in Figures 2.1 to 2.6. The corresponding values for a sinß and b appear in Table 2.3.

The results from powder photographs taken with the X-ray beam parallel to the planar surface of the specimen gave results similar to those when the

beam was at a normal angle in all but nascent scale wax. For completeness, these diffraction patterns are included in the Appendix (Figures 2.A.2 to 2.A.7). The X-ray photograph for untreated virgin wax, taken with the beam parallel to the plane, clearly demonstrated the existence of crystallographic texture (Figure 2.7).

2.3.2 Long Spacings

Diffraction lines corresponding to long spacings were only observed after melted wax solidified between glass slides. A comparison of the recorded long spacings for native scale and comb wax are given in Table 2.4.

Table 2.1 The d values, Miller indices (hkl) and Intensities (I) for scale wax preparations.

	scale wax untreated		scale wax sheeted		scale wax sheeted & soxhletted	
	d(nm)	I	d(nm)	I	d(nm)	I
hkl indices						
?	0.466	w	0.470	w	.	
110	0.423	s	0.430	vs	0.415	vs
020	0.378	s	0.386	s	0.373	s
120	0.302	w	0.309	w	0.302	w
200 030	0.252	w	0.256	w	0.249	w
210	0.238	vw	0.230	w	.	
130	0.224	w	0.220	w	0.223	w
220	0.209	w	0.213	w	0.209	vw
?	0.196	vw	0.190	vw	.	
140 230	0.170	vw	.		.	

For I, vs=very strong, s=strong, w=weak, vw=very weak

Table 2.2 The d values, Miller indices (hkl) and Intensities (I) for comb wax preparations.

	comb wax untreated		comb wax sheeted		comb wax sheeted & soxhletted	
	d(nm)	I	d(nm)	I	d(nm)	I
hkl indices						
?	0.474	s	0.450	w	0.481	w
110	0.425	vs	0.423	vs	0.439	vs
020	0.382	s	0.381	s	0.393	s
120	0.306	w	0.302	w	0.310	s
200 030	0.254	w	0.252	w	0.256	w
210	0.239	w	.		.	
130	0.217	w	0.240	w	0.229	w
220	.		0.210	w	0.210	w
?	.		.		.	
140 230	.		.		.	

For I, vs=very strong, s=strong, w=weak, vw=very weak

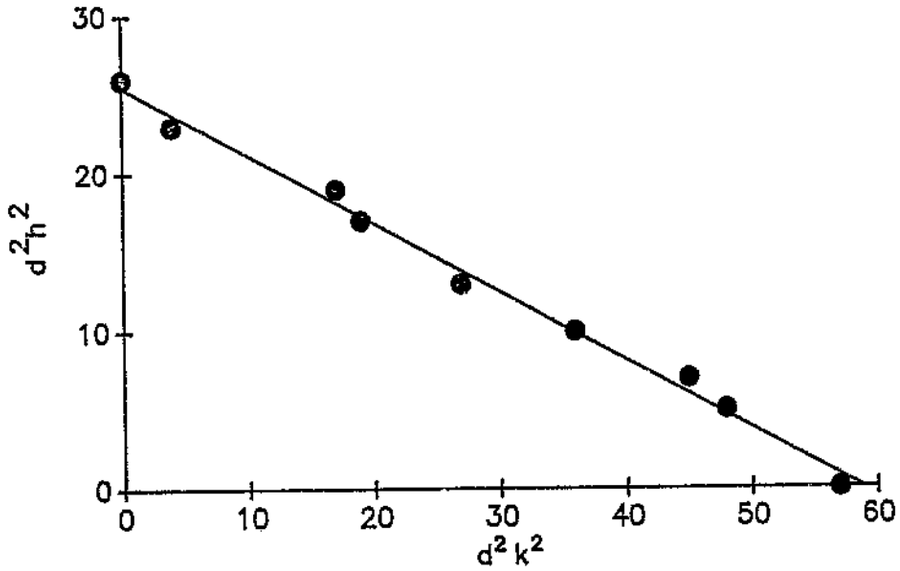


Figure 2.1 Plot of d^2h^2 against d^2k^2 for nascent scale wax.

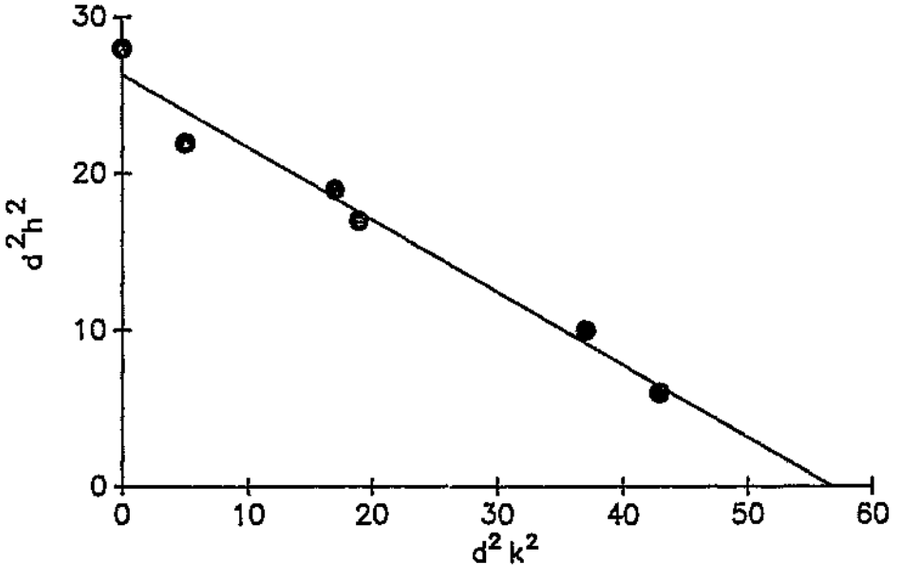


Figure 2.2 Plot of d^2h^2 against d^2k^2 for sheeted scale wax.

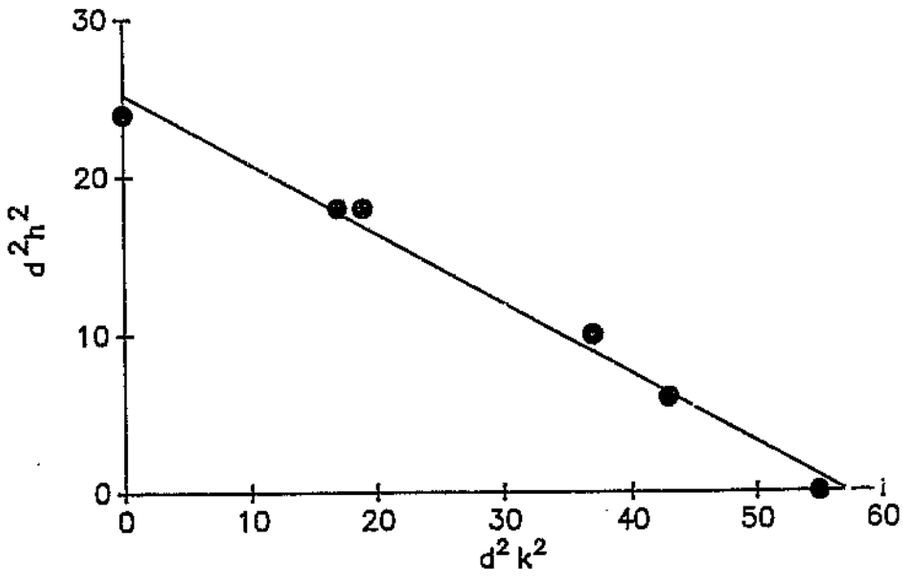


Figure 2.3 Plot of d^2h^2 against d^2k^2 for Soxhlet-extracted and sheeted scale wax.

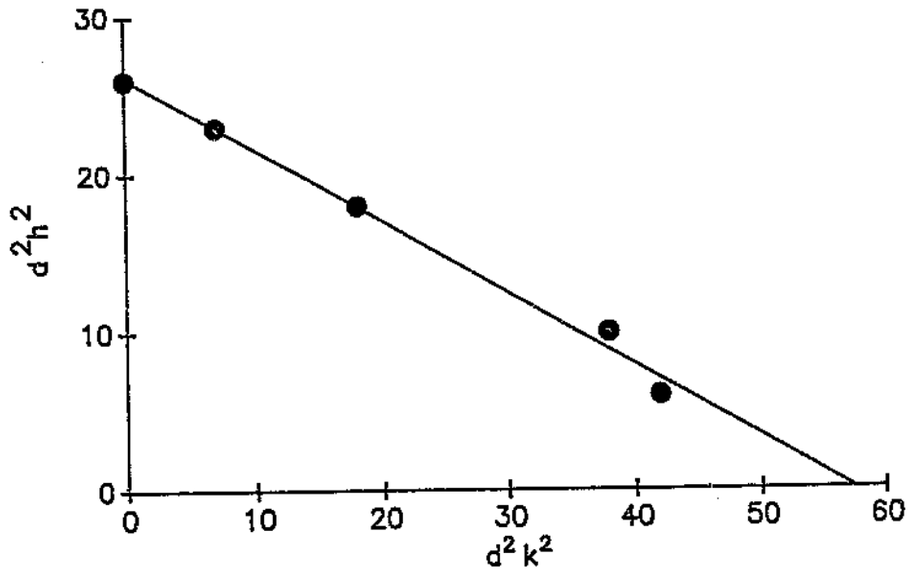


Figure 2.4 Plot of d^2h^2 against d^2k^2 for untreated comb wax.

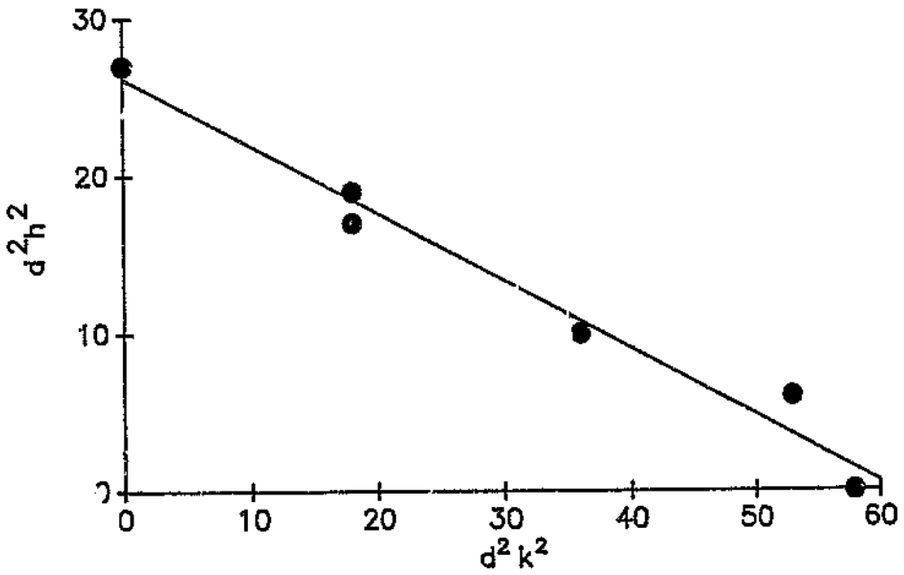


Figure 2.5 Plot of d^2h^2 against d^2k^2 for sheeted comb wax.

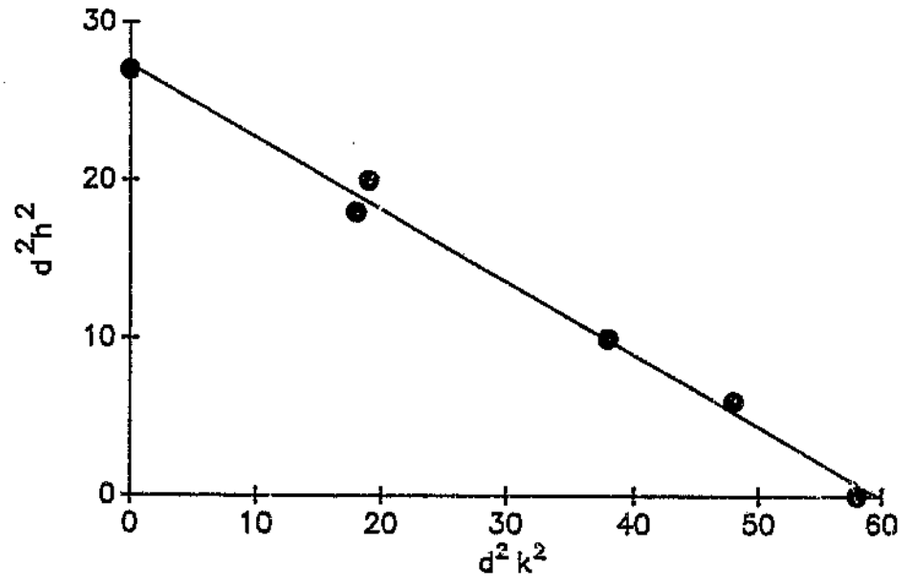


Figure 2.6 Plot of d^2h^2 against d^2k^2 for Soxhlet-extracted and sheeted comb wax.

Table 2.3 Crystallographic parameters of the scale and comb wax preparations.

Wax Sample	a sinB (nm)	b (nm)
1) Comb Wax		
nascent	0.506	0.756
sheeted	0.503	0.772
sheeted + soxhletted	0.513	0.776
2) Scale Wax		
nascent	0.502	0.750
sheeted	0.519	0.781
sheeted + soxhletted	0.506	0.762

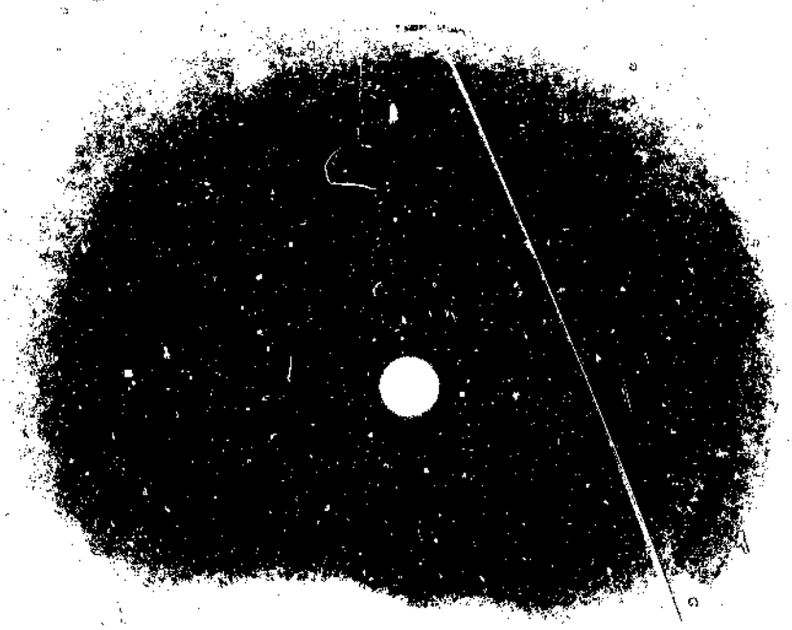


Figure 2.7 Powder photograph of scale wax, taken with the beam parallel to the planar surface of the specimen.

Table 2.4 A comparison of the long spacing reflections of wax scales and comb wax.

	Strong reflection d (nm)	Secondary overlay d (nm)
Comb wax:	7.06 ± 0.05 (n=9)	6.40 ± 0.05 (n=6)
Virgin scale wax:	7.07 ± 0.04 (n=15)	6.22 ± 0.04 (n=13)

Means ± SEM

2.4 Discussion

Basic empirical studies have demonstrated a difference in chemical composition between scale and comb wax. From his solubility experiment, Huber (1814) deduced that virgin scales were "less compounded" then newly constructed comb. He concluded that bees impregnate scale wax with a substance giving it the ductility and whiteness of comb wax. Casteel (1912) similarly ascribed the changes in colour, pliability and opacity, from transparent to translucent, to the addition of a salivary secretion to the scale during mastication. These changes in mechanical properties may, in part, be explained by the rearrangement of crystallites in the process of comb construction.

Powder photographs for all but untreated virgin scale samples showed full concentric rings (Figures 2.A.2 to 2.A.7) indicative of a random orientation of the wax constituents. Freshly secreted 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 therefore revealed continuous powder rings (Figure 2.A.2). With the beam parallel to the plane of the scale however, diffraction patterns with pronounced arcs typical of an ordered molecular arrangement were obtained

(Figure 2.7). The a sinß and b parameters calculated for all specimens (Table 2.3) compared well with those previously obtained for monoesters (cetylpalmitate, a sinß = 0.492 nm, b = 0.742 nm; Kohlhaas, 1938).

The long spacings recorded for virgin wax and nascent comb wax are presented in Table 2.4. Before these results can be compared, two issues confounding their interpretation need to be addressed. Firstly, the chain axis may be inclined to the reflecting planes, producing a spacing shorter than the length of the molecule, and secondly two molecules may join end to end in which case the observed spacing will be double the molecular length. This ambiguity was resolved by comparing the spacings with the results of Tulloch (1980). Subsequently a good correlation between both primary and shoulder reflections and the diester component of beeswax, as reported by Tulloch, was obtained. The shoulder reflection is attributable to an inclined form (angle of inclination from 62 to 65 degrees).

Thus virgin wax scales are textured, the molecular c-axis of the crystallites arranged perpendicular to and inclined at 62 to 65 degrees to the planar surface. Newly constructed comb wax, and all the variously treated waxes, exhibit no diffraction texture, implying a random crystallographic arrangement. These findings confirm and extend previous investigations employing polarised light techniques (Dujardin, 1850; Schmidt, 1924) and X-ray diffraction (Woog and Yanniquis, 1935). Brewster (1815) had previously defined the cell walls

of combs as crystals in which the neutral and depolarising axes of adjacent layers are not coincident. Woog and Yanniquis (1936 a,b) however, reported the presence of very weak X-ray reflections in the comb cell walls of French wax. These probably arose from patches of incompletely masticated scales which are occasionally included in the comb (Casteel, 1912).

Crystallite orientation in w is influenced by two major factors: temperature and deformation forces. In a series of diffraction studies on waxes prepared by extrusion over a range of temperatures, Woog and Yanniquis (1935, 1936a) demonstrated augmentation of texture at higher temperatures. They further established the presence of enhanced crystallinity, and as a consequence tensile strength, in comb w stored at 38 °C over those kept at 15 °C. By inference then, newly constructed combs subject to the warmth of the honeybee nest are, with the passage of time and subsequent progression of texture, structurally superior.

Tensile deformation has been identified as an important determinant of crystal texture. Gaubert (1910 a,b) observed that in beeswax compressed between glass plates, the crystals align in the plane of the compression force. Indeed, this phenomenon has been employed in sample preparation for the recording of long spacings in beeswax (Schoening, 1980). Schoening (1980) performed X-ray and optical studies on deformed wax specimens. He concluded that in beeswax specimens fractured in the ductile mode, the needle

axis aligns along the tensile axis producing an imperfect [100] fibre texture. In compressed wax, the needle axis and the molecular axis line up in the plane of compression. It would be interesting to speculate that the latter process is responsible for the exture observed in freshly secreted wax scales, the compression force occurring between the ventral wax plates of the worker bee (Hepburn, 1986).

Finally, the role of texture in maintaining the structural integrity of the nest can be alluded to. Virgin scales are fused, laminated structures (Huber 1814) in which the crystallites are vertically inclined to the plane of the scale. In uniaxial tensile tests in the plane of the scale (Chapter 5), the crystallites have their c-axis normal and inclined to the direction of load. This implies that they flow passively in the amorphous matrix of the scale, contributing little to strength or stiffness. The random orientation of the crystallites in sheeted scale wax, and indeed comb wax, suggests that at least some of the crystallites will have their molecular axis pointing in the direction of loading. By process of mastication, and hence textural rearrangement then, both the strength and resistance to deformation of the honeycomb nest are improved.

2.A Appendix

Crystals are composed of sheets of molecules running in all directions. The members of each set of planes are distributed a constant distance apart - the "spacing" of the set.

A beam of X-rays is shown incident on a single pair of planes in Figure 2.A.1(a). From this figure it is evident that there will be a maximum in the diffraction pattern only if

$$n \lambda = 2d \sin \Theta \qquad \text{(Bragg's Law)}$$

where n is an integer, λ is the wavelength, d is the spacing of the planes under consideration, and Θ is the angle which the incident X-ray beam makes with the planes. In order that every set of planes has an equal chance to reflect the incident beam, the crystal must be rotated.

When an X-ray pattern is obtained, the first requirement is to measure the d-spacings. These are obtained by calculating the Θ angles for the reflections from the camera geometry and then applying Bragg's Law. The diameter 2R

of each circle is measured and used to calculate the Bragg angle θ in the relation

$$\tan 2\theta = R/D$$

where D is the specimen-to-film distance (Figure 2.A.1(b)).

The measured d-spacings are then compared with those for the various sets of hkl planes of unit cells of different dimensions. The Miller indices, hkl, define the number of intersections of the particular set of planes with the a, b, and c axes, respectively, of a single unit cell. Equations for monoclinic and orthorhombic unit cells are presented below:

Monoclinic:

$a \neq b \neq c \qquad \alpha = \gamma = 90° \quad ß \neq 90°$

$$d_{hkl} = \sqrt{\frac{\frac{h^2}{a^2} + \frac{l^2}{c^2} - 2hl\frac{\cos\beta}{ac}}{\sin^2\beta} + \frac{k^2}{b^2}}$$

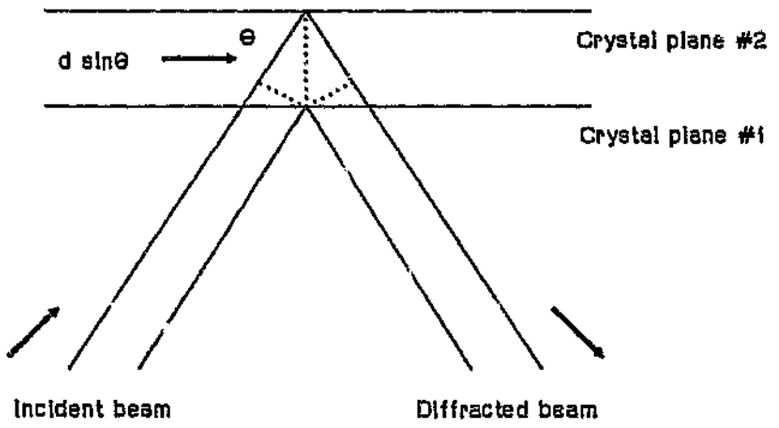
Orthorhombic:

$a \neq b \neq c \qquad \alpha = \beta = \gamma = 90°$

$$d_{hkl} = \sqrt{\frac{h^2}{a^2} + \frac{k^2}{b^2} + \frac{l^2}{c^2}}$$

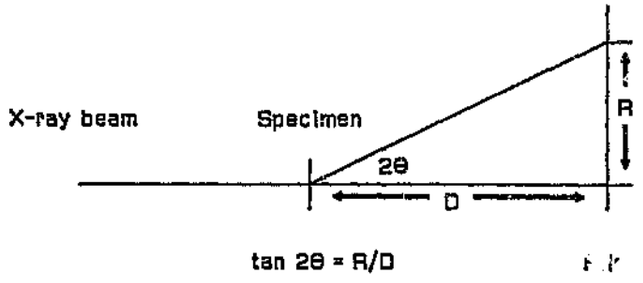
Most indexing is performed either manually or with the aid of a computer. The latter rapidly calculates the d-spacings for a number of trial unit cells. When a correlation of 1% exists between the observed and calculated spacings, the hkl values are assigned to the observed data and the approximate unit cell parameters are refined using a least-squares procedure (Kreger, 1951).

For poorly crystalline polymer systems, often all that can be obtained is a powder pattern consisting of about 10 lines or less. In such a case only a rough idea of the unit cell may be obtained.



Ray reflected at plane #2 has travelled 2d sinθ further than ray reflected at plane #1.

1(a)



tan 2θ = R/D

1(b)

Figure 2.A.1(a) Demonstration of the Bragg relationship. (b)Schematic of method of recording a powder photograph.

Figure 2.A.2 X-ray diffraction pattern of virgin scale wax, taken with the beam normal to the plane of the specimen.

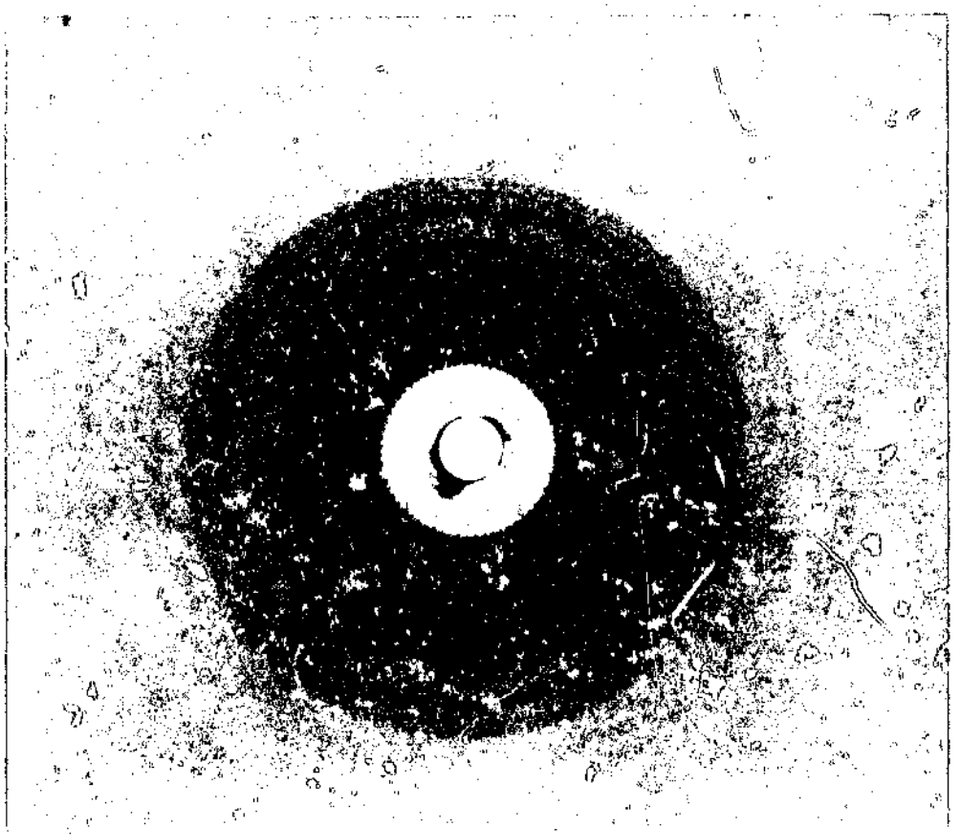


Figure 2.A.3 X-ray diffraction pattern of sheeted scale wax, taken with the beam normal to the plane of the specimen.

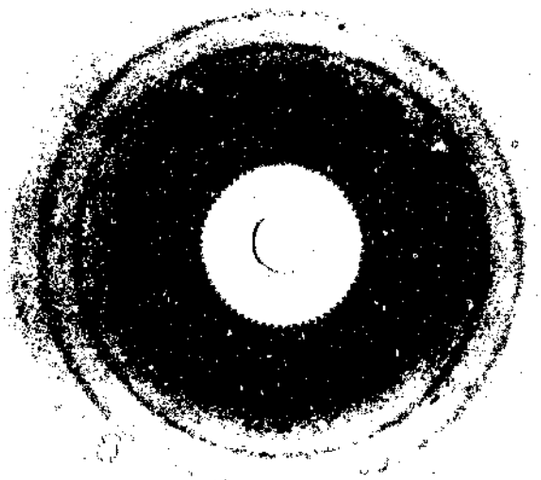


Figure 2.A.4 X-ray diffraction pattern of Soxhlet-extracted and sheeted scale wax, taken with the beam normal to the plane of the specimen.

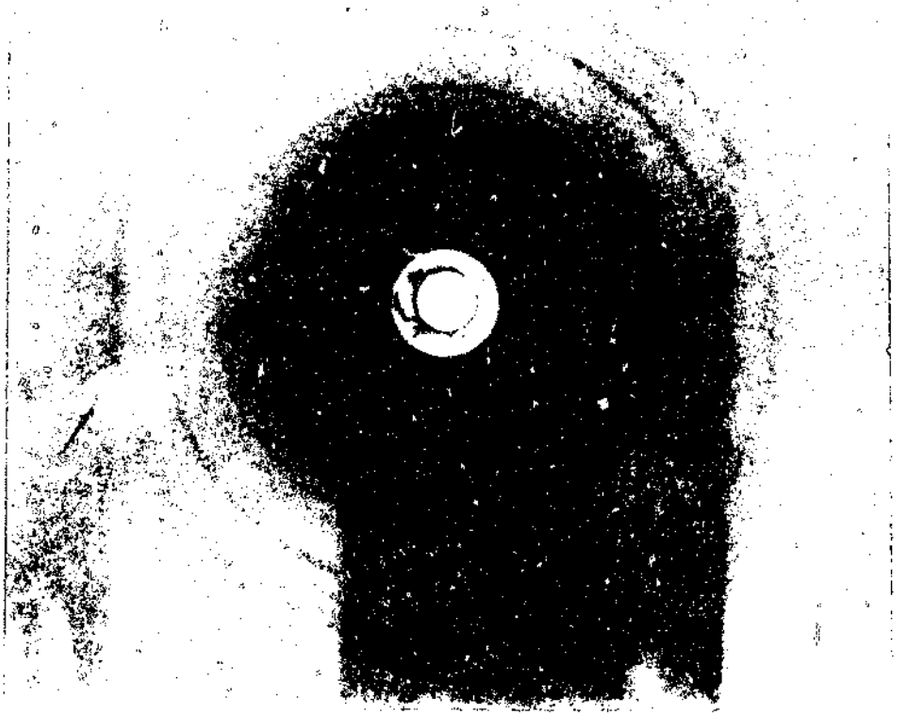


Figure 2.A.5 X-ray diffraction pattern of comb wax, taken with the beam normal to the plane of the specimen.

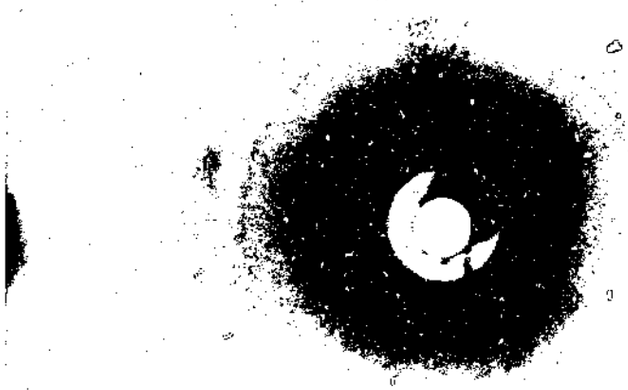


Figure 2.A.6 X-ray diffraction pattern of sheeted comb wax, taken with the beam normal to the plane of the specimen.

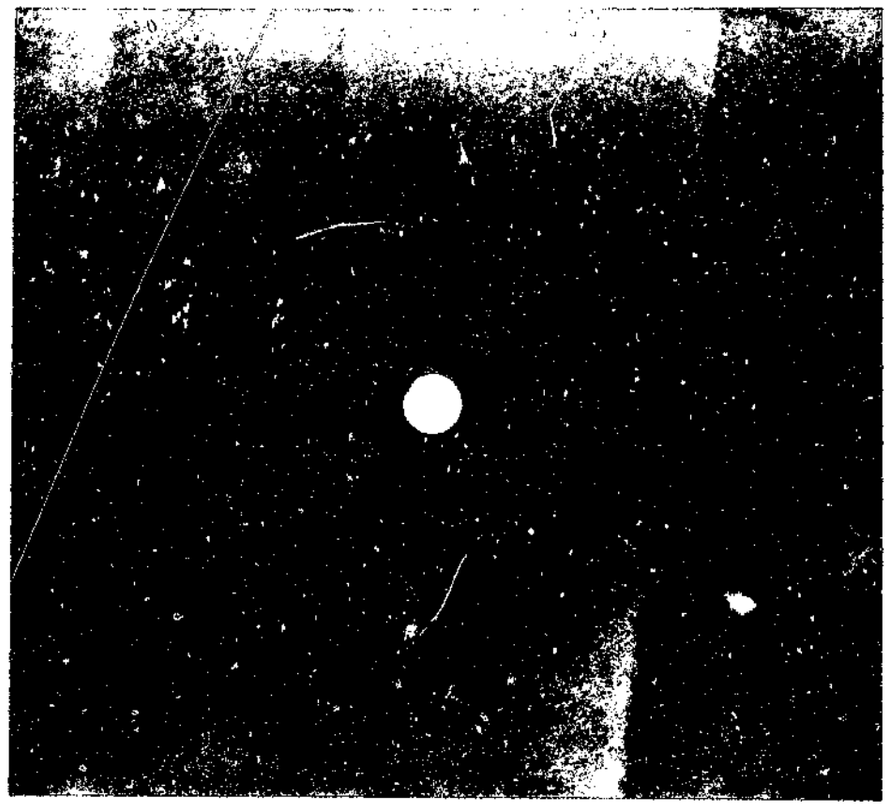


Figure 2.A.7 X-ray diffraction pattern of Soxhlet-extracted and sheeted comb wax, taken with the beam normal to the plane of the specimen.

Chapter 3

3. Alterations in the Lipid Composition of Beeswax following Mastication

3.1 Introduction

Most plants and insects typically possess an integumental water-proof barrier. A thin film of wax, either deposited on the surface or impregnated within the cuticle, is primarily responsible for this effect. In addition to this waxy cuticle, certain insects produce larger amounts of wax which are employed in structural or protective roles. Cuticular waxes are complex compounds. In plants and insects, hydrocarbons are one of the most prevalent components. They usually comprise a mixture of n-alkane, n-alkenes, and branched alkanes, both internal and external. In plants, n-alkanes constitute the principal hydrocarbon fraction (10 to 50 percent) and range in length from 21 to 37 carbon atoms with an overwhelming preponderance of odd numbered chains (Hadley, 1981). Very often, one or a few chains predominate. In Brassica oleracea, for example, more than 90% of the paraffin fraction is a C_{29} alkane, while in Senecio odoris a C_{31} alkane prevails (Kolattukudy, 1968). Branched hydrocarbons are widely distributed in plant waxes. In only a few cases, such as in tobacco, do they constitute a significant portion (50%) of the hydrocarbons (Tulloch, 1976). The major branched hydrocarbons are iso-(2-methyl) and anteiso-(3-methyl) hydrocarbons, although internal and multiple methyl branches as well as cyclical structures have been described as

minor components (Kolattukudy and Walton, 1972). Unsaturated hydrocarbons are rare.

The hydrocarbon content of insect cuticular lipids varies from 0.5% to greater than 90% (Coudron and Nelson, 1978; Jackson and Blomquist, 1976). The alkane pattern of insects is very similar to that of plants: n-alkanes of insect surface lipids are generally in the range 21 to 36. Again, alkanes having an odd number of carbon atoms predominate. Branched alkanes are more common in arthropods and account for over 90% of the total hydrocarbon fraction (Hadley, 1977). Alkenes and alkadienes, with up to three double bonds and with variable positions in the chain, have been reported in the cuticles of numerous insects (Mody et al., 1975).

The non-hydrocarbon component of cuticular lipids is composed predominantly of oxygenated derivatives of alkanes. Wax esters, free fatty acids, aldehydes, alcohols and ketones are present in both plant and insect surface lipids in varying proportions. Wax esters are ubiquitous in most plant waxes. They usually comprise saturated, even chain fatty acids in the range C_{12} to C_{32} esterified to alcohols having an even number of carbon atoms from C_{22} to C_{30} (Kolattukudy, 1975). Conversely, wax esters are not common in insect cuticular lipids, with the exception of those identified in the grasshoppers Melanoplus sanguinipes and Melanoplus packardii. The wax esters of these

two insects comprise 18 and 28% respectively of the total cuticular lipids (Blomquist, G. et al., 1972).

In both plants and arthropods, free fatty acids and free alcohols are seldom major contributors of the surface waxes. The free alcohols resemble esterified alcohols in their chain length distribution and rarely may predominate. Saturated, unsaturated and even branched fatty acids have been observed. These often exceed the length of the esterified equivalents. Finally, cyclic compounds are also represented in the cuticular lipids of plants and arthropods. Triterpenes comprise a significant proportion (up to 40%) of cuticular waxes of fruits and berries (Martin and Juniper, 1970). Further, flavols, sterols and aromatic hydrocarbons have also been reported. In arthropods, dietary cholesterol is sequestered in the cuticle, and its presence has been postulated as mandatory for the efficient functioning of the epicuticle (Toolson and Hadley, 1977).

There are two principal groups of wax producing insects, the Coccidae and the Apidae. Of these, the scale insect and the honeybee are the most common examples. In the former, the wax is employed as a protective shield or scale, while the honeybee uses wax as a structural material in comb construction.

Scale insects derive their name from the waxy secretions produced by the female. This usually hardens and forms a protective scale beneath which the

insect lives. Although some species are serious plant pests, certain scale insects produce enough wax to be commercially important. These include the Chinese wax insect Coccus ceriferus, the Japanese Ceroplastes species, the lac insect, Tachardia lacca and the cochineal insect Coccus cacti (Warth, 1956).

Faurot-Bouchet and Michel (1964, 1965) have separated and analysed by gas-liquid chromatography the waxes of seven species of scale insects. Hydrolysis of these waxes yielded varying proportions of hydrocarbons, alcohols, acids and hydroxy acids. Significant amounts of hydroxy acids were isolated from Gascardia madagascariensis (family Lacciferidae), Icerya purchasi (family Margaroididae) and Pulvinaria flocifera (family Coccidae). Those of G. madagascariensis were shown to be a mixture of C_{30} to C_{34} acids. These three species plus that of the Chinese wax insect, C. ceriferus, further contained approximately equal amounts of alcohols and acids. Acids were shown to predominate in Quadraspidiotus perniciosus (family Diaspididae) and Ceroplastes rusci (family Coccidae), while alcohols occurred in excess in Tachardia lacca. These acids and alcohols were found mainly to be C_{26} to C_{30} compounds. Those of the Chinese insect and of lac wax, however, were C_{26} to C_{28} and C_{28} to C_{34} respectively, implying primary esters of C_{52} and C_{56} to C_{62}. The hydrocarbons of the waxes comprised odd-numbered carbons in the range C_{25} to C_{35}, with C_{27}, C_{29}, C_{31} or C_{33} predominating.

As beeswax is possibly the most important of the insect waxes, its chemical composition has been extensively researched. A detailed composition of the wax has, however, only recently been elucidated through modern chromatographic techniques.

In 1961, Downing et al. separated the components of hydrolysed beeswax and analysed them by gas-liquid chromatography. The hydrolysis products yielded hydrocarbons (C_{25} to C_{33}), alcohols (C_{24} to C_{34}), acids (C_{16} to C_{32}), hydroxy acids (C_{16} to C_{28}) and diols (C_{24} to C_{32}). This study served to confirm the X-ray crystallographic findings of Chibnall et al. (1934) which demonstrated that although the hydrocarbons were odd numbered C_{25} to C_{31} compounds, the alcohols and long chain acids were in fact even numbered with 24 to 34 carbons. However, in order . extrapolate back to biosynthetic pathways and to compare the properties of waxes, a knowledge of the constitution of the natural unhydrolysed wax is imperative.

The composition of unadulterated comb wax produced by Apis mellifera was reviewed in detail by Tulloch (1980). His analyses revealed the following constituent fractions : hydrocarbons (14%), monesters (35%), diesters (14%), triesters (3%), hydroxy monoesters (4%), hydroxy polyesters (8%), acid esters (1%), acid polyesters (2%), free acids (12%), free alcohols (1%) and unidentified (6%). Each fraction further comprised numerous major (forming more than 1% of each fraction) and minor (forming less than 1%)

components. Tulloch reported a total of 74 major and 210 minor components. Of these only a few formed more than 1% of the unfractionated wax : three saturated hydrocarbons, C_{27} (4%), C_{29} (2%) and C_{31} (1%); two unsaturated hydrocarbons, $C_{31:1}$ (1%) and $C_{33:1}$ (2.5%); five saturated monoesters, C_{40} (6%), C_{42} (3%), C_{44} (3%), C_{46} (8%) and C_{48} (6%); two unsaturated monoesters $C_{46:1}$ (2%) and $C_{48:1}$ (2%); five diesters, C_{56}, C_{58}, C_{60} (all 2%), C_{62} (3%) and C_{64} (1%); one hydroxy ester, C_{46} (1%); and three free acids, C_{24} (6%), C_{26} and C_{28} (both 1%). Together these 21 components account for 56% of the wax.

The monoesters (C_{40} to C_{50}) may be separated into 85% saturated and 15% mono-unsaturated esters. They are mainly C_{16} esters of C_{24} to C_{32} alcohols, although oleic and palmitic acid were revealed on hydrolysis (Stránsky et al., 1971). Diesters, C_{56} to C_{66} components, yield acids and alcohols as well as hydroxy acids and diols on hydrolysis. They also contain saturated (80%) and unsaturated (20%) fractions (Tulloch, 1971). The major hydroxy acid is 15-hydroxyhexadecanoic acid (15-hydroxy palmitic acid) (Horn et al., 1964), while C_{24} to C_{32} diols predominate (Tulloch, 1971). Triesters are similar in structure to diesters, with the addition of two hydroxy acid units or a hydroxy acid and diol unit in the middle of the molecule (Tulloch, 1980). Hydroxy monoesters occur as long chain alcohols esterified by hydroxy acids or esterified diols. Hydroxy polyesters are more complex and probably consist of chains of hydroxy acids, although diols may also be incorporated (Tulloch, 1971). Acid monoesters (C_{32} to C_{44}) consist of hydroxy acids esterified by

mainly palmitic acid, while the free acids comprise C_{22} to C_{36} acids (Tulloch, 1971; 1980). The free alcohols are similar to those found combined (Stránsky et al., 1972).

Inspite of these detailed analyses of comb wax, very few reports appear on the chemistry of freshly secreted virgin wax scales. Huber (1814) first demonstrated that the properties of fresh wax scales differed from those of newly built comb. He noticed that although scales immersed in turpentine readily dissolved, worked wax did not, with many particles remaining suspended in the liquid. When placed in sulphuric ether, however, the comb fragments disintegrated and fell in powder to the bottom of the vase, while the wax scales preserved their size and shape and lost only their transparency. From these observations Huber concluded that wax scales were less compounded than nascent comb. He deduced that bees must impregnate the scales with a substance to give it the ductility and whiteness of comb wax. In his study of the manipulation of wax scales by the honeybee, Casteel (1912) described the transfer of wax scales to the mouthparts of the bee, where the wax was masticated and mixed with saliva. He further corroborated the altered chemical composition by noting changes in colour, pliability and translucency of the wax following chewing. More recently, as an aside to their investigation of wax synthesis in the honeybee, Lambremont and Wykle (1979) have reported a thin-layer chromatographic pattern of scale wax similar to that reported for honeycomb cappings (Tulloch, 1970), with the exception of

activity in the region of beeswax diesters. In this study then, the lipid composition of virgin scale wax is examined, and contrasted with that of freshly constructed comb in an endeavour to explain their behaviourial differences.

3.2 Materials and methods

3.2.1 The waxes

The waxes analysed in this study were produced by the African honeybee, Apis mellifera scutellata. Comb wax was retrieved from apiaries kept in the Transvaal, while virgin wax scales were obtained directly from the wax mirrors of numerous bees.

3.2.2 Chemicals

All chemicals used in this study were analytical grade, available from SAAR Chem., unless otherwise stated.

3.2.3 Thin-layer chromatography (TLC)

TLC is one of the most versatile and effective techniques for the separation of intact complex lipids (phospholipids, glycolipids, etc) and their lipid moieties, as well as for neutral lipids. As with other chromatographic techniques, in TLC the separation and purification of compounds is a function of their distribution coefficient between the stationary and mobile phases.

The gross lipid composition of three samples of both virgin scale and comb wax were analysed by thin-layer chromatography.

3.2.3.1 Sample preparation

The waxes were first extracted according to Folch et al. (1957). Samples were thoroughly homogenized in chloroform-methanol (2:1) to which the antioxidant butylated hydroxytoluene had been added (0.01% weight for volume (w/v)). The extracts were then washed in 20% (volume for volume (v/v)) 0.85% (w/v) saline and the lower chloroform phase collected. These phases were reduced under vacuum and aliquots taken for lipid dry weight determination. The extracts were then applied to TLC plates.

3.2.3.2 Preparation of thin-layer plates and sample application

A uniform slurry of 50 g Silica Gel G (E. Merck) in 90 ml of water was added to the reserve chamber of an adsorbent applicator. The spreader was adjusted to the desired layer thickness of 0.5 mm, and the applicator pulled at steady rate across a series of 20 x 20 cm plates on the mounting board. The plates were then left at room temperature for 1 hour and subsequently dried at 120° C for 2 hours. Trace lipids were removed from all plates by running them twice in the chromatographic chamber with chloroform-methanol (1:1), and then drying them as before.

The samples were applied as a row (0.5 to 1 cm long) of slightly overlapping small spots, each of 0.5 to 2 μl and containing 20 to 80 μg of lipids. A Hamilton micro-syringe was employed for this purpose.

3.2.3.3 Development of TLC plates and detection of the lipids

Preparative as well as standard analytical plates were developed. In both cases, the chromatographic procedure was identical. Sixty to eighty milliliters of solvent was added to the chromatographic chambers and 1 to 2 hours allowed for equilibration. This prevents poor separation due to irregular running of the solvent. Preloaded thin layer plates were placed vertically in

the tank, with the end of the plate bearing the sample standing in the solution. The solvent was then allowed to ascend to within 1 to 2 cm of the top of the plate (a procedure requiring 50 to 60 minutes). The plates were removed from the tank, and the solvent allowed to evaporate for a short period of time. The chromatogram was immediately stained. Detection reagents were applied to the plates by spraying with an all-glass atomizer operating off a compressed air line. Analytical plates were developed with hexane-diethylether-acetic acid (80:20:2, v/v) after Storry and Tuckley (1967). A mixture of triacylglycerol, diacylglycerol and monoacylglycerol standards were employed as markers. The developed plates were sprayed with 50% sulphuric acid and heated at 120 °C until all lipids were charred and appeared as black spots. The preparative plates were similarly developed with hexane-diethylether-acetic acid (80:20:2). However, these bands were visualised after spraying with a 0.2% (w/v) solution of 2',7'-dichlorofluorescein in 95% ethanol and viewing under ultraviolet light (366 μm). This general stain is non-destructive and allows the bands to be removed for further analyses.

Acyl-lipids separated on TLC plates were quantitated by scraping them from the plate and subjecting them to acid methanolysis to form fatty acid methyl esters (FAME).

3.2.4 Transmethylation of lipids (after Moscatelli, 1972)

Bands extracted with chloroform-methanol (2:1) were dried to completion at 37 °C under nitrogen. To each aliquot, 0.2 ml of petroleum ether and 1 ml of 14% (w/v) boron trifluoride in methanol was added. Each tube was then flushed briefly with nitrogen, sealed and incubated at 100 °C for thirty minutes to facilitate transmethylation of the fatty acids. The vials were then cooled, and 1 ml of saturated aqueous sodium chloride followed by 5 ml of petroleum ether was added to each. After mixing, the tubes were centrifuged at moderate speed on a bench top centrifuge, and the petroleum ether layer extracted twice by the same procedure.

The fatty acid profiles of the isolated fatty acid methyl esters were ascertained by gas-liquid chromatography. The glycerol content of the aqueous residue from the transmethylation reaction mixtures was also determined.

3.2.5 Glycerol determination

The glycerol content of scale and comb wax as well as their respective diacylglycerol and monoacylglycerol constituents was determined by an

enzyme-catalyzed ultraviolet method (Boehringer Mannheim, Cat. No. 148270).

The principle of this assay is as follows (Figure 3.1). Glycerol is phosphorylated by adenosine-5'-triphosphate (ATP) to glycerol-3-phosphate in a reaction catalyzed by glycerokinase (GK). The adenosine-5'-diphosphate formed is subsequently reconverted by phosphoenolpyruvate (PEP) to ATP and pyruvate by pyruvate kinase (PK). Finally, in the presence of lactate dehydrogenase (LDH), pyruvate is reduced to lactate by reduced nicotinamide-adenine dinucleotide (NADH), with the oxidation of NADH to NAD. The amount of NADH consumed, as determined by means of its absorption at 340 nm, is stoichiometric with the amount of glycerol.

3.2.6 Gas-liquid chromatography (GLC)

GLC is the method of choice for rapid, quantitative analysis of compounds of relatively low polarity. The method involves partitioning of the components of a mixture in the vapour state between a mobile gas phase and a stationary non-volatile liquid phase dispersed on an inert support. The column of liquid material is maintained at an elevated temperature which volatizes the compounds to be analysed. The basis for their separation is the difference in the partition coefficients of the volatilised compounds between the liquid and

gas phases as they are carried through the column by a gas (usually nitrogen or argon). As the compounds leave the column they pass through a detector linked to a recording device.

One microliter of the solution of fatty acid methyl esters in petroleum ether was injected into a 6 m x 3 mm ID gas-liquid chromatograph column. A Varian 3400 Gas Chromatograph unit with flame ionisation detectors was employed. The column temperature was set isothermally at 210 °C with both detector and injector set at 250 °C. A liquid phase of 10% SP2330 on Chromosorb 100/120 mesh with a carrier gas of helium constituted the stationary and mobile phases of the system respectively. Peaks were identified by comparison to known standards run under identical conditions. A Varian 4270 electronic integrator provided a digitised printout of retention times and peak areas. The stationary phase of this column was, however, unable to separate geometrical isomers of fatty acids.

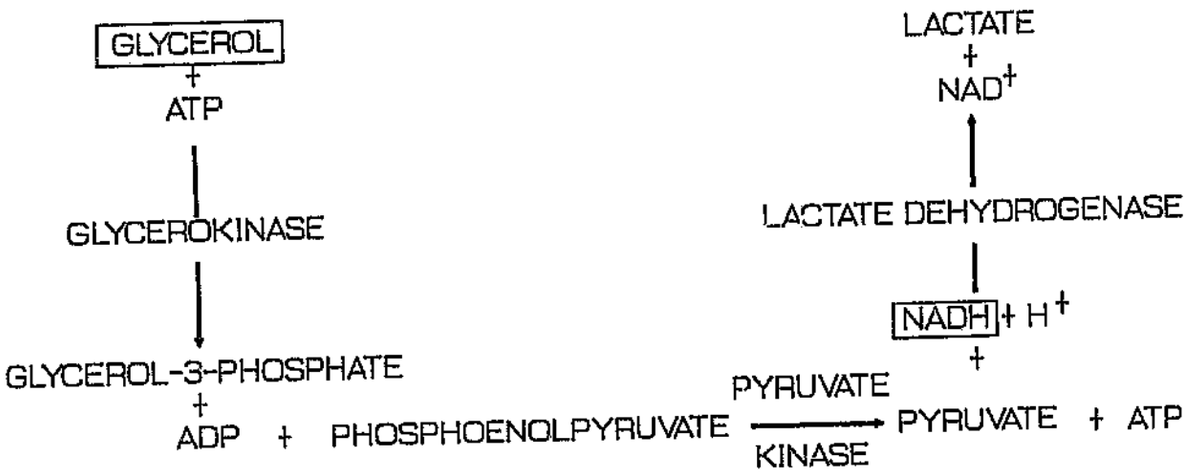


Figure 3.1 Ultraviolet method for glycerol determination.

3.3 Results

A typical Silica Gel-G thin-layer chromatogram of virgin scale wax (lanes 2, 3 and 4) and freshly constructed comb wax (lanes 6, 7 and 8) is presented in Figure 3.2. Standards for mono-, di-, and triacylglycerols (from bottom to top) were co-separated in lanes 1, 5 and 9. Bands were visualised by charring with 50% sulphuric acid

A comparison of the glycerol content of scale and comb wax is given in Table 3.1. Further, that of the constituent monoacylglycerol and diacylglycerol fractions of each are also provided.

Finally, in Table 3.2, the fatty acid content of scale and comb wax, as determined by gas-liquid chromatography, is contrasted. This is graphically represented in Figure 3.3. Again, the comparison is extended to include the mono- and diacylglycerol component of each wax (Tables 3.3 and 3.4, and Figures 3.4 and 3.5 respectively).

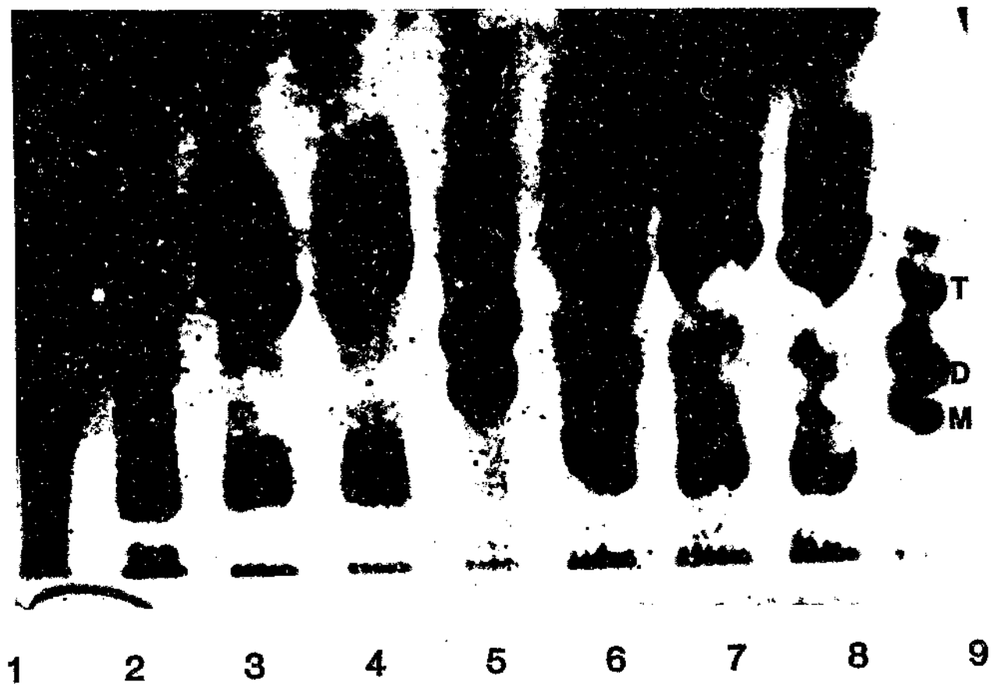


Figure 3.2 Thin- layer chromatogram on silica gel G of scale wax (lanes 2,3,4), comb wax (lanes 6,7,8) and standards (lanes 1,5,9) for mono- (M), di- (D) and triacylglycerols (T).

Table 3.1 Comparison of glycerol content of scale and comb wax.

	Scale Wax (ug/mg)	Comb Wax (ug/mg)
Total	5.72 ± 0.32	5.35 ± 0.31
Diacylglycerol	4.26 ± 0.19	2.70 ± 0.16
Monoacylglycerol	0.97 ± 0.09	2.16 ± 0.13
Calculated total	5.23	4.86
Percentage recovery	91.5%	90.9%

Results expressed as mean ± S.D., n=6.

Table 3.2 Fatty acid analyses of scales and comb wax.

Fatty acid	Virgin scales	Comb wax
12:0	nd	nd
14:0	8.3 ± 0.36	4.7 ± 0.25
16:0	15.2 ± 0.30	20.2 ± 0.64
18:0	4.9 ± 0.25	1.4 ± 0.12
20:0	1.8 ± 0.09	1.8 ± 0.10
22:0	5.0 ± 0.23	3.3 ± 0.31
24:0	28.3 ± 0.79	35.8 ± 0.90
26:0	3.0 ± 0.08	2.9 ± 0.05
28:0	1.9 ± 0.20	2.1 ± 0.22
Total	68.4	72.1
16:1w9	0.9 ± 0.11	1.9 ± 0.10
18:1w9	18.2 ± 0.45	15.0 ± 0.30
20:1w9	nd	nd
Total	19.1	16.9
18:2w6	7.2 ± 0.23	6.9 ± 0.21
18:3w6	3.5 ± 0.15	2.0 ± 0.16
20:4w6	nd	nd
Total	10.7	8.9
18:3w3	1.8 ± 0.09	2.1 ± 0.10
20:5w3	nd	nd
Total	1.8	2.1

Results expressed as means ± S.D., n=6. nd = not detected.

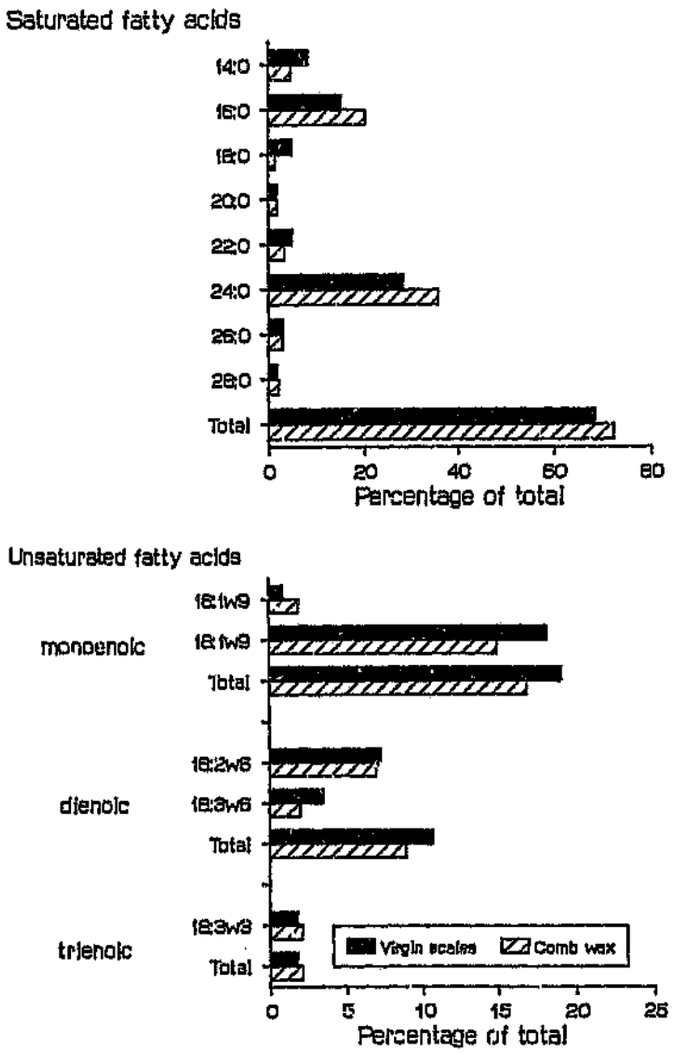


Figure 3.3 Comparison of fatty acid profiles of comb and scale wax.

Table 3.3 Fatty acid analyses of the monoacylglycerol (MAG) fractions of scale and comb wax.

Fatty acid	MAG Virgin scales	MAG Comb wax
12:0	nd	nd
14:0	1.7 ± 0.13	1.9 ± 0.25
16:0	21.0 ± 0.50	21.7 ± 0.42
18:0	12.1 ± 0.42	12.5 ± 0.49
20:0	3.5 ± 0.31	3.9 ± 0.29
22:0	3.6 ± 0.14	3.3 ± 0.11
24:0	10.2 ± 0.27	10.9 ± 0.27
26:0	nd	nd
28:0	nd	nd
Total	52.1	54.2
16:1w9	3.2 ± 0.11	2.5 ± 0.13
18:1w9	25.4 ± 0.33	23.8 ± 0.61
20:1w9	nd	nd
Total	28.6	26.3
18:2w6	11.9 ± 0.33	12.1 ± 0.22
18:3w6	5.2 ± 0.44	5.5 ± 0.19
20:4w6	nd	nd
Total	17.1	17.6
18:3w3	2.3 ± 0.16	2.2 ± 0.11
20:5w3	nd	nd
Total	2.3	2.2

Results expressed as means ± S.D., n=6., nd=not detected

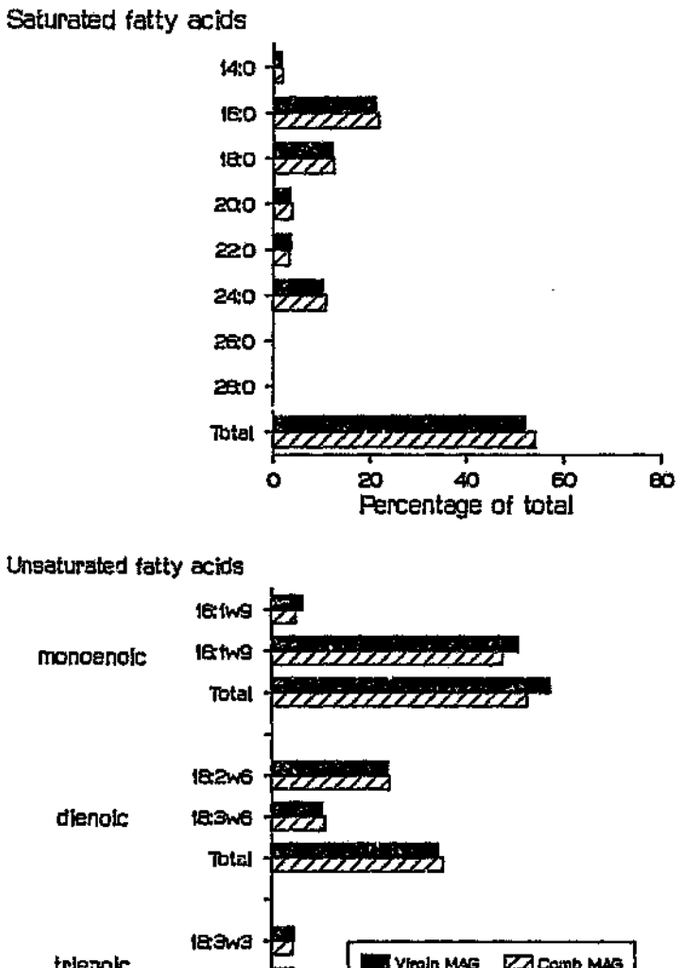


Figure 3.4 Comparison of the fatty acid profiles of the monoacylglycerols of comb and scale wax.

Table 3.4 Fatty acid analyses of the diacylglycerol (DAG) fractions of scale and comb wax.

Fatty acid	DAG Virgin scales	DAG Comb wax
12:0	nd	nd
14:0	1.1 ± 0.11	1.4 ± 0.16
16:0	23.2 ± 0.44	19.9 ± 0.63
18:0	12.8 ± 0.50	12.4 ± 0.55
20:0	3.9 ± 0.26	3.6 ± 0.26
22:0	3.2 ± 0.17	3.9 ± 0.16
24:0	10.4 ± 0.33	10.0 ± 0.42
26:0	nd	nd
28:0	nd	nd
Total	54.6	51.2
16:1w9	2.0 ± 0.18	3.0 ± 0.20
18:1w9	17.6 ± 0.37	19.3 ± 0.40
20:1w9	nd	nd
Total	19.6	22.3
18:2w6	12.2 ± 0.29	11.9 ± 0.30
18:3w6	8.1 ± 0.41	8.7 ± 0.30
20:4w6	nd	nd
Total	20.3	20.6
18:3w3	5.6 ± 0.16	5.9 ± 0.15
20:5w3	nd	nd
Total	5.6	5.9

Results expressed as means ± S.D., n=6, nd=not detected.

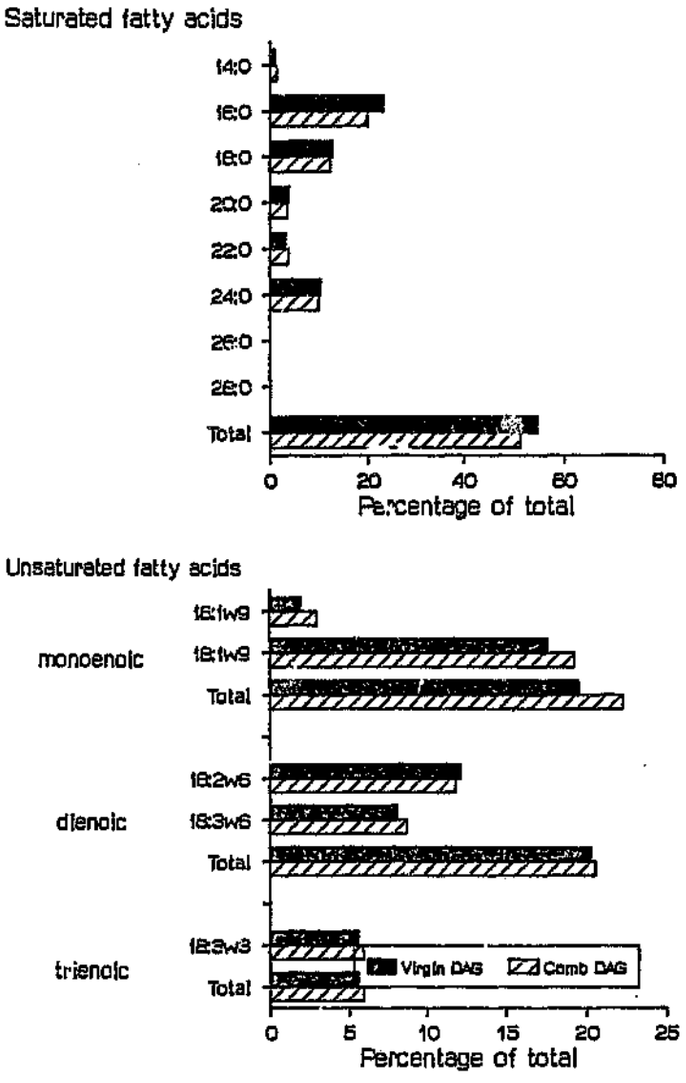


Figure 3.5 Comparison of the fatty acid profiles of the diacylglycerols of comb and scale wax.

3.4 Discussion

Comb wax is a highly complex compound. It is composed of a great diversity of components, each varying in primary constituents and thus chain length. Predictably, the properties of beeswax are directly dependent upon its chemistry. Indeed, Tulloch (1980) ascribes the relatively low melting point (63 to 65 °C (Warth, 1956)) and plasticity of honeycomb wax to the preponderance of numerous minor components, the presence of hydroxy compounds with the hydroxyl group on the penultimate carbon, the occurrence of significant quantities of unsaturated oleic acid derivatives, and the contributions made by a mixture of racemic and optically active compounds.

The hexagonal honeycomb is moulded from thin slivers of scale wax secreted on the ventral surface of the bee. Huber's solubility experiments (1814) implied a basic chemical disparity between scale and comb wax. A knowledge of the composition of wax scales may thus provide a partial explanation for the change in physical properties of the wax following mastication, as well as allow for analysis of the biosynthesis of the wax.

In Figure 3.2, the gross lipid composition of scale wax is contrasted with that of comb wax by thin-layer chromatography. The chromatogram of these two waxes, run alongside the appropriate mixed standards, shows that although virgin scales have a relatively large diacylglycerol pool, no significant monoacylglycerol fraction can be demonstrated. Comb wax, conversely, contains detectable amounts of monoacylglycerols in addition to a diacylglycerol component that is less intense than that of wax scales.

These differences in acylglycerol distribution were more clearly demonstrated by comparing the glycerol content of scale and comb wax (Table 3.1). Here, the mono- and diacylglycerol fractions are found to comprise approximately 91% of the total glycerol for both waxes. As seen in Table 3.1, the diacylglycerol content of scale wax is about 4.5 times that of the monoacylglycerols, whereas in comb the ratio is close to 1:1 (di- : monoacylglycerol). In comb construction then, there is a significant reduction in the diacylglycerols from 4.26 μg/mg scale wax to 2.70 μg/mg comb wax, with a concomitant increase in the monoacylglycerol fraction from 0.97 μg/mg scale wax to 2.16 μg/mg comb wax. Notably, the overall levels of acylglycerols remain similar in the conversion of scale to comb.

The mono- and diacylglycerols were further characterised by gas-liquid chromatographic analyses of their fatty acid content. Examination of these results reveals the following trends in the transformation of virgin scales to

comb wax. Firstly, in the monoacylglycerol fraction there is an increase in the saturated fatty acid content of comb, which is accompanied by a decrease in the unsaturated component, predominately the monoenoic acids (16:1ω9 and 18:1ω9) (Table 3.3, Figure 3.4). Following mastication of the wax, the diacylglycerol component shows a decrease in saturated acids, especially of the 16:0 constituent, with a concomitant increase in the polyenoic acid content, again involving the 18:1ω9 and 16:1ω9 acids (Table 3.4, Figure 3.5). Considering the relative preponderance of monoacylglycerols in comb wax, the net effect of manipulation of the scales would thus appear to be an increase in the saturated fatty acids. This is confirmed in a comparison of the fatty acids of comb and scale wax (Table 3.2, Figure 3.3). Here comb demonstrates a higher proportion of saturated fatty acids, predominantly in the 16:0 and 24:0 elements, with a reduced contribution from the monoenoic (18:1ω9) and dienoic (18:3ω6) unsaturates.

The effect of these changes in lipid composition on the physical characteristics of the wax is revealed in a comparison of the deformation properties of Soxhletted virgin scale wax and its comb equivalent (Chapter 5). Here the former is shown to be significantly stronger though not as stiff as the latter. From the above chemical findings, it would thus appear that although the strength of the wax is compromised through mastication by the subsequent reduction in the diacylglycerol pool, the associated relative increase in saturated monoacylglycerols results in a stiff structural material. These

alterations in lipid composition further suggest the presence of enzymatic lipase activity in the salivary emulsion added to the scales during mastication. Considering the complex composition of beeswax, however, it would be naive to regard these changes in acylglycerols as solely responsible for the observed mechanical alterations. Indeed, Tulloch (1970) has speculated that unsaturated hydrocarbons in comb wax may act as plasticisers.

Finally, the synthesis of beeswax can be considered. The subepidermal oenocytes and fat body play a prominent role in wax synthesis, and together with the epidermal cell may be considered the "wax gland system" of the honeybee (Hepburn, 1986). In one of the first reports on the biosynthesis of beeswax, Piek (1961, 1964) found that when worker honeybees were fed with deuterated water, (1-^{14}C)-acetate or (U-^{14}C)-glucose, the labels were incorporated into the hydrocarbons and free acids, but not into the wax esters or their component acids and alcohols. In addition, micro-autoradiographic assays showed a 30% increase in uptake in oenocytes compared with fat cells after feeding acetate-^{14}C but no distinct difference after feeding uniformly labelled glucose-^{14}C. He thus postulated that oenocytes synthesize wax acids and hydrocarbons from acetate originating from glycolysis in the adipocytes, but as the fat cells themselves are unable to incorporate acetate, the wax esters remain unlabelled. Piek consequently proposed that esters are produced by the fat cells from monoses sequestered from the hemolymph. This suggestion, that fat body cannot metabolize acetate, was brought into dispute

by Young (1963), who successfully recovered labelled wax esters and free acid fractions after injection of sodium acetate-2-^{14}C into the body cavity of honeybees. Lambremont and Wykle (1979) have suggested that Piek's failure to see incorporation of labelled precursors into the ester fractions may have been due to the very low specific activities of the labelled compounds fed to the bees.

Utilising a cell-free preparation, Lambremont and Wykle (1979) have described an enzyme-containing system, isolated from active wax glands, capable of incorporating (1-^{3}H) tetracosanol into wax esters. They characterised the wax-synthesizing enzyme as not highly specific for long-chain fatty alcohols, detergent sensitive, and ATP, CoA, magnesium and pH dependent. They have thus suggested that the monoester component of beeswax is synthesized by the reaction of a long-chain alcohol with a fatty acyl-CoA. Blomquist and Ries (1979) similarly examined monoester synthesis in a microsomal preparation from Apis mellifera L., and confirmed the mechanism of monoester synthesis to involve the transfer of an acyl group from acyl-CoA to a primary alcohol.

In a significant study on the synthesis of wax in the honeybee, Blomquist et al. (1980) compared the lipid composition of comb and cuticular wax. They report the major component of cuticular lipids to be hydrocarbon (58%), while monoesters predominate in comb wax (35%), thus establishing that epidermal

cells and "wax glands" each produce a wax with a distinct composition. They further observed age and seasonal differences in wax composition. Radio-labelled acetate was recovered from the hydrocarbon fraction of the wax of worker bees not actively producing comb wax, while a higher percentage of label was observed in the monoester component of those actively producing comb. Further, bees eleven to eighteen days following emergence synthesized monoesters as a major wax component, while younger and older insects produced hydrocarbons. Blomquist et al. (1980) also showed that in insects actively producing wax, the ventral abdominal cuticle, which contains the wax glands, preferentially incorporated labelled acetate into monoesters, as opposed to hydrocarbons as incorporated by the thorax tissue. They therefore concluded that the production of large amounts of monoesters is dependent upon the wax gland.

In the final analyses then, although a substantial body of knowledge supports the role of oenocyte, fat body and epidermal cell in the production of wax, no definitive work has provided irrevocable evidence of their individual biosynthetic function. Indeed, a detailed study involving an integration of cellular morphology and biochemical synthetic pathways is required to fully elucidate the contribution of the "wax gland" to scale wax production.

Chapter 4

4. The Proteins of Beeswax

4.1 Introduction

Insects are distinguished wax producers. The waxy secretions which they accumulate on their body surfaces are amalgamates of various organic substances. They comprise not only a wide assortment of lipids ranging from long-chain hydrocarbons, fatty acids, alcohols, to true wax esters, but also non-lipoidal substances such as natural resins, free amino acids, and proteins (Waku and Foldi, 1984).

These "waxes" provide an extensive variety of critical functions. Thin layers of cuticular wax on the outer layer of the integument play an important role in protection against desiccation by forming a waterproof layer (Hadley, 1980). Insect cuticular lipids provide chemical communication; as sex pheromones and as species and caste recognition cues in several social insects and as kairomonal cues for parasites (Nelson, et al., 1981; Blomquist, et al., 1979; Lewis, et al., 1976). The filamentous bloom of some insects affords camouflage, thermal protection and may serve as predator deterrents (Hadley, 1985). Moreover, cuticular lipids may affect the absorption of insecticides from the environment (Brooks, 1976), and have been suggested to serve as a barrier to the penetration of micro-organisms (Koidsumi, 1957). In addition to cuticular lipids, some insects secrete large quantities of wax. In scale insects

the female is protected by a shield which consists of a mixture of cast skins and wax. Honeybees use wax as structural materials to build combs for raising brood and storing honey.

Most insect waxes are manufactured by modified epidermal cells. These can be grouped into three categories according to the relation between the secretory cells and the cuticle covering them (Noirot and Quennedy, 1974). In class 1, the cell is simply covered by the cuticle as in the common epidermal cells. The secretory cell thus has a dual role in producing both cuticle and secretory substance. In class 2, the secretory cells are enclosed by the epidermal cells and the secretion must first be transferred to the adjoining epidermal cells to reach the surface. In the most complex case, class 3, a long cuticular duct penetrates the gland cell and the canal runs into a ductule cell which has secreted it. The canal is in continuity with the cuticle.

Smooth endoplasmic reticulum (SER) is thought to be responsible for wax or lipid production (Waku and Foldi, 1984). Cuticle-secreting general epidermal cells may produce very small quantities of cuticular waxes. Accordingly, these cells contain trace amounts of SER. Complex wax glands occur commonly in scale insects. Such glands are composed of ductule and secretory cells. The central cell, which is present at the basal extremity of the gland, possesses cytoplasm rich in rough endoplasmic reticulum (RER), mitochondria, Golgi apparatus and secretory globules. The secretion of the cell is stored in a large

reservoir along with proteins and glycoproteins. Around the ductule cell are accessory cells. These secrete the lipoidal moiety of the covering subst of the insect. The cytoplasm of these cells contains extensive networks of SER (Foldi, 1981).

In honeybees, the wax gland comprises epidermal cells, together with adjoining oenocytes and fat body (Rösch, 1930). This functional unit lies directly beneath the wax mirror cuticle. Electron microscopic studies of the epithelial layer has demonstrated an absence of cytoplasmic organelles essential for protein secretion (Sanford and Dietz, 1976). In addition, there is no evidence of SER. The cytoplasm of the oenocytes are, however characterised by tubular SER (Wigglesworth, 1972). Diehl (1973) has further demonstrated the role of oenocytes in lipid synthesis in the locust, Schistocerca gregaria. Fat body is the principal synthetic source of hemolymph proteins, and also plays an integral role in lipid synthesis and storage (Dean, et al., 1985). These three cell types are intimately related in the wax gland of the honeybee, and exhibit synchronous development, reaching a maximum at the peak of wax secretion (Boehm, 1965). In addition, both oenocyte and fat cell appear to discharge their contents into the underlying epidermal cell of the wax mirror. The wax gland complex of Apis can therefore tentatively be classified as a class 2 functional unit according to Noirot and Quennedy (1974).

How synthesized lipids (waxes) are transported from the epithelium and deposited on the surface of the cuticle remains a mystery. A number of theories attempting to explain the movement of a relatively hydrophobic compound through the aqueous phase of the endocuticle have be proposed (Locke, 1974).

(1). The wax may be secreted in a solvent which later evaporates. Beament (1955) suggested that volatile paraffins and alcohols in the surface grease of the cockroach provide a mechanism for wax secretion, although such were shown to be absent by Gilby (1962).

(2). The wax may be synthesized near the surface from its constituent fatty acids and alcohols which might themselves diffuse across the endocuticle. The presence of an epicuticular esterase in Calpodes, which can synthesize wax in situ, supports this hypothesis (Locke, 1959; 1961a).

(3). Insect wax secretion may occur by the growth of tubular crystals. The filaments of a pure long-chain wax secreted by the woody alder aphid represent a tubular, crystal habit for paraffins. This tubular form is consistent with well known crystallisation behaviour, requiring only a circular closure at the wax secretion site. Further growth behaviour is entirely controlled by van der Waal's intermolecular actions (Dorset and Ghiradella, 1983).

(4). The wax may pass through a complex, transverse canal system that connects the epidermal cells with the outer region of the epicuticle (Locke, 1961b). Despite the presence of this interconnecting system, no explanation for the transport of lipids to the surface has yet been provided. In addition, pore canals are absent from the cuticles of many wax-secreting insects, as in Calpodes (Locke, 1961a). In the wax mirror cuticle of the honeybee, however, the inner epicuticle is penetrated by wax canal pores (Locke, 1961b). These in turn are tightly packed with filaments of about 100 to 300 Å in diameter (Sanford and Dietz, 1976). Similar tubules are evident in the wax gland epithelium (Reimann, 1952) providing a conduit for the transport of wax precursors from the fat body and oenocyte to the surface of the cuticle (Hepburn, 1986).

(5). The wax may be secreted in a water soluble form in combination with a protein. The egg-waxing organ of ticks appears to utilize such a mechanism (Lees and Beament, 1948). As the external wax deposits appear to be protein-free, the authors suggest that the wax-protein complex dissociates at the apex of the pore canals thus releasing the wax. Diehl (1975) noted that the presence of hemolymph in incubation medium greatly stimulated the release of labelled hydrocarbons from oenocytes. He speculated that the presence of hemolymph lipoproteins, as lipid acceptors, was required for the functioning of the release mechanism. Chino and Gilbert (1964) first demonstrated the presence of a specific hemolymph lipoprotein (lipophorin) that functions as

a true carrier and acts as a reusable shuttle in transporting a variety of lipid classes. Subsequently, cockroach lipophorin was shown to specifically load hydrocarbons at the oenocyte and unload this lipid at the cuticle for deposition (Katase and Chino, 1982). Although the entire lipophorin molecule does not appear to migrate to the cuticular surface, further modification of the lipid-protein complex may occur. Lipophorin has been shown to be present in a wide variety of insect species including the honeybee Apis mellifera linguistica (Ryan, et al., 1984).

To date, the vast majority of investigations into the chemical composition of insect waxes have focused on their lipid content. Nevertheless, Hanrahan et al. (1987) have described the presence of a protein component in the wax bloom of the desert tenebrionid Zophosis testudinaria. This appears to originate from the reservoir of the wax secreting dermal glands (Hanrahan et al., 1984). These proteins may be responsible for maintaining the structural integrity of the wax bloom and are probably essential for the morphological changes observed at various extremes of humidity (Hadley, 1979). They may also play a role in wax secretion.

In this study, the protein content of freshly secreted wax scales from the honeybee Apis mellifera was investigated. Furthermore, modification of this component by mastication and moulding in the process of comb construction was also characterized.

4.2 Materials and methods

4.2.1 The waxes

The comb waxes used in this study were collected from apiaries in the Transvaal and Cape. Freshly constructed, white combs without processed beeswax foundation templates, were the materials of choice. However, more aged yellow comb waxes were also included. These waxes were rinsed thoroughly in distilled water to remove possible extraneous contaminants.

Virgin wax scales were collected directly from the wax mirrors of the Cape bee, Apis mellifera capensis.

4.2.2 Chemicals

All chemicals used in this study were analytical grade, available commercially from SAAR Chem., unless otherwise stated. Double distilled, deionised water was used throughout.

4.2.3 Protein isolation

A variety of delipidation methods were attempted in isolating purified wax proteins. These are described in some detail below. Firstly however, general methodological recommendations for most preparative procedures are characterized (Herbert et al., 1977).

(1) The organic solvents should be precooled, preferably to below 0° C.

(2) If dialyses is employed as part of the procedure, it should be against 0.15 M sodium chloride (NaCl), and not distilled water.

(3) The precipitated protein should be collected by centrifugation at the lowest feasible speed to avoid compaction of the pellet.

(4) The protein should be dried as a thin film not as a pellet. Here, other authorities recommend that the protein not be dried at all, nor should it come into contact with pure hydrophillic solvents (Olofsson et al., 1980). Rather, they suggest that the protein be stored as a suspension in peroxide-free diethyl-ether.

In most biological systems, lipoproteins are bound only by non-covalent forces, that is by hydrophobic, electrostatic, hydrogen and van der Waals bonds. The

protein and lipid moieties can therefore be separated by extraction with a mixture of polar and non-polar organic solvents. This principle forms the basis for a series of delipidation processes.

4.2.3.1 The ethanol-ether process (after Scanu and Edelstein, 1971)

Twenty milliliters of ice-cold ethanol (-15 to -20°C) was added to 3 g of wax and homogenized thoroughly using a high speed Ultraturrex. Thirty milliliters of similarly pre-cooled ether was then added, mixed and allowed to stand for 24 hours at -15°C. The precipitate was collected by centrifuging for 20 minutes at 850xg (approximately 2 500 r.p.m. using a Beckman JA-20 rotor) at -15°C. Care was taken not to compact the protein into a pellet. If some of the precipitate adhered to the side of the tube, it was resuspended and centrifuged a second time.

The supernatant was carefully aspirated off and the protein pellet dispersed in 50 ml of cold ethanol-ether mixture (3:1 volume for volume (v/v)) and kept for 24 hours at -15°C. The precipitate was collected by centrifugation as before. The pellet was subsequently re-extracted with 50 ml ethanol-ether (1:1 v/v) for 30 minutes, and again with 50 ml ice cold ethanol-ether (1:3 v/v). Finally, the precipitate was washed twice with 50 ml of peroxide-free ether at -15°C. After the last wash, the protein was left suspended in 1-2 ml of ether, or dissolved immediately in the desired aqueous solvent.

4.2.3.2 The acetone-ethanol process (after Warnick et al., 1979)

One gram of wax was homogenized in 25 ml of an equivolume mixture of acetone and ethanol at -15 °C. The tube contents were mixed thoroughly and allowed to stand at -15 °C for 4 hours before the protein was sedimented by centrifugation (850xg for 15 minutes) and the supernatant aspirated. The pellet was resuspended in another 25 ml portion of solvent mixture, and incubated at -15 °C for 2 hours, after which the protein was again isolated. The pellet was then treated with diethyl-ether at -15 °C for 1 hour, re-isolated and dried under nitrogen.

4.2.3.3 The chloroform-methanol process (after Folch et al., 1957)

Two variants of this method were attempted. Both are based on the insolubility of virtually all wax proteins in this solvent.

In the first, the more "classical" Folch extraction, 10 g of washed wax was homogenized in 200 ml of chloroform-methanol (2:1 v/v). This follows the accepted solvent to tissue ratio of 20:1. The slurry was kept overnight at 4 °C. It was then filtered through Whatman no. 1 filter paper into separating funnels. To this, 20% of the extraction volume of 0.8% (weight for volume

(w/v)) NaCl (40 ml) was added and the solutions mixed vigorously. After phase separation was complete, the lower chloroform-rich component was discarded. The more polar upper phase was aliquoted, and dried to completion under nitrogen at 37°C.

The pellet was then resuspended in distilled water by severe mechanical agitation; 3 ml per pellet yielded from 5 g of wax. To this 5 volumes (15 ml) of ice cold acetone was added. The mixture was incubated for 30 minutes at -20°C, and the precipitant collected by centrifugation at 10 000xg for 5 minutes (11 500 r.p.m in a Beckman JA-20 rotor). Often initially an oily, micellular collection was observed at the bottom of the test-tube. After removing the supernatant, the addition of 1 volume (5 ml) of ice-cold acetone to this "pellet" allowed for rapid precipitation to occur. The supernatants were themselves extracted by similar acetone precipitation. The final pellets were stored in a dried form at -20°C.

The alternative chloroform-methanol extraction method is based on Olofsson et al. (1980). One gram of wax was homogenized in 15 ml of pre-cooled (-20°C), dry, analytical grade chloroform. To this, 30 ml of ice-cold methanol was added. The solution was well mixed, and allowed to stand for 30 minutes at -20°C. The protein residue was collected by centrifugation at low speed (850xg for 20 minutes) and the solvent aspirated off. The process was then repeated. Then under similar conditions, the protein pellet was first extracted

twice with 45 ml of cold chloroform-methanol (1:1), and then twice with cold chloroform-methanol (2:1). Finally, the protein was washed twice with 45 ml of cold peroxide-free diethyl-ether. After the final wash the protein was left suspended in a small volume of ether.

A simplified version of the latter, is to homogenize 1 g of wax in 30 ml of chloroform-methanol (2:1) and allow to stand for a minimum of 2 hours at -20°C, although overnight is preferable. The precipitate is recovered by centrifugation at low speed as above. The protein pellet is washed once in chloroform-methanol (2:1) and is stored in a small volume of ether.

Of all these isolation methods, the chloroform-methanol procedure was found the most suitable. Not only was it the most efficient extraction process, but the high solvent density allowed for easy separation of the bulk of the lipoidenous wax constituents from the protein pellet. After centrifugation, the wax residue was easily removed from the top of the solution, where it formed a compacted aggregate. In the other delipidation processes, often a combined wax-protein pellet was obtained, especially in the early stages of the extraction. This often proved difficult to purify, requiring more than the recommended washing steps and thus increasing the probability of protein loss.

Once purified, the protein was virtually insoluble in all but the most powerful detergents, making it inaccessible to the majority of physico-chemical

identification methods. This occurred despite addition of the desired solvent to a fine suspension of the protein in ether, a preparation that, with subsequent evaporation of the ether, should have favoured the slow hydration and solution of the protein (Olofsson et al., 1980). This problem was partially circumvented by using the "classical" Folch extraction, as described above. Although this method encouraged the solubility of the proteins, the addition of acetone to the reconstituted polar upper phase residue induced co-precipitation of small polar molecules (such as phospholipids). These in turn increased the minimum volume of solvent required for solubilization.

A satisfactory amount of protein was, however, dissolved by heating the precipitate at between 85 and 90°C for 4 to 5 minutes in the presence of 2% (w/v) sodium dodecyl sulphate (SDS) (BDH, England) and 5% (v/v) 2-mercaptoethanol (BDH, England) (Chow, et al., 1987). This only procured samples suitable for electrophoretic characterization under denaturing conditions. The proteins solubilized by the above "classical" Folch extraction were employed for native polyacrylamide gel electrophoresis.

4.2.4 Protein quantification

The protein concentration after extraction was determined by the Bradford microprotein assay (Bradford, 1976).

4.2.4.1 Preparation of the Bradford protein reagent

One hundred milligrams of Coomassie Brilliant Blue G-250 (Serva, Germany), or the equivalent PAGE-Blue 83 (BDH Chemicals, England) was dissolved in 50 ml 95% ethanol. To this solution, 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. This produced final reagent concentrations of 0.01% (w/v) Coomasie Brilliant Blue / PAGE - Blue, 8.5% (w/v) phosphoric acid, and 4.7% (w/v) ethanol.

4.2.4.2 The microprotein assay

Standard curves were constructed using bovine serum albumin (BSA) (BDH Chemicals, England). Protein solutions containing 1-10 μg of BSA in a volume of 0.1 ml of the appropriate buffer were added to 1 ml of the protein reagent and the contents mixed by vortexing. The absorbance at 595 nm was measured after 3 minutes. Suitable dilutions of the wax protein extracts were similarly treated. All samples were prepared in duplicate to ensure greater accuracy.

Due to the relative insolubility of the wax proteins, only the concentration of protein extracted by boiling in 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol could be determined. However, trace quantities of detergents such as SDS, as well as reducing agents like 2-mercaptoethanol,

interfere with the Coomasie Brillant Blue-Protein Complex Assay, producing spuriously high readings (Bradford, 1976). This problem was eliminated by incorporating the proper buffer control in the assay. In addition, the yellowish colouration of some of the waxes used in the study (possibly due to carotenoid pigments) was also responsible for falsely elevated measurements. This interfering colouration was removed by washing the pellet in acetone prior to protein determination (Chino et al., 1977).

4.2.5 Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of the purified wax proteins was performed on non-denaturing discontinuous gels. In addition, proteins were separated on the basis of molecular weight by SDS gel electrophoresis.

4.2.5.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polypeptides when treated with the anionic detergent SDS receive an equal charge per unit length. In SDS-PAGE therefore, migration is not determined by intrinsic electrical charge, but rather by the molecular weights of the polypeptides (Shapiro, et al., 1967). Co-electrophoresis with standard proteins

of known molecular weights allows for easy calculation of the molecular weight of the polypeptide to be characterized.

The Laemmli discontinuous SDS system (1970), a modification of Davis (1964), was employed to determine the molecular weights of the wax proteins. Here the pretreated proteins are first stacked in a stacking gel before being resolved in the separating gel. All solutions used in electrophoresis were passed through 0.2 μm filters (Millipore) prior to usage. Polyacrylamide gels were prepared from a stock solution of 30% (w/v) acrylamide, with a ratio between monomer, acrylamide (BDH Chemicals, England), and crosslinker, N,N'-bis-methylene acrylamide (Merck, Germany), of 60 : 1.6. The stacking gel mixture contained 3% acrylamide and 0.1% (w/v) SDS in 0.125 M Tris (hydroxymethyl) methylamine - hydrochloric acid (HCl) (pH 6.8). The resolving gel solution contained 10% acrylamide and 0.1% (w/v) SDS in 0.375 M Tris-HCl (pH 8.8). Both gel solutions were degassed for a few minutes prior to usage, since molecular oxygen inhibits the polymerization reaction.

Gels were polymerized chemically by the addition of a catalyst accelerator-chain initiator mixture. This mixture consisted of freshly prepared ammonium persulphate as catalyst (0.05% and 0.1% (w/v) for stacking and resolving gels respectively) together with 0.06% (v/v) N,N,N',N'-tetramethylethelynediamine (TEMED) (Riedel de Haen, Germany) as initiator.

Electrophoresis was performed on a dual, cooled, vertical slab gel unit (Hoefer, Scientific Instruments, SE 600). Gels of 1.5 mm thickness were employed. The resolving gel was poured first, overlayed with 400 µl of distilled water to ensure a uniform stacking-separating gel interface, and left for approximately 60 minutes to polymerize. Thereafter a 1.5 cm stacking gel was similarly prepared. A fifteen-well comb was inserted into the stacking gel immediately after it had been poured. The electrode buffer (pH 8.3) contained 0.192 M glycine, 0.1% (w/v) SDS and 0.025 M Tris.

Wax proteins samples were prepared as described above. In addition to the 2% (w/v) SDS and the 5% (v/v) 2-mercaptoethanol, the treatment buffer also comprised 62.5 mM Tris-HCl (pH 6.8), 5% (v/v) glycerol and 0.001% (w/v) bromophenol blue (SERVA, Germany) as the tracking dye. Approximately 8 to 10 µg of protein in 75 µl sample buffer was loaded into each well. Molecular weight marker proteins (BDH, England, no. 44264 2L) were co-electrophoresed with the experimental samples. These included hen egg ovotransferrin (76-78 kilodaltons), bovine serum albumin (66.25 kD), hen egg ovalbumin (45 kD), bovine erythrocyte carbonic anhydrase (30 kD), equine myoglobin (17.2 kD) and equine cytochrome c (12.3 kD). Electrophoresis was carried out at a constant current of 20 milliamperes (mA) through the stacking gel and 30 mA through the resolving gel, until the bromophenol blue marker reached the bottom of the gel (approximately 6 hours). The system was kept

at a constant temperature of 10°C for the duration of each run by means of a refrigerated circulating water bath.

After electrophoresis, gels were fixed and stained overnight with 0.125% Coomasie Brilliant Blue R-250 or the equivalent PAGE-Blue 83 in 50% (v/v) methanol and 10% (v/v) acetic acid (C. mbrath, et al., 1967). The optimal degree of destaining was attained by immersing the gel initially in a solution containing 50% (v/v) methanol and 10% (v/v) acetic acid for between one and three hours. Gels were the stored in 7% (v/v) acetic acid and 5% (v/v) methanol. Destained gels were scanned densitometrically on a Zeineh SL-2D/1D UV/VIS Soft Laser scanning densitometer (Biomed Instruments,

4.2.5.2 Native gel electrophoresis

Separation of proteins on non-denaturing discontinuous gels depends on the sample, its intrinsic charge, size and shape, the strength of the electric field, as well as the ionic strength, viscosity and temperature of the medium in which the molecules are moving. It is a useful system when dealing with a mixture of proteins.

Native gel electrophoresis was performed on 10% acrylamide gel slabs. Gel preparation was identical to that described above. The resolving gel (in 0.375

M Tris-HCl, pH 8.8) was overlayed with a 3% stacking gel containing 0.125 M Tris-HCl, pH 6.8. The tank buffer (pH 8.3) contained 0.025 M Tris and 0.192 M glycine. Electrophoresis was performed at 4 °C at a constant current of 10 mA through the stacking gel and 15 mA through the resolving gel.

Protein isolated by the "classical" Folch extraction method was solubilized directly in sample buffer (10% (v/v) glycerol and 0.001% (w/v) bromophenol blue in 62.5 mM Tris-HCl, pH 6.8). Between 0.1 and 1 μg of protein was electrophoresed per lane.

After electrophoresis the polypeptides were stained with silver according to Merril et al. (1981).

4.2.5.3 Silver staining procedure

All manipulation of gels were done with gloved hands. After the tracking dye had reached the bottom of the gel, the gel was removed and fixed overnight in a solution of methanol : water : acetic acid (5:4:1, v/v). The following day the gels were rinsed three times, for 10 minutes each, in 200 ml 10% (v/v) ethanol and 5% (v/v) acetic acid. Gels were then soaked for 5 minutes in 250 ml of oxidizing solution (3.4 mM potassium dichromate and 0.0032 N nitric acid). They were washed twice, for 10 minutes in 200 ml deionised water and

placed in 250 ml of 20 mM silver nitrate (J.M. Chemicals, England). Excess silver was removed by two 5 minute washes in distilled water. This was followed by rapid rinsing with two 300 ml portions of image developing solution, which contained 0.28 M sodium carbonate and 0.0037% (v/v) formaldehyde. The gels were gently agitated in a third portion of this solution until the image had reached the desired intensity. Development was stopped by discarding the developer and adding 100 ml of 1% (v/v) acetic acid for 5 minutes. Finally, the gels were washed in distilled water and stored in 50% (v/v) methanol.

4.3 Results

4.3.1 Native gel electrophoresis

Inherent protein composition of comb waxes produced by Transvaal (Apis mellifera scutellata) and Cape (Apis mellifera capensis) honeybees was determined by non-denaturing PAGE. As seen in Figure 4.1, three major and two minor bands were visualized on the gel. No difference in gross protein constituents was noted between Transvaal and Cape honeycomb wax (lanes A and B respectively).

Using the "classical" Folch extraction procedure, large quantities of wax were required to yield enough protein for a single electrophoretic lane (10 g). It was thus impractical to conduct the experiment with virgin wax scale protein - a single scale weighs on average between 0.4776 and 1.7022 mg (Gwin, 1936). Vast numbers would therefore have been required.

4.3.2 SDS-polyacrylamide gel electrophoresis

Information regarding the substructure of the wax proteins was provided by electrophoresis under reduced, denaturing conditions. In Figure 4.2 a typical SDS-electrophoretogr .m comparing virgin scale wax protein (lane 2) with that of Transvaal and Cape comb waxes (lanes 3 and 4 respectively) is presented. Molecular weight marker proteins were co-electrophoresed in lane 1.

Eleven wax scale and thirteen comb wax protei.: bands were resolved on electrophoresis. Seven of these bands were common to both preparations. Again, no difference existed between Transvaal and Cape comb wax proteins.

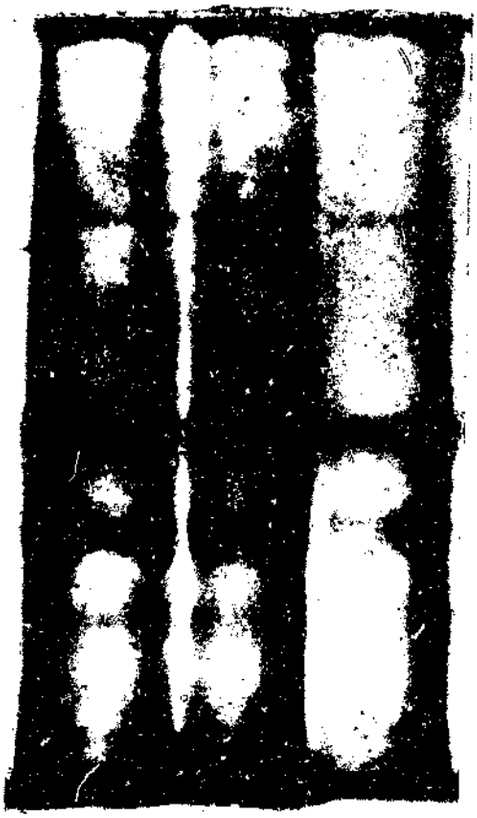
4.3.3 Molecular weight determination

To determine the molecular weight of the wax proteins, known molecular weight standard proteins were run on the same gel. The migration distances of the bands of the calibration proteins and that of the wax samples, as well as the total length of the gel were measured. R_f values for each individual band was then calculated (migration distance/gel length). The logarithms of the molecular weights of the calibration proteins were then plotted as ordinates against their respective R_f values as abscissae. From this working curve, the molecular weights of the wax proteins were calculated.

In Figure 4.3 the molecular weights of the proteins characterized in comb wax and virgin scales are compared. Electrophoretic band number refers to the sequence of the protein bands from the top of the gel, considering the scale and comb wax lanes as a composite. The molecular weights of the proteins range between 19 and 100 kD. Bands 1, 2, 6 and 17 (approximate molecular weights - 97, 89, 66 and 19 kD) occur only in scale wax, while proteins 3, 4, 10, 11, 13 and 15 (87, 82, 54, 52, 47 and 43 kD respectively) are unique to comb wax (Table 4.1).

The densitometric scans of the gel are presented in Figures 4.4 and 4.5. Here the relative proportions of the component proteins are demonstrated. The

most prevailing constituent in virgin wax scale protein (Figure 4.4) is electrophoretic band number 17, or the 19 kD protein. In comb wax, bands 7 to 12 predominate (Figure 4.5).



A B

Figure 4.1 Non-denaturing electrophoretogram of the comb wax proteins of A. m. scutellata (lane A) and A. m. capensis (lane B).

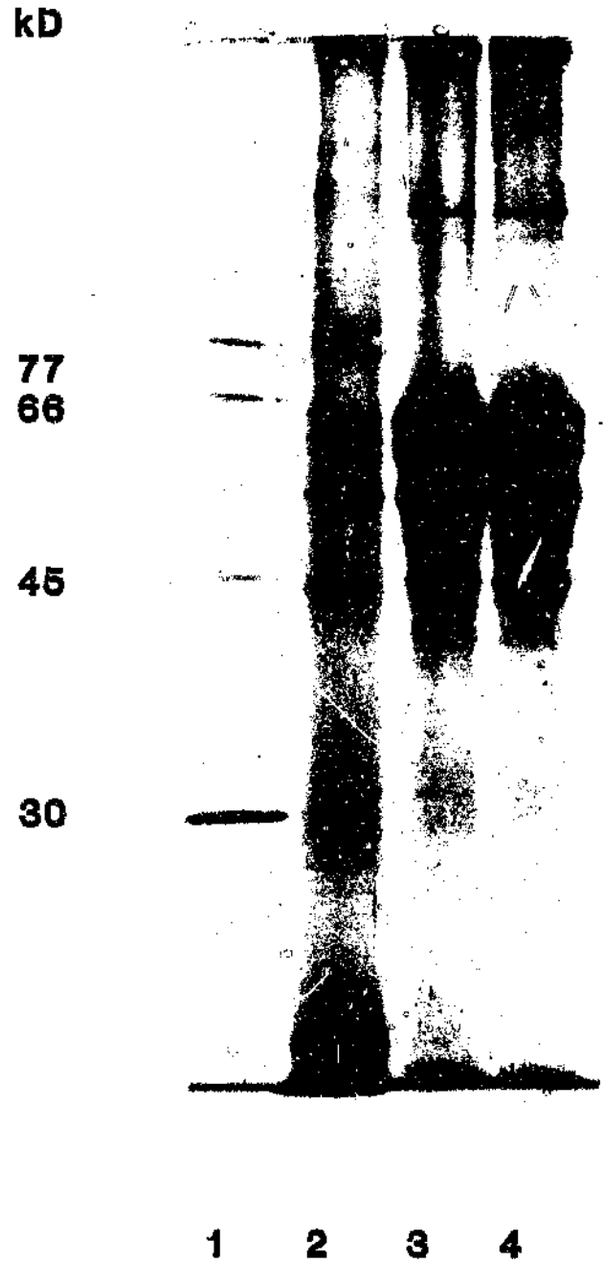


Figure 4.: 10% SDS-polyacrylamide gel of beeswax protein. Standard (lane 1), scale wax of A. m. capensis (lane 2), comb wax of A. m. scutellata (lane 3) and A. m. capensis (lane 4).

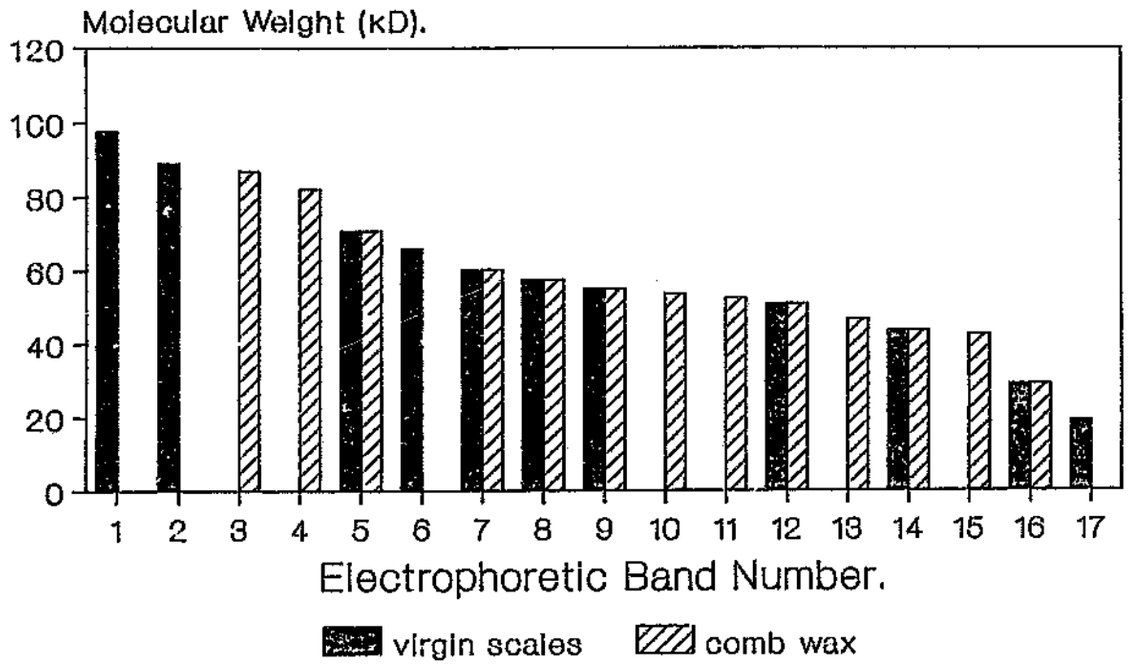


Figure 4.3 Molecular weights. Comb wax protein versus scale wax protein.

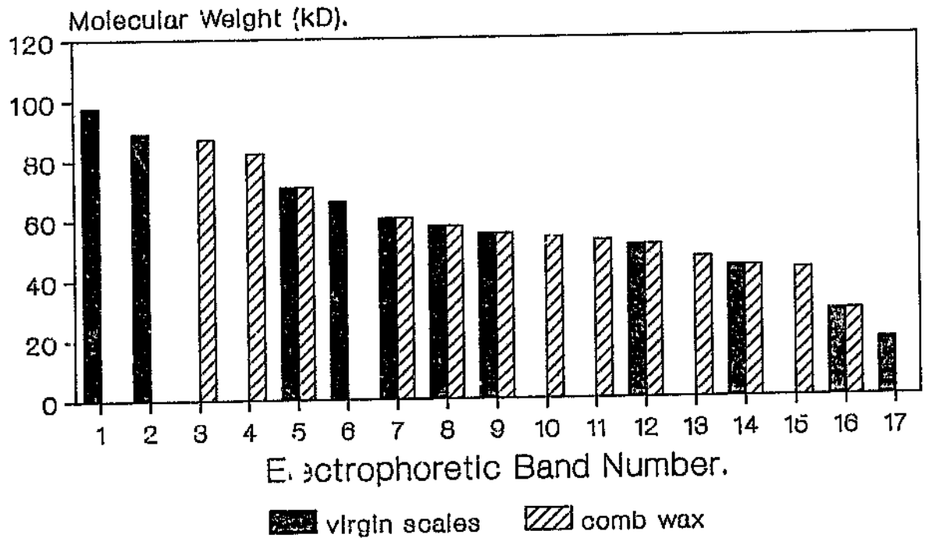


Figure 4.3 Molecular weights. Comb wax protein versus scale wax protein.

Table 4.1 Comparison of comb and scale wax protein.

Characteristic.	*Molecular Weight (Daltons).*
Specific to comb wax:	87 000, 82 000, 54 000, 52 000 47 000, 43 000.
Specific to virgin scales:	97 000, 89 000, 66 000, 19 000
Common to both comb wax and wax scales:	70 000, 60 000, 57 000, 55 000 51 000, 44 000, 29 000

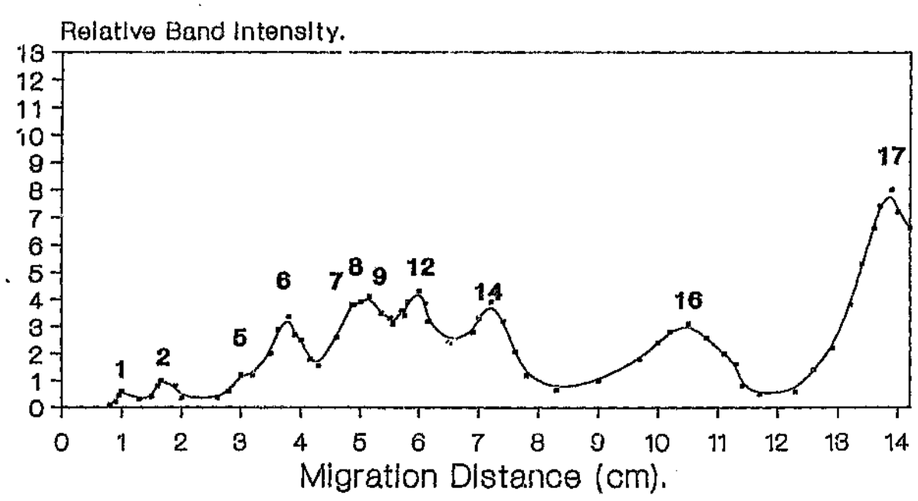


Figure 4.4 Densitometric tracing of scale wax protein.

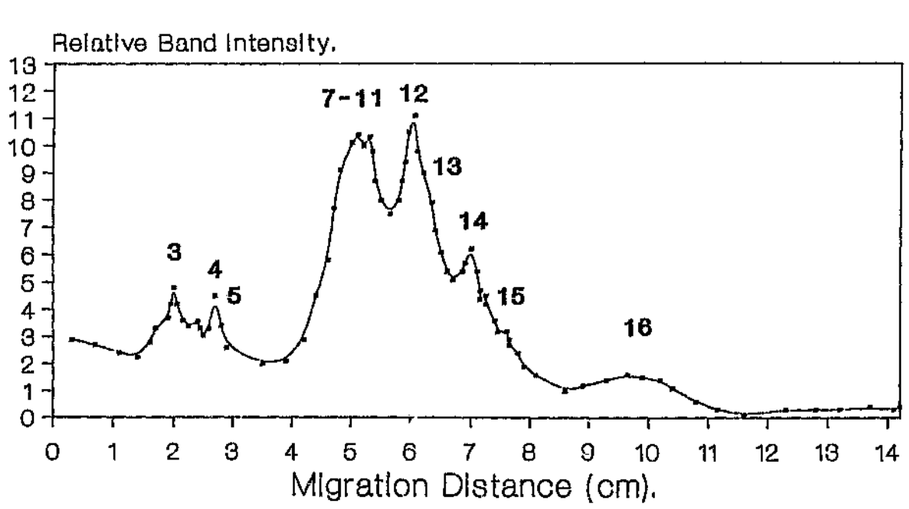


Figure 4.5 Densitometric tracing of comb wax protein.

4.4 Discussion

Both wax scales and newly constructed comb wax contain a protein component. Each of these waxes possess unique protein fractions in addition to polypeptide species common to both (Table 4.1). At present the role of these proteins remains unknown, although they may be implicated in variety of processes.

The secretion of wax presents a problem in transport. Chino and Gilbert (1964) have described a specific hemolymph lipoprotein, lipophorin, that acts as a lipid carrier. Lipophorin is involved in conveying hydrocarbons to the cuticle (Katase and Chino 1982). Analysis of the apoprotein components of Apis mellifera lipophorin has revealed two apolipophorins, type I (MW approximately 250 000) and type II (MW approximately 78 000). Although the intact lipophorin molecule is reported not to migrate to the cuticular surface (Katase and Chino, 1982), this does not preclude involvement of individual apolipoprotein species, be they in a primary or modified state. In fact, apolipophorin II is similar in molecular weight to the 82 kD protein of comb wax and the 70 kD fraction common to both waxes. Logically, the presence of

protein species common to both wax scales and comb wax implies the persistence of integral proteins, such as transport proteins, despite further manipulations as in comb construction. The incorporation of fat body, a principal site of protein synthesis, with oenocytes, the most probable locus for lipid synthesis, in the wax gland complex of the honeybee further supports the concept of a lipoprotein wax precursor.

Besides its possible involvement in wax secretion, the protein fraction is positively associated with enhancing the structural potential of wax. The tensile properties of comb wax and virgin wax scales as Soxhlet-extracted and sheeted versus sheeted wax preparations were compared (Chapter 5). The presence of protein significantly augments the stiffness in both scales and comb, improving their resistance to deformation.

The difference in protein profiles between comb and wax scales may, at least in part, be explained by manipulations evoked in comb construction. In comb building, wax scales produced on the abdomen of the worker honeybee are transferred, via the hind tarsus, to the mouth and thus mandibles of the bee (Casteel, 1912). Here the scale is masticated and mixed with saliva. Casteel (1912) noticed that in this process the scale wax became translucent, changed colour, and behaved in a more pliable fashion. Chemically these alterations produce marked differences in the solubility characteristics of the two wax types (Huber, 1814). Not all wax deposited on the comb are, however, treated

in such a manner. Small unchewed portions of scales are intermingled with the masticated wax, and even near perfect scales can be found mixed with the wax of newly constructed comb. This latter observation lends further support for a protein species common to both waxes. The changes observed in masticated wax represent considerable alterations in lipid composition. Specifically, the saliva added to the wax appears to reduce the diacylglycerol pool of scale wax with a corresponding increase in the monoacylglycerol fraction of comb wax (Chapter 3). It seems reasonable therefore to suppose the presence of a lipase in the salivary secretions. Proteins peculiar to comb wax may therefore include those of such an enzyme, amongst other salivary proteinaceous additions.

Enzymatic action may similarly contribute toward altering the protein content of virgin scale wax. The high molecular weight components specific to scale wax (97, 89 and 66 kD) may undergo digestive proteolysis yielding the lower molecular weights unique to comb (54, 52, 47, and 43 kD). The disappearance of the 19 kD protein, one of the major contributors of scales wax protein is more difficult to explain. However, a number of possibilities exist. Mastication may further reduce this fraction to peptides, undetectable by polyacrylamide gel electrophoresis. The protein may be innately labile, degrading under the conditions which prevail in the honeybee colony. Alternatively, the protein may assume a paradoxically high molecular weight by glycosylation.

Finally, an association exists between protein intake, and wax gland development as manifest by comb production. In an extensive series of experiments, Goetze and Bessling (1959) and later Freundenstein (1960) demonstrated diminished wax production in pollen-deprived bees. These observations were paralleled by a degeneration of the wax gland epithelium and reduction in oenocyte size. In addition, fat body protein granules, first described by Koehler (1921), were absent in pollen-deprived bees. The amount of wax produced appears to depend directly on the quantity of pollen made available. At present no detailed explanation for this correlation exists. In plants, however, Kolattukudy (1968) has proposed a pathway for the biosynthesis of branched chain hydrocarbons from the three amino acids valine, leucine and isoleucine. Together, these results tentatively suggest a further role for proteins in lipid synthesis.

These newly characterised wax proteins therefore appear to be fundamental to the biology of beeswax, be it in a biosynthetic, secretory or structural capacity.

Chapter 5

5. The Tensile Characteristics of Beeswax, Cocoon Silk and Propolis

5.1 Introduction

The honeybee nest serves a variety of functions, from brood nursery to storage facility for pollen and nectar. In addition, it provides domicile for vast numbers of bees which perform a range of duties on the comb face. The comb wax must therefore be structurally competent to support both these internal and external loads.

Comb wax has its origin in the fine wax scales produced on the abdomen of worker honeybees (Hornbostel, 1744). Following secretion, these scales are manipulated and moulded into honeycomb (Huber, 1814; Casteel, 1912). The wax scales are removed from the wax mirror cuticle and transferred, via the medial surface of the hind leg, to the mouth. Here they are masticated, either entirely or piecemeal, and applied to the comb. In the chewing process, a frothy salivary addition is incorporated into the wax.

The properties of fresh wax scales differ form those of newly constructed comb. Huber (1814) observed that when wax scales were dropped into spirits of turpentine they dissolved completely and disappeared without making the solvent turbid. An equal amount of spirit could not dissolve, either as quickly or as completely, similar sized fragments of new white comb. In a subsequent

experiment, Huber (1814) demonstrated that comb fragments disintegrated in sulphuric ether and fell in powder to the bottom of the vessel. Scales, on the other hand, preserved their size and shape and lost only their transparency. Casteel (1912) further observed differences in colour and ductility between comb and scale wax. In addition, he noted comb wax to be translucent compared to the transparency of scales.

These differences in properties between the two waxes have been ascribed , at least in part, to the liquid added by the wax worker during manipulation of the scales. The origins and composition of this salivary emulsion remain unknown. Cruz Landim (1963) has suggested it to be derived from the mandibular gland. 2-Heptanone isolated from this gland does partially dissolve comb wax, although it has no effect on scales (Shearer and Boch, .965).

Besides these preliminary observations on the mechanical and chemical disparities, the crystallographic arrangement also varies between comb wax and scale wax (Chapter 2). In the process of mandibulation, the highly crystalline virgin scale is converted from a texturally anisotropic body into an isotropic one. Together, these changes in crystal texture and chemical behaviour associated with the addition of salivary secretions may affect the structural potential of wax. Although beeswax constitutes the principal structural component, with the passage of time other adjuvant materials, in the

form of silk and propolis, are incorporated into the nest. In this chapter the mechanical advantages of these alterations are explored.

Huber (1814) observed that newly completed cells were white and brittle, but within a few days they became darker, more pliable, stronger and heavier. Their orifices became coated with a reddish varnish chemically identical to the propolis used to fill gaps and crannies in the wall of the hive. Propolis, or bee glue, is derived from the gums and resins of various plants (Hornbostel, 1744; Huber, 1814). Its colour varies depending on the botanical origin of the source; poplar yields red and pine white propolis (Alfonsus, 1933). Although its more familiar function is in filling gaps in order to protect the colony from weather and enemies, propolis is also utilized in comb construction.

Huber (1814) observed the distribution of a propolis load within the hive. Some bees deposited it onto the comb frame, while others placed it into crannies in the walls of the hive. Another group applied it to the inside of the cells, embedding it into the comb wall. Finally, bees were observed substituting cell walls with much heavier pillars composed of a mixture of wax and propolis. Huber noted that the time at which the structure was so strengthened depended upon circumstances. It was more likely to be required when comb was constructed in the absence of foundation. Manipulation of the propolis, especially in a dry hardened form, appeared problematic for the bees. Huber speculated that they impregnated it with the same frothy matter

used for softening wax. Further, warmth improves the handling of the propolis. Bees returning laden with propolis were often observed sunning themselves until their loads had softened and could be removed.

Just before pupation, honeybee larvae cover the walls of their cells with silk (Huber, 1814; Arnhart, 1919). The fibres are randomly dispersed, so that by the end of spinning the walls are covered by thin sheets in which the individual fibres are readily discernible (Jay, 1964). Subsequently the larvae produce, from the anus, a colourless pollen-free material and then a yellow pollen-bearing one, both of which are applied in turn to the silk base (Verlich, 1930; Jay, 1964). Although nothing further is known of these substances, they invite the analogy of a size as in paper manufacture.

Successive generations of brood apply more silk to the walls, the cells become smaller, and the mass ratio of silk to wax greater (Chauvin, 1962). Thus old brood combs are heavily impregnated with silk which is inseparable from the wax except by chemical and heat treatments. The development and maturation of broodcomb proceeds then from a single phase material of pure white wax to a coloured fibre-reinforced composite. In this chapter the material properties of the individual phases of the honeybee nest as well as their possible contributions to the composite are described.

5.2 Materials and methods

5.2.1 The waxes

The waxes used in this series of studies were produced by the African honeybee, Apis mellifera scutellata, that were either feral or kept in hives on the Transvaal highveld. Virgin wax scales were collected directly from the wax mirrors on the abdomens of many bees. The individual combs ranged in colour from white through to dark, brownish black. Colour is roughly correlated with age and use. In brood comb, this is evidenced by the mass ratio of silk to wax and the number of layers of silk incorporated into each cell wall.

Three different preparations of both virgin wax scales and white, freshly constructed comb wax were investigated. These included (1) sheeted, (2) chloroform Soxhlet-extracted and sheeted, as well as (3) untreated samples of native comb cell walls and wax scales. The Soxhlet extraction process and preparation of sheeted waxes is described in the Appendix (5.A.1 and 5.A.2 respectively).

Tensile test specimens of brood comb comprised intact comb samples as well as rectangular slabs cut from individual cell walls. In addition, the silk component was also investigated. Sheets of silk were separated from the beeswax by soaking the composite in chloroform.

5.2.2 The propolis

Propolis was collected from the hives of colonies of Apis mellifera scutellata kept at an apiary in Pretoria. Although the precise botanical origin of the samples was unknown, the major sources of propolis near the apiary included Populus deltoides and Acacia karroo. The samples consisted of two major phases: a yellowish and very tacky component that remained molten at about 50 °C and a darker reddish-brown phase that softened greatly at about 70 °C. Appropriate mechanical test specimens were cut from sheets of rolled propolis.

5.2.3 Tensile testing

Specimens of intact comb samples were prepared from native brood combs (without foundation). These were cut to form two isosceles contiguous at their apices. In the final specimen, the bases of the triangle were five cells wide and

their apices one. These samples were held for testing in solid wood grips patterned in the female form to accommodate the male test specimens.

All other tensile specimens were cut to uniform dimensions to minimize variation in specimen geometry (length = 0.6 mm and width = 1.4 mm). The sides of the wax samples were trimmed parallel to obtain a consistent width. Thickness was left unchanged. Gauge length was kept constant with the use of a vernier microscope stage. Shims of polyester film were clamped flush with the stage, and the verniers moved to obtain the desired length of specimen. Small amounts of cyanoacrylate fixative were then applied to each shim, and the wax test specimen positioned and glued into place. All proportions were subsequently confirmed with a micrometer graticule.

Tensile tests were performed on the extensometer of Joffe and Hepburn (1974) at an extension rate of 53.6×10^{-6} ms^{-1} (Appendix 5.A.3). The resulting force-time curves were transformed into their corresponding stress-strain relationships which yielded engineering values for stress, strain, stiffness and energy to fracture (Appendix 5.A.4). These conventions were only applicable to wax specimens. Because there is no measured value for silk denier, nor could the cross-sectional area of a broken piece of silk be determined with any confidence, values for stress and stiffness are given as relative, in units of N mm^{-1} and not N mm^{-2}. Values for intact comb are similarly presented due to the complex nature of the fracture cross-section. After the completion of

a tensile test, the resulting fracture was checked microscopically to make sure of its location in the test region and thus exclude any grip-break specimens.

Studies were performed to establish whether the tensile test specimens exhibited planar isotropy. In these cases, specimens were cut and tested at the required angles. In addition, the mechanical properties of the preparations were examined as a function of ambient temperature. To accommodate this, a temperature controlled water-bath was fitted to the lower grip, and all specimens were submerged before and during testing. It is assumed that water had no effects on the deformation behaviour of the waxes. Silk, however, is extremely hygroscopic (Denham and Lonsdale, 1933) so that immersion in water obviated differences that may otherwise have arisen with natural changes in relative humidity. The test temperatures, 25 to 45 °C, were meant to reflect a range that might impinge on the honeybee nest in nature.

5.3 Results

5.3.1 The tensile properties of the various comb and scale wax preparations

The tensile strength, strain and stiffness of both native and modified waxes are presented in Table 5.1. Deformation was performed in the surface plane at an

ambient temperature of 23 °C. All waxes were shown to be planar isotropic, except for virgin wax scales, which could only be tested along their greatest length. Results are expressed as mean ± standard error of the mean (SEM), and for each value given, n equals 6.

In Table 5.2, the mechanical parameters observed for all six wax preparations are compared. Statistical analyses was carried out using the Student's t-test, and a P value of less than 0.05 was accepted as significant.

5.3.2 Temperature relations

In Figure 5.1, the mechanical behaviour of white, newly constructed cell walls and virgin wax scales is expressed as a function of temperature. The influence of temperature on the strength, strain, stiffness and workability of propolis and brown composite comb as compared with freshly constructed comb wax is demonstrated in Figures 5.2.1 to 5.2.4 and Figures 5.3.1 to 5.3.4 respectively. Finally, the tensile properties of honeybee cocoon silk over the temperature range 25 to 45 °C are presented in Figure 5.4. For completeness, all three series of recorded values are tabulated and appear in the Appendix (Tables 5.A.1. 5.A.2 and 5.A.3). For each value (mean ± SEM), n equals 6.

5.3.3 The contribution made by silk content to the behaviour of the composite comb

The mechanical behaviour of both individual comb cell walls and whole brood combs of differing silk content is shown in Figures 5.5 and 5.6 respectively. All tensile tests were performed at 20 °C, and for each point, n equals 6. Means are given with SEM. Again, this data is presented in table form in the Appendix (Tables 5.A.4 and 5.A.5).

Table 5.1 Tensile properties of the wax preparations.

Wax sample	Strength (MPa)	Strain (%)	Stiffness (MPa)
Virgin scales (native)	1.488 ± 0.178	61.3 ± 9.8	2.578 ± 0.329
Virgin scales (sheeted)	2.050 ± 0.136	56.7 ± 1.8	3.593 ± 0.269
Virgin scales (soxhletted)	1.868 ± 0.036	85.6 ± 4.3	2.202 ± 0.086
Comb wax (native)	1.455 ± 0.147	35.5 ± 3.2	4.175 ± 0.425
Comb wax (sheeted)	1.111 ± 0.068	23.5 ± 2.4	4.855 ± 0.446
Comb wax (soxhletted)	1.286 ± 0.184	37.6 ± 1.6	3.366 ± 0.406

For each value, n = 6

Table 5.2.1 Statistical comparison of tensile strength.

Wax Sample	Strength					
	A	B	C	D	E	F
Virgin scale wax:						
A Native	+	–	–	–	–	
B Sheeted		–	+	+	+	
C Soxhletted			+	+	+	
Comb wax:						
D Native				–	–	
E Sheeted					–	
F Soxhletted						

Significance is defined as $P < 0.05$
+ = significant, – = not significant

Table 5.2.2 Statistical comparison of tensile strain.

Wax Sample	Strain					
	A	B	C	D	E	F
Virgin scale wax:						
A Native		–	+	+	+	+
B Sheeted			+	+	+	+
C Soxhletted				+	+	+
Comb wax:						
D Native					+	–
E Sheeted						+
F Soxhletted						

Significance is defined as $P < 0.05$

+ = significant, – = not significant

Table 5.2.3 Statistical comparison of stiffness.

Wax Sample	Stiffness					
	A	B	C	D	E	F
Virgin scale wax:						
A Native		+	−	+	+	−
B Sheeted			+	−	+	−
C Soxhletted				+	+	+
Comb wax:						
D Native					−	−
E Sheeted						+
F Soxhletted						

Significance is defines as $P < 0.05$

+ = significant, − = not significant

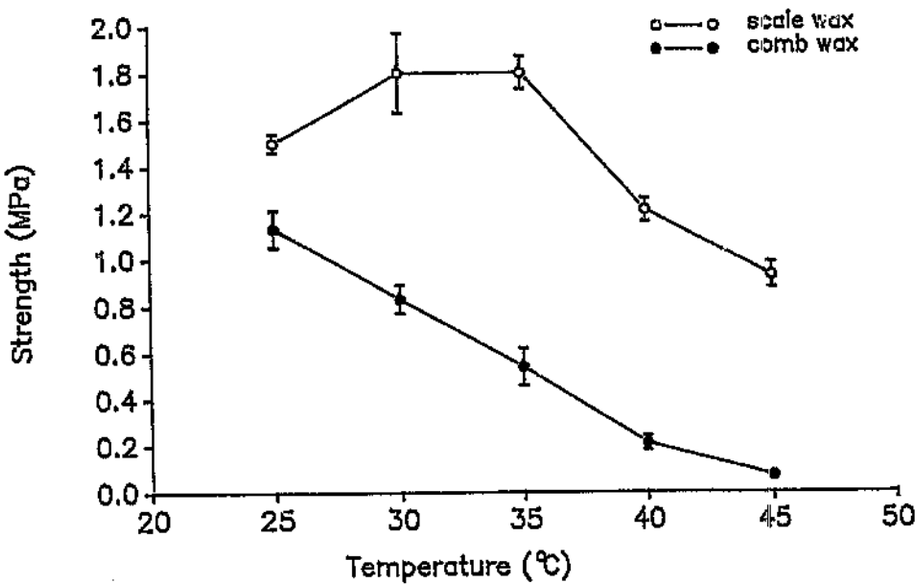


Figure 5.1.1 Tensile strength of scale and comb wax over the range 25 to 45 °C.

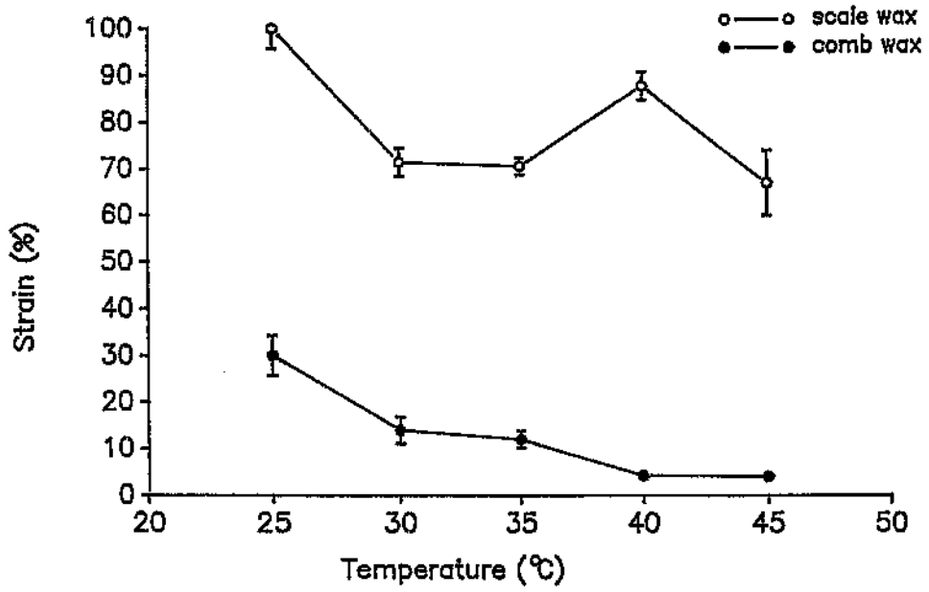


Figure 5.1.2 Breaking strain of scale and comb wax over the range 25 to 45°C.

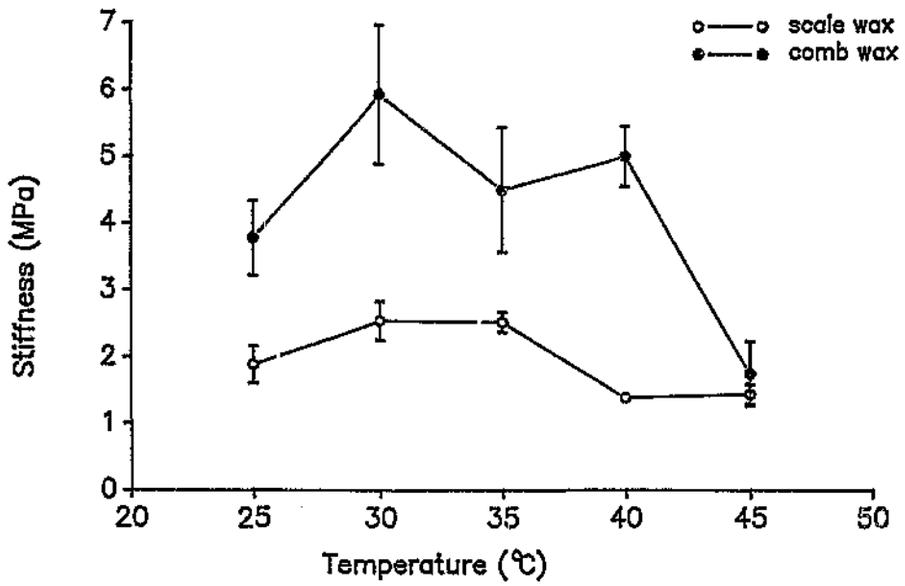


Figure 5.1.3 The stiffness of scale and comb wax over the range 25 to 45 °C.

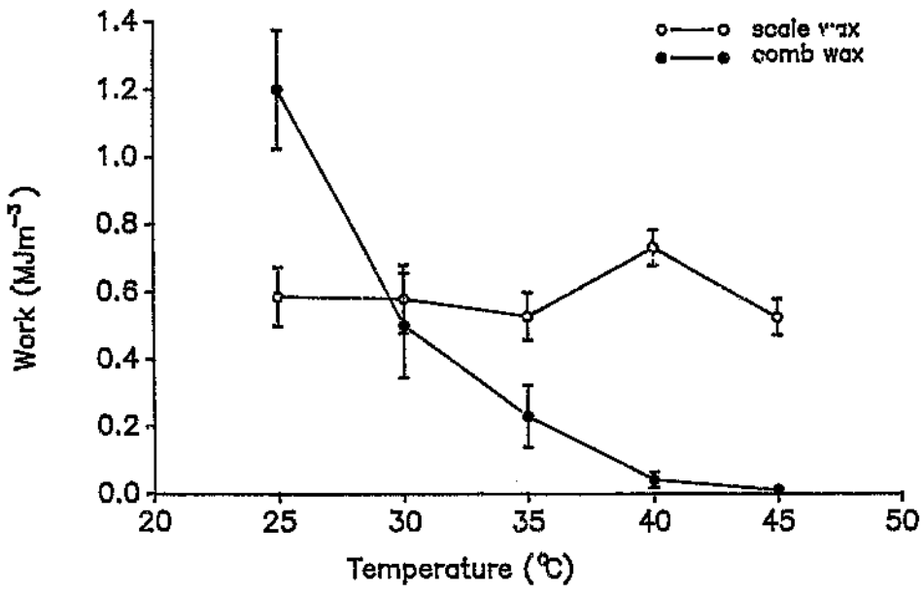


Figure 5.1.4 The work to fracture of scale and comb wax over the range 25 to 45°C.

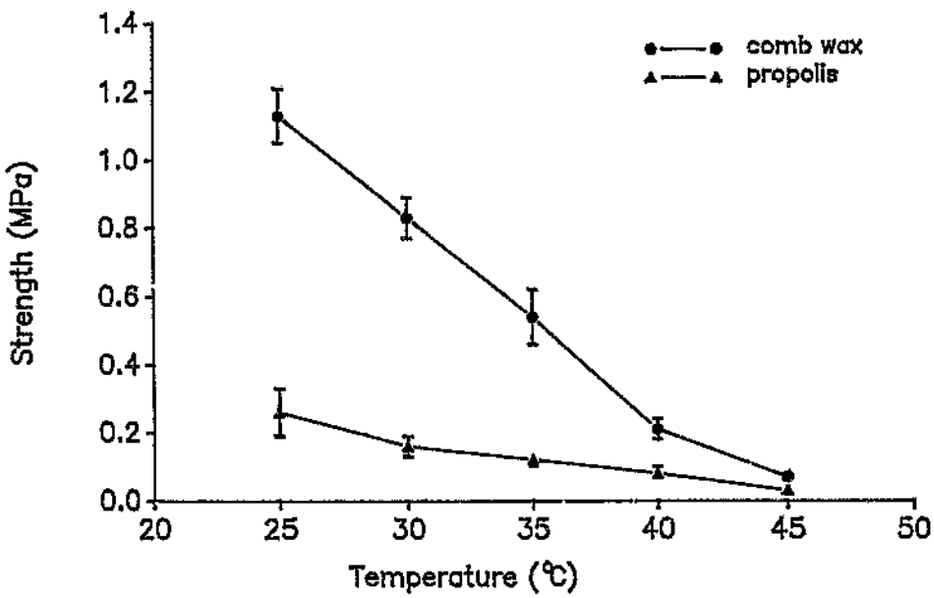


Figure 5.2.1 Tensile strength of propolis and comb wax over the range 25 to 45 °C.

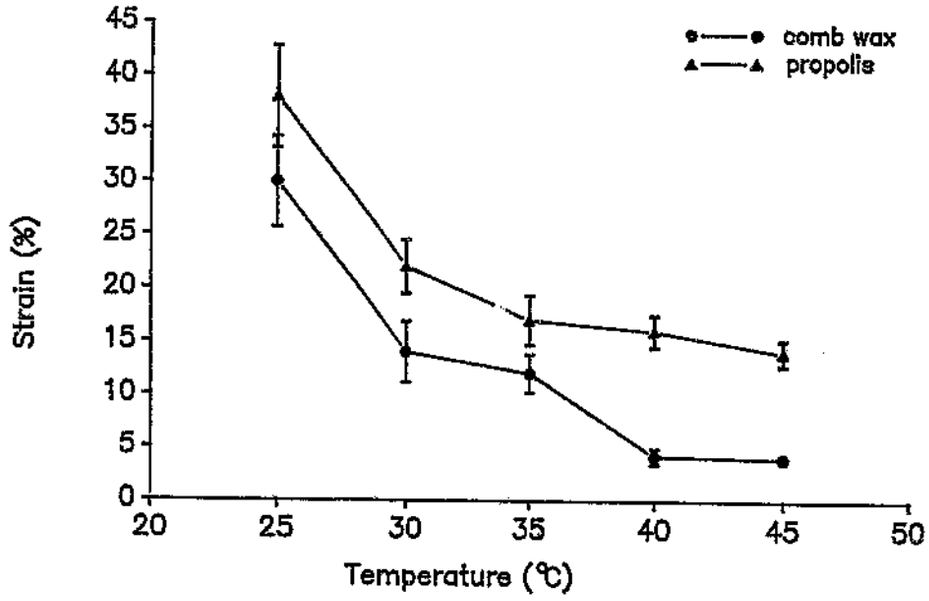


Figure 5.2.2 Breaking strain of propolis and comb wax over the range 25 to 45 °C.

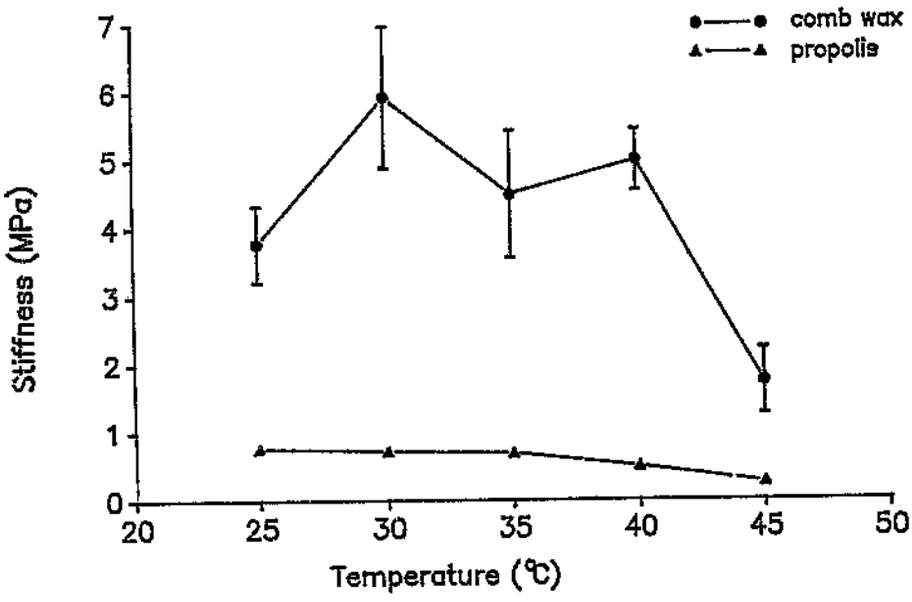


Figure 5.2.3 The stiffness of propolis and comb wax over the range 25 to 45 °C.

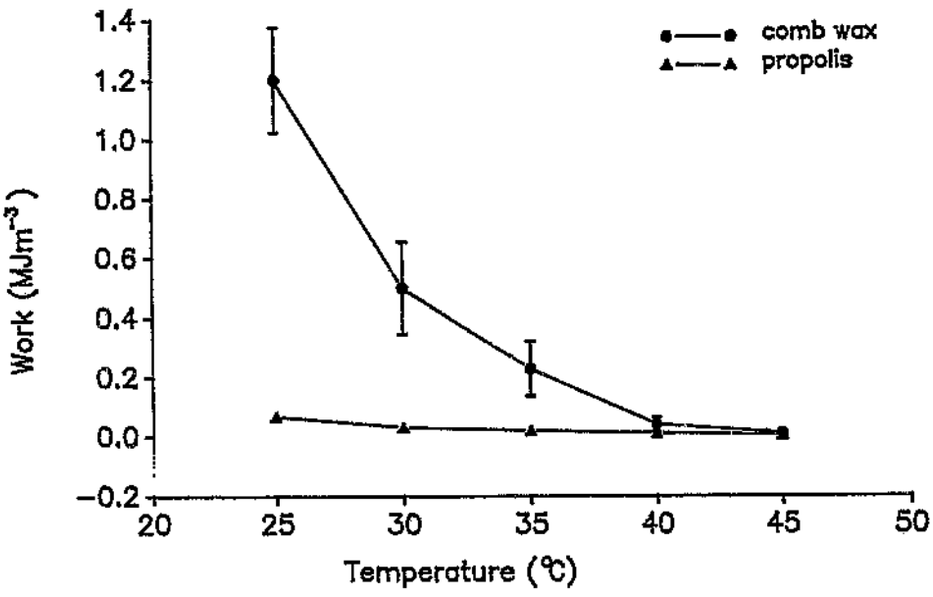


Figure 5.2.4 The work to fracture of propolis and comb wax over the range 25 to 45 °C.

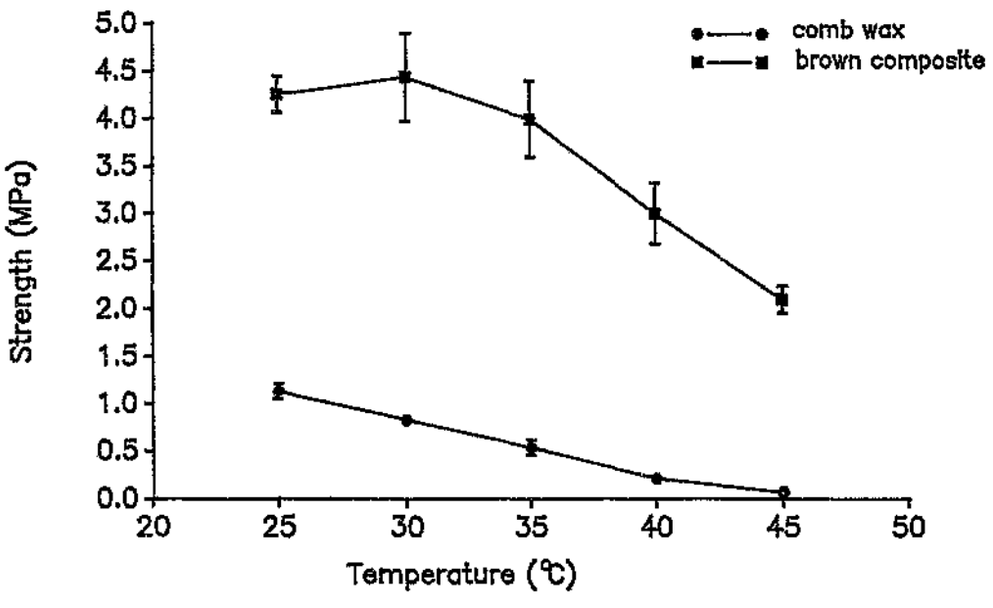


Figure 5.3.1 Tensile strength of brown, composite wax and comb wax over the range 25 to 45 °C.

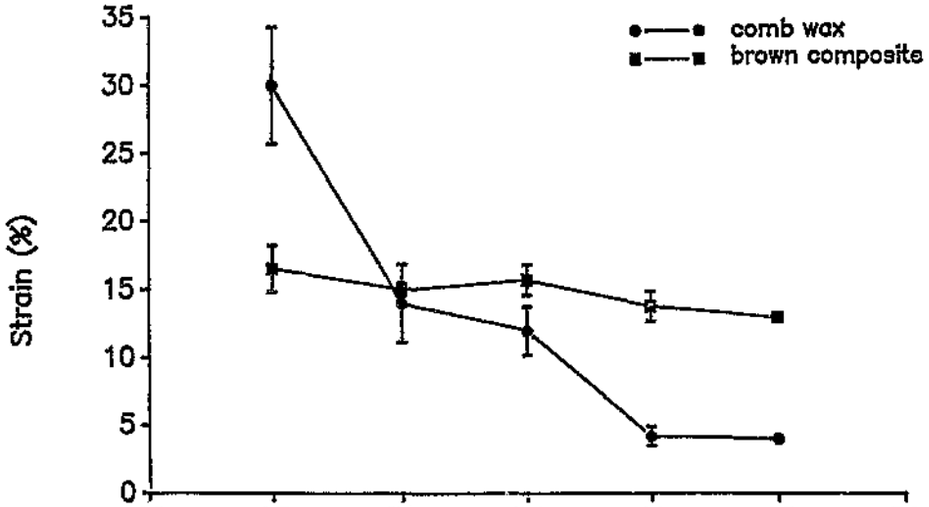


Figure 5.3.2 Breaking strain of brown, composite wax and comb wax over the range 25 to 45 °C.

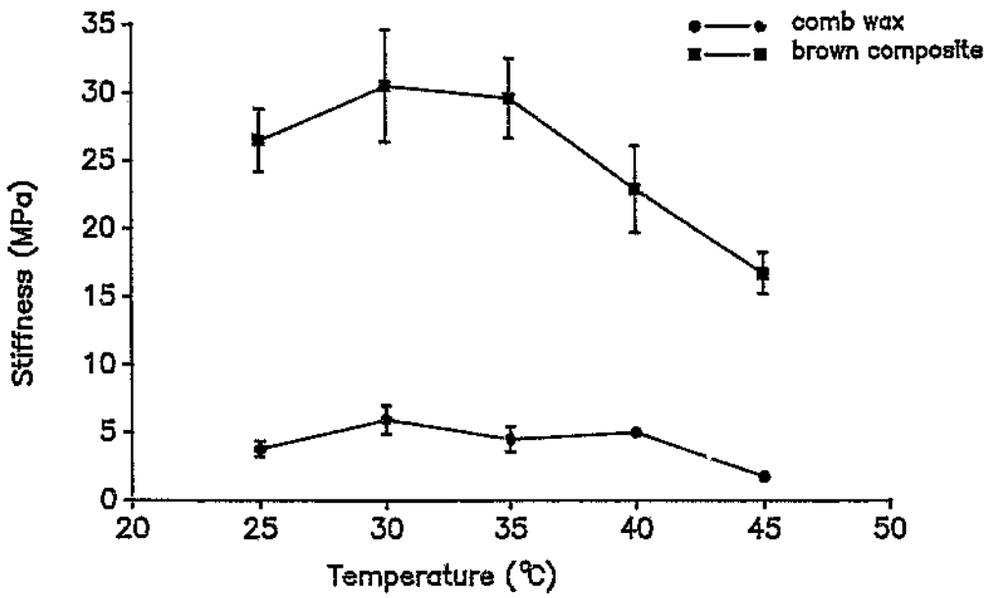


Figure 5.3.3 The stiffness of brown, composite wax and comb wax over the range 25 to 45 °C.

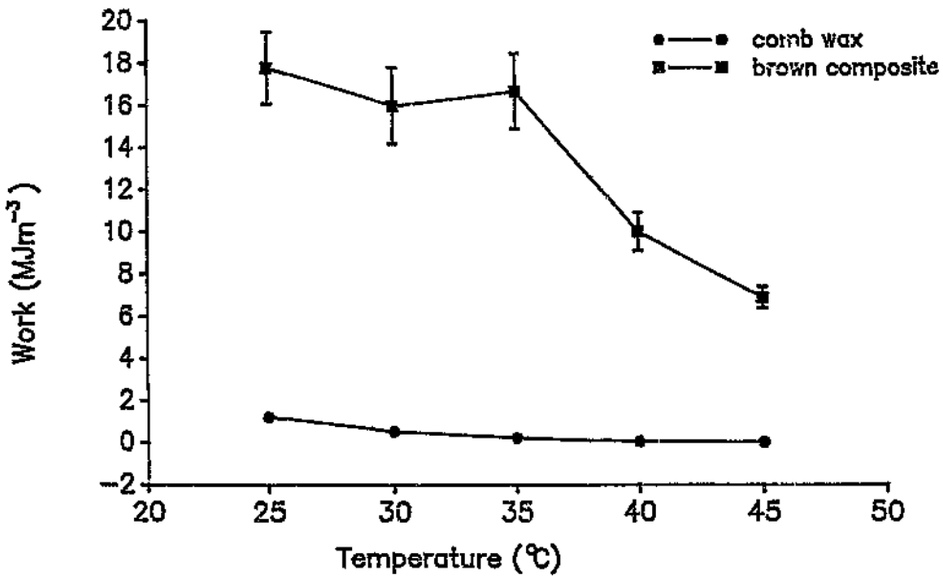


Figure 5.3.4 The work to fracture of brown, composite wax and comb wax over the range 25 to 45 °C.

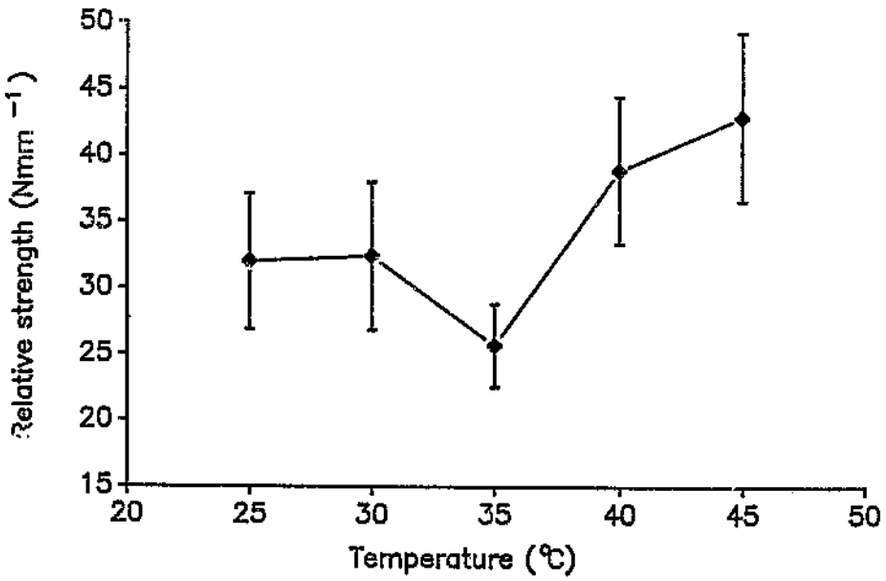


Figure 5.4.1 The relative strength of cocoon silk over the range 25 to 45 °C.

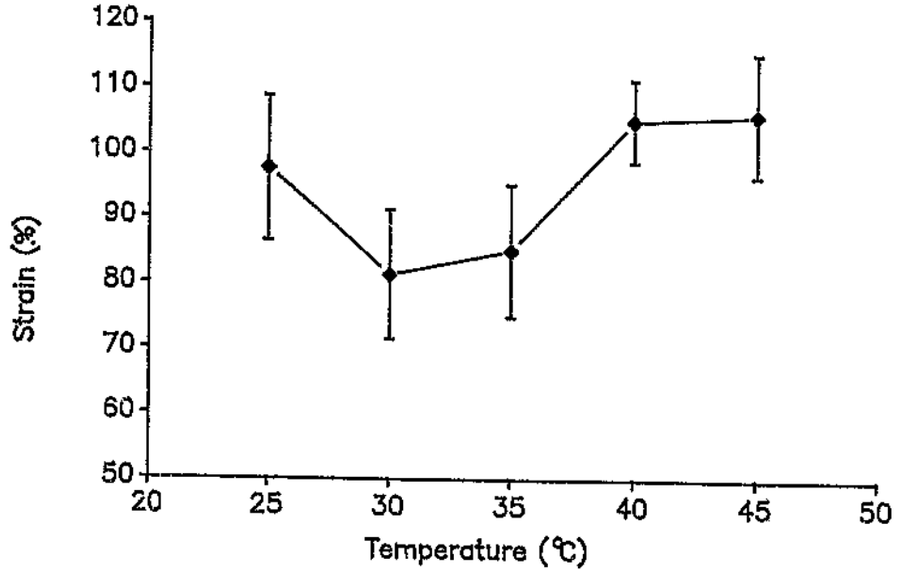


Figure 5.4.2 Breaking strain of cocoon silk over the range 25 to 45 °C.

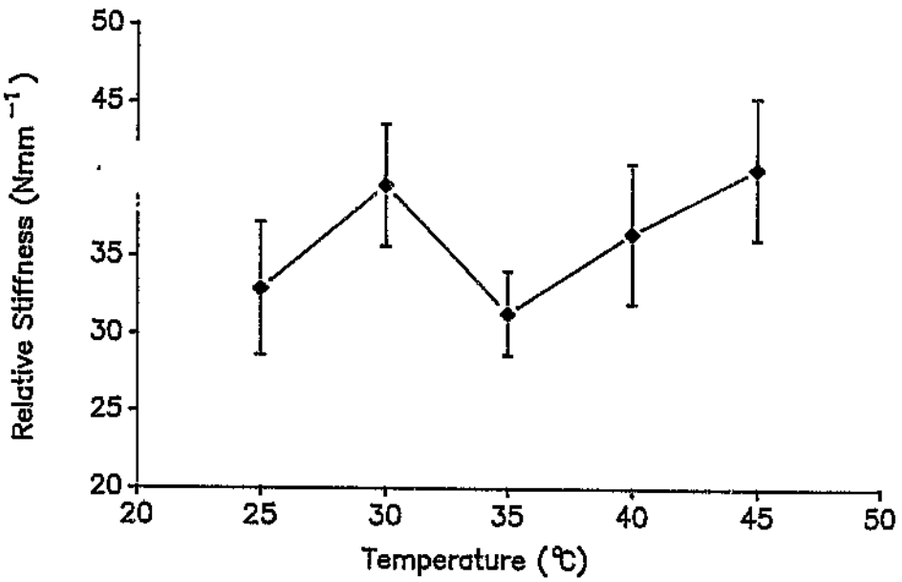


Figure 5.4.3 The relative stiffness of cocoon silk over the range 25 to 45 °C.

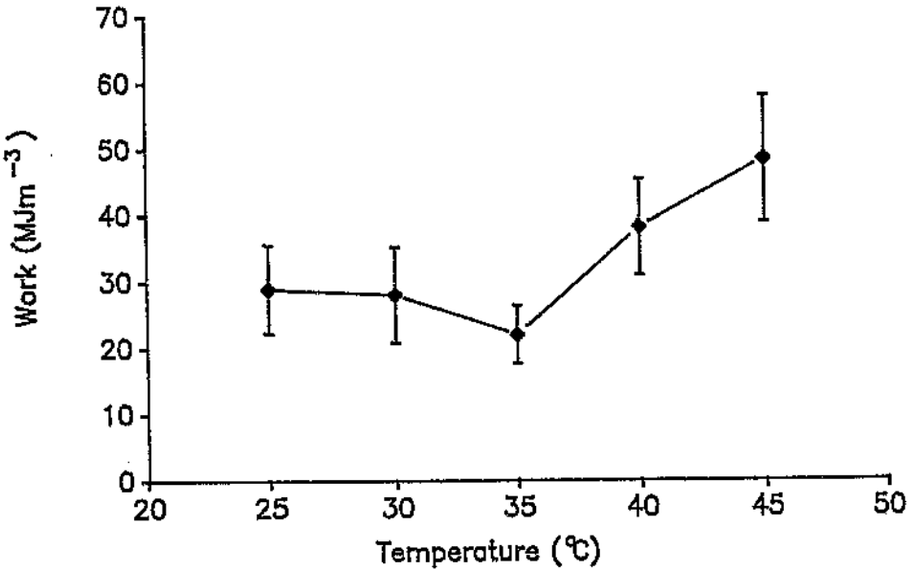


Figure 5.4.4 The work to fracture of cocoon silk over the range 25 to 45 °C.

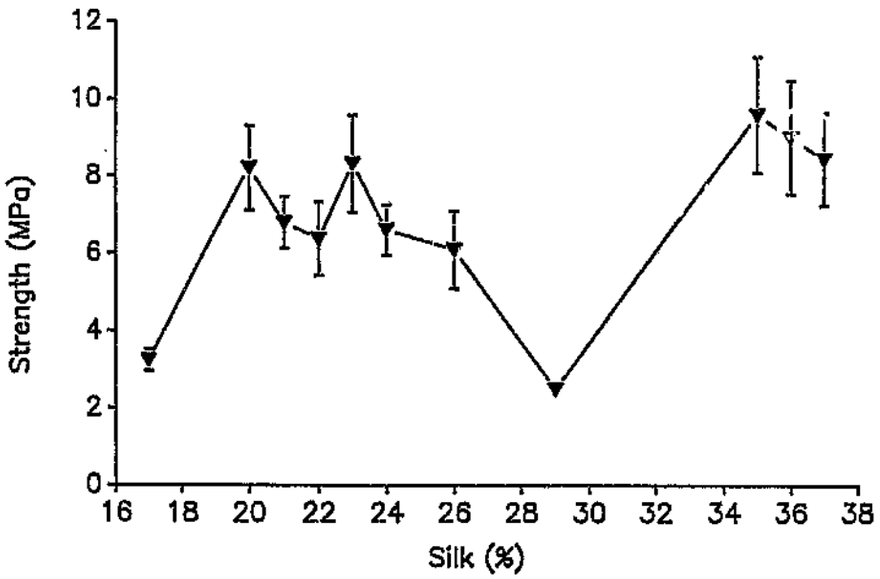


Figure 5.5.1 Tensile strength of individual comb cell walls of differing silk content.

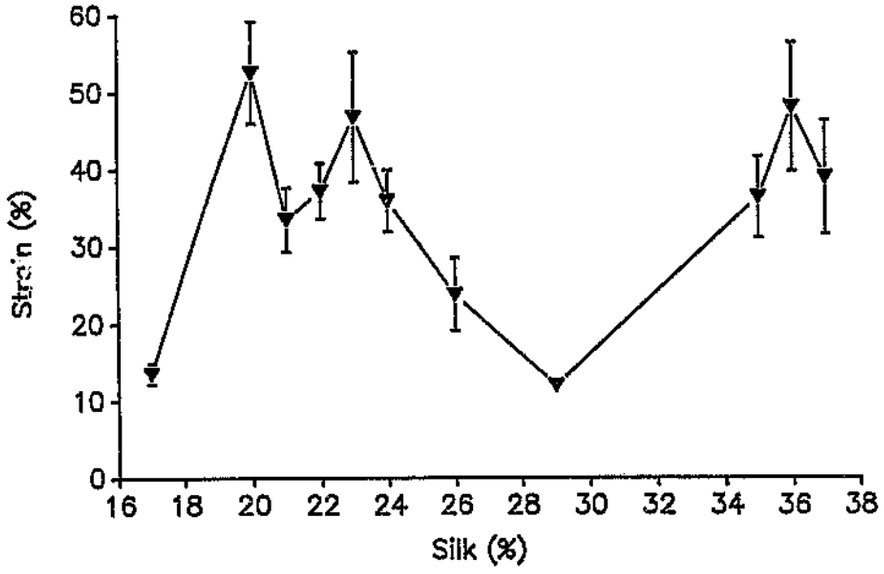


Figure 5.5.2 Breaking strain of individual comb cell walls of differing silk content.

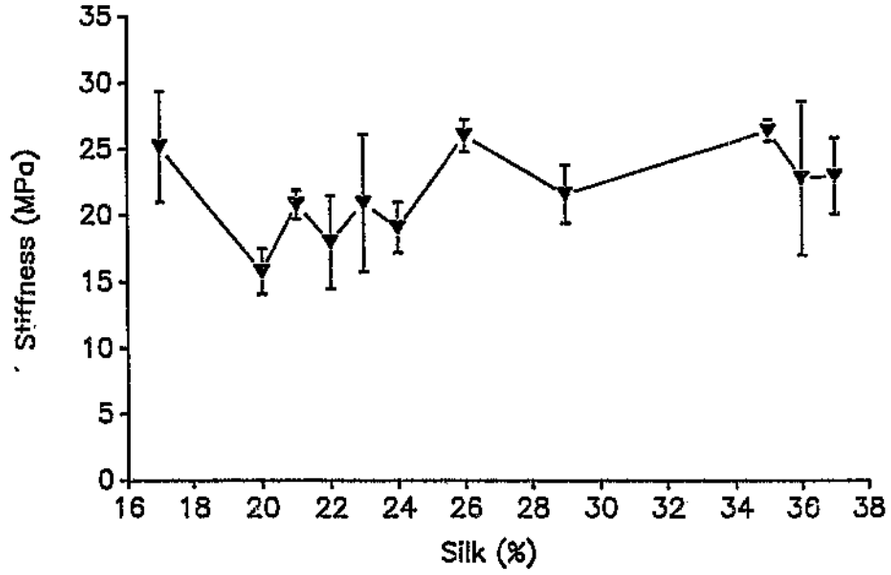


Figure 5.5.3 Stiffness of individual comb cell walls of differing silk content.

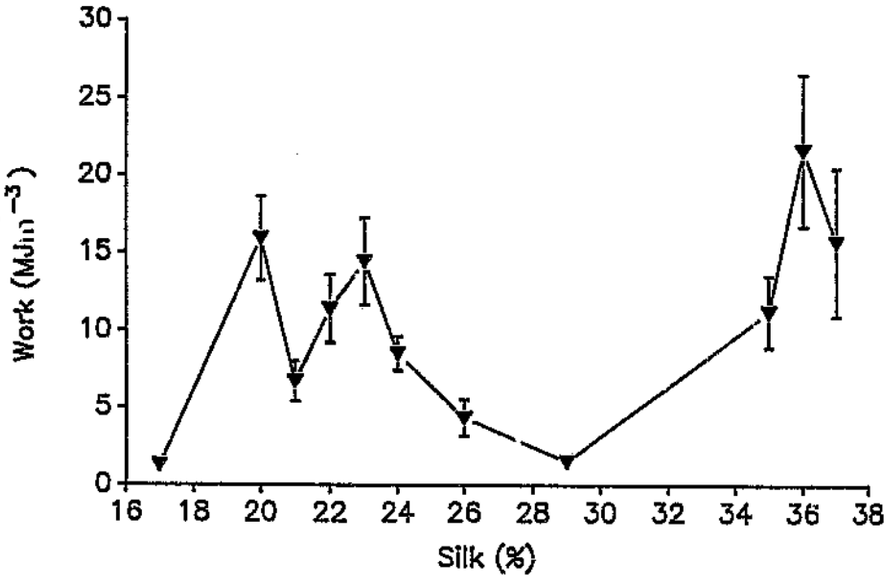


Figure 5.5.4 Fracture energy of individual comb cell walls of differing silk content.

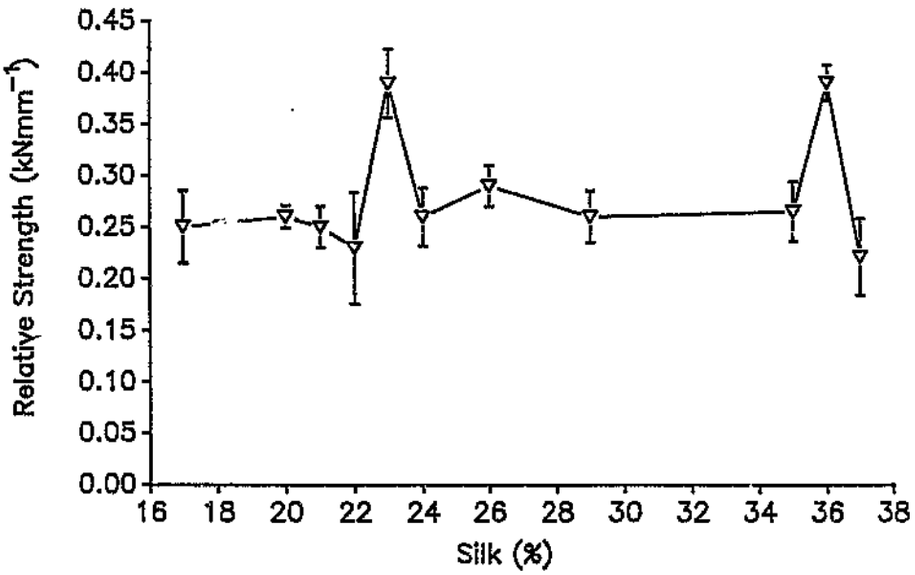


Figure 5.6.1 Tensile strength of whole brood combs of differing silk content.

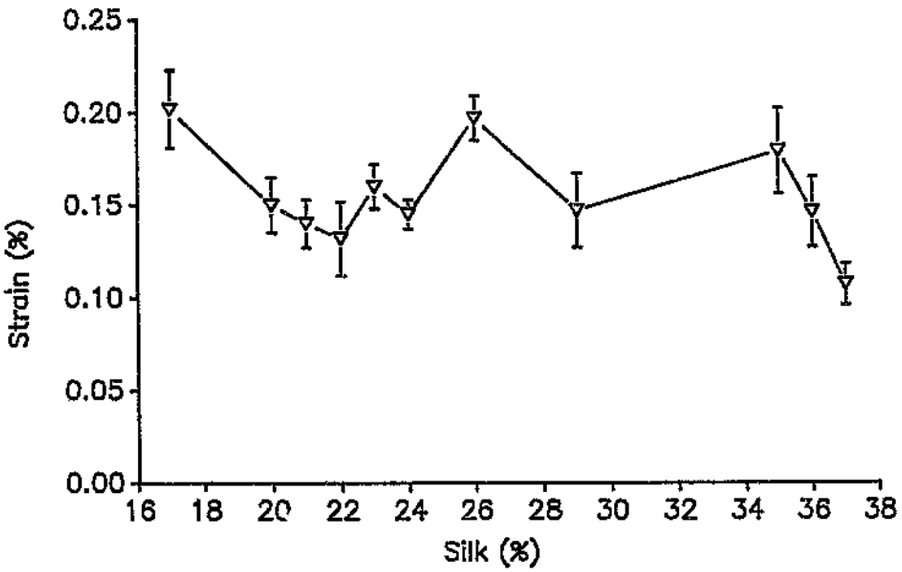


Figure 5.6.2 Breaking strain of whole brood combs of differing silk content.

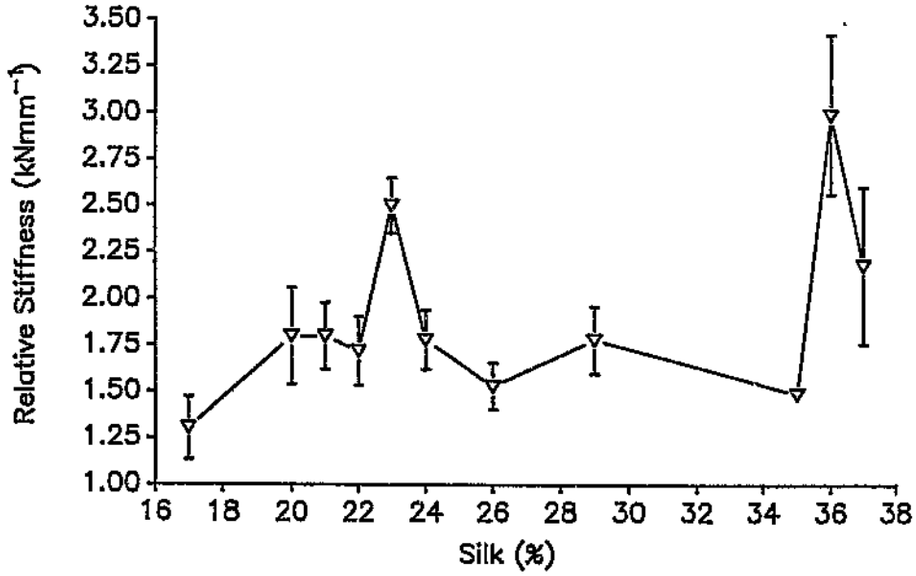


Figure 5.6.3 Stiffness of whole brood combs of differing silk content.

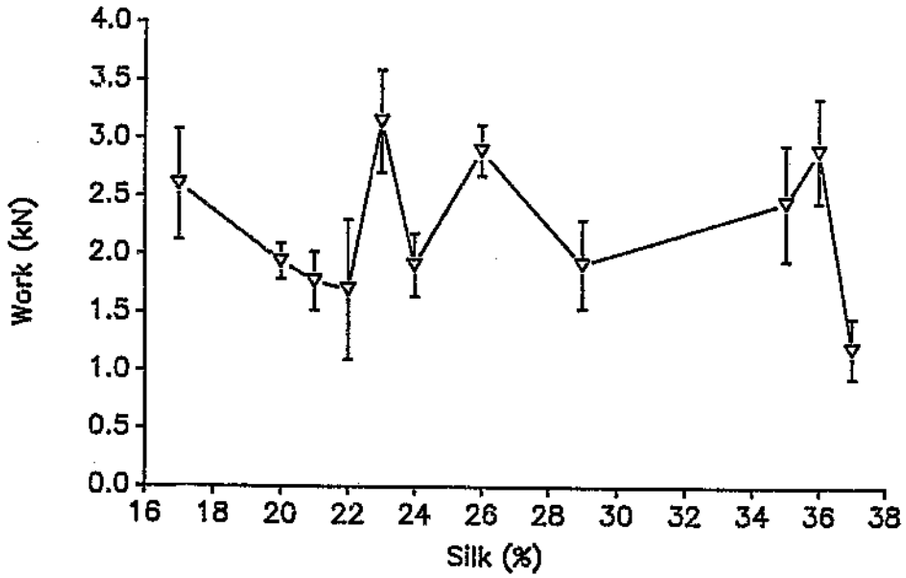


Figure 5.6.4 Fracture energy of whole brood combs of differing silk content.

5.4 Discussion

5.4.1 The transformation of virgin wax scales into comb wax

In the process of comb construction, wax scales are thoroughly masticated in the presence of a frothy salivary secretion (Huber, 1814; Casteel, 1912). This manipulation imparts an alteration in both the texture and chemical nature of the scale wax.

Crystallographic analyses (Chapter 2) revealed virgin wax scales to be highly textured. The molecular c-axes of the crystallites are arranged both perpendicular and inclined at 62 to 65 degrees to the planar surface. Freshly constructed comb wax, on the other hand, exhibits no diffraction texture, implying a random crystallographic arrangement. The mechanical significance of this textural rearrangement can be sought in a comparison of the tensile properties of native and sheeted virgin scales (Tables 5.1 and 5.2).

In the course of sheeting (Appendix 5.A.2), a negligible degree of alignment of the wax crystallites occurs (Chapter 2). Indeed, X-ray analyses demonstrated the absence of crystallographic texture in all four sheeted wax

preparations. The significant difference in both strength and resistance to deformation (stiffness) between sheeted and native scales is thus a function of textural variation alone. In tensile testing, the native scales are loaded normal to the c-axes of their crystallites, which flow passively in the amorphous matrix of the scale contributing little to strength or stiffness. Sheeted virgin wax, however, comprises a random crystallite orientation, in which a proportion have their molecular axis aligned in the direction of loading. In addition, the relatively slow extension rate may promote further recruitment 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).

Unlike scale wax, native comb lacks texture. It does, however, possess a loosely particulate structure (Casteel, 1912), the significance of which is seen in a comparison of native cell walls and sheeted comb wax. While both are planar isotropic and of equal strength and stiffness (Table 5.1 and 5.2), they differ significantly in extensibility. The greater extensibility of native comb wax suggests that there is an incomplete fusion of the new pieces as they are added to the comb by the bees in building.

The extent to which mastication alters the chemical composition of scale wax in its conversion to comb may now be considered. The possibility of

modifications in both lipid and non-lipoidal (proteinaceous) constituents are examined. By comparing sheeted preparations of Soxhlet-extracted comb wax and virgin scales, a difference in lipid composition is revealed. This is facilitated by the very nature of the specimens tested; possible contributions from variatic in texture and non-lipoidal constituents are eliminated by sheeting and Soxhlet-extraction respectively. The results (Tables 5.1 and 5.2) show that Soxhletted virgin scale wax is both stronger and more distensible then the comb equivalent. The former is consequently not as stiff as the latter.

The presence of differing quantities and species of proteins in virgin and comb wax might well effect the structural properties of the waxes. This possibility can be assessed in a comparison of Soxhlet and sheeted waxes. Here the preparations are matched for texture and lipid composition. Sheeted scale wax is significantly stiffer but less distensible than Soxhletted scales (Tables 5.1 and 5.2). Identical trends are demonstrated in a comparison of sheeted and Soxhlet-extracted comb wax (Tables 5.1 and 5.2). The incorporation of protein into both wax fractions therefore appears to markedly improve their resistance to deformation.

The precise nature of these chemical alterations involved in the metar phosis of wax scales are detailed in Chapters 3 and 4.

The overall implications of these changes in texture and chemical composition may now be assessed, and the resultant tensile properties of the native waxes compared. Virgin wax scales are highly textured, fused laminated structures (Huber, 1814; Philipp, 1935; Jordan, 1962) which, by process of mandibulation, are converted into texturally isotropic cell walls, thus increasing both strength and resistance to deformation. The incorporation of a salivary secretion into the wax further serves to greatly augment the stiffness of the final product, by altering both the basic lipid content as well as further supplementing the protein fraction.

For the bee, these alterations in mechanical properties are of great significance. While native wax scales are ideal moulding materials due to their high distensibility and relatively low stiffness (Tables 5.1 and 5.2), these same properties make them unsuitable structurally. Comb wax, on the other hand, with its significantly greater resistance to forces of deformation and considerably lower potential for extension (Tables 5.1 and 5.2) is a superior structural material.

The relevance of this transition in mechanical behaviour is further illustrated when considering the tensile properties of comb and scale wax over the range of temperatures likely to impinge on the honeybee nest (Figure 5.1, Table 5.A.5). The tensile strength of comb wax decreases almost linearly with incremental increase in temperature (correlation coefficient = -0.99). Virgin

wax scale strength, although virtually constant between 25 and 35 °C, declines markedly from 35 to 45 °C. Over the entire range of temperatures comb wax is considerably weaker than wax scales (Figure 5.1.1).

In a comparison of extensibility (Figure 5.1.2) scales flow between three and six times more readily than comb above 35 °C, below which there exists an average 16-fold difference. This variation is due to the exponential reduction in comb strain (exponential correlation coefficient = -0.97) while scale wax remains in the range of 70 to 100 % elongation. The stiffness, or resistance to deformation, of the waxes also differs markedly (Figure 5.1.3). Comb wax is on average twice as stiff as scales over the order of temperatures at which they were tested.

Finally, the energy expended in working comb and scale wax may be considered (Figure 5.1.4). Over the range 25 to 45 °C, the work required to manipulate wax scales remains relatively constant. With comb wax, however, the ergonomic effort required decreases radically with increase in temperature (exponential correlation coefficient = -0.99).

Hunter (1792) first demonstrated that honeybee colonies actively regulate nest temperature. He speculated that the warmth generated by the bees kept the wax soft enough as to allow them to model it with ease. Although a temperature of 35 °C has been suggested as essential for wax secretion and

comb construction (Weiss, 1965), core temperatures of between 30 and 34 °C have been recorded in clusters of building bees (Darchen, 1962; Hepburn et al., 1984).

At "optimal" nest temperature (35 °C) wax scales are stronger, more distensible but less stiff then comb wax. The energy required to work the scale wax is, however, over twice that needed to manipulate comb. This finding may, in part, explain the vast extent to which previously constructed comb is reworked (Darchen, 1980).

In conclusion, these temperature profiles highlight a delicate balance between the structural properties of the waxes and the environmental constraints of the wax worker. At temperatures in excess of 40 °C, the advantage of conserving energy in comb construction is negated by compromising the mechanical stability of the nest and exposing the bees to lethal temperatures (Free and Spencer-Booth, 1958). At lower temperatures, however, the cost of wax manipulation is prohibitive.

5.4.2 The comb as a composite material

The last few days of honeybee larval life are spent constructing a cocoon within the cell. The main substance used in the cocoon is silk secreted from

what will later become the thoracic salivary glands. To spin the cocoon, the larvae uncurl and stretch out fully in the cells with their heads toward the capped end and begin weaving with their spinnerets (Jay, 1963). Dark brown faeces as well as a lighter-coloured substance from the excretory tubules make up most of the other materials used in cocoon construction (Jay, 1964).

Honeybee silk is an alpha-helical fibroin (Rudall, 1962) the micelles of which form a four-stranded array of coiled-coils parallel to the fibre axis (Atkins, 1967). The honeybee fibroin is crystalline relative to other insect silks (Lucas and Rudall, 1968) but the hydrated fibre is only half as stiff as when dry, although they are equal in strength (Hepburn et al., 1979). The fibroin is hygroscopic and when solvated is highly distensible largely owing to its conformation (Lucas and Rudall, 1968). These structurally undesirable properties of the fibroin are substantially suppressed by the cocoon-spinning larvae.

The fact that the silk is impacted in the wax of the cell wall, possibly aided by the anal secretions, immediately checks the susceptibility of fibroin to solvation. Thus it is likely that inter-micellar friction is enhanced (Warwicker, 1960) and the conformational change restricted (Rudall, 1962), effects consistent with a good stiffness and reduced distensibility (Hepburn et al., 1979). The silk fibres are spun and randomly arranged in the cell wall, thus

overcoming the basic anisotropy of the material : dewaxed sheets of cocoon silk are planar isotropic on tensile deformation.

The tensile properties of dewaxed sheets of honeybee cocoon silk, deformed in tension at a fixed rate between 25 and 45 °C are presented in Figure 5.4 and Table 5.A.2. The mechanical behaviour of the honeybee fibroin appears independent of variation in temperature. Sheets of silk thus maintain the same relative strength and distensibility (Figures 5.4.1 and 5.4.2). Consequently, no variation in stiffness (Figure 5.4.3) or energy required to fracture the sheet (Figure 5.4.4) is observed over the range of temperatures.

Newly formed combs of white wax soon yellow, pass through shades of brown and finally become almost black with age. Huber (1814) suggested that white and yellow combs differed mechanically and that propolis (not the source of yellowness), added to wax, colours it darkly.

Propolis is the general name for the resinous material gathered from plants by honeybees. Besides its purely mechanical role, as in cementing and strengthening the comb bases and sealing cracks in the nest wall, propolis is also suggested to provide colonial defense against infection and mould (Ghisalberti, 1979; Grange and Davey, 1990).

Inasmuch as propolis is a common constituent of dark combs (Chauvin, 1962) its contribution toward their final extensometric properties should be evaluated by considering the material in isolation (Figure 5.2, Table 5.A.3). Propolis exhibits an unusual behaviour on tensile deformation : there is a linear relationship between load and elongation from the origin of the curves to the maximum sustainable load. Thus the yield stress coincides with the ultimate strength of the material. But propolis, like beeswax is an entirely plastic material in the range 25 to 45 °C so that this linearity is not an elastic one. On yield, propolis is highly ductile and flows about 200 % before the necking thread finally fails.

The tensile strength of propolis decreases 6-fold over the range of temperatures tested (Figure 5.2.1), and the yield strain some 3-fold (Figure 5.2.2). These temperature dependent trends, linear for stress (correlation coefficient = -0.979) and exponential for strain (exponential correlation coefficient = -0.94), are similar to those exhibited by newly constructed white comb. The stiffness of propolis remains virtually constant at lower temperatures, with a major transition between 35 and 45 °C (Figure 5.2.3). Finally, the energy required for moulding decreases exponentially (exponential correlation coefficient = -0.99) with increasing temperature (Figure 5.2.4). On a comparative basis, over the range of temperatures tested propolis at its strongest just matches beeswax at its all but weakest (Figure 5.2.1).

In summary, the mechanical integrity of white comb wax (Figure 5.1) and propolis (Figure 5.2) is diminished with increasing temperature. These properties are in contrast to those of honeybee silk (Figure 5.4) which appear temperature independent within the range tested.

Finally the net contributions of the plastic (wax and propolis) and elastic (silk) fractions may be considered by examining the characteristics of mature combs.

The tensile behaviour of brood comb, a two-phase composite material, is well illustrated by comparing the walls of silk-free white comb with similar specimens of brood comb having a substantial amount (34 %) of silk (Figure 5.3, Table 5.A.3). Between 25 and 45 °C, both preparations decrease in strength and very rapidly so, near 40 °C (Figure 5.3.1). The fibre reinforcement of silk in the propolis-bearing brown comb is an order of magnitude stronger than white wax alone at all of the assay temperatures. On average, the distensibility of reinforced comb is greater than that of white wax (Figure 5.3.2). Combined with the greater loads required to break combs reinforced with silk, this degree of extensibility provides a safety factor of 1 over that of white comb wax.

The contribution of the silk fibre to the composite is also reflected in the initial resistance of the material to deformation. A decrease in stiffness with rising temperature is common to both freshly constructed white wax and the

comb composite (Figure 5.3.3). However, the composite at its least tenacious is still significantly stiffer than white wax at its most unyielding. The energy fracture profile for reinforced comb and white wax (Figure 5.3.4) is similar to that of stiffness. The very rapid rate of decline in the structural integrity of comb wax with rising temperature is thus greatly moderated by reinforcement with fibroin.

The above behaviour of composite comb is specific for specimens of brood comb having 34 % silk by weight. The mass ratio of silk to wax does, however, change with successive generations or brood cycles. Chauvin (1962) showed that in a series of combs ranging from pale yellow to brownish black, the mass of wax per unit area doubled, while that of silk increased from 2 to 42 %. So, while the absolute mass of wax increased over time, the relative amount of wax actually decreased. This occurred in addition to unspecified chemical changes associated with comb colour.

The tensile performance of silk-bearing comb cell walls in relation to changing silk wax ratios are presented in Figure 5.5 (Table 5.A.4). The strength of such combs varies biphasically with change in silk content, peaking at 21 to 23 % and 35 to 37 % (Figure 5.5.1), as does distensibility (Figure 5.5.2). As strength and extensibility change at virtually the same rate with increasing silk content, stiffness remains constant over a range of 17 to 37 % silk content (Figure

5.5.3). However, the energy to fracture profile is similar to that of stress and strain (Figure 5.5.4).

These results suggest that the greater the silk to wax ratio the stronger the cell wall and the greater the energy required to break the material. However, the interactions between silk and wax are difficult to define. The silk fibres are randomly displaced in the plane of the cell wall, and thus there may be large variations in the number of fibrils participating in the deformation process. Indeed, their degree of involvement may depend upon the percentage of silk incorporated, as the biphasic mechanical profiles suggest.

Tests of whole combs provide data (Figure 5.6, Table 5.A.5) whose origins are obscured by complex specimen geometry. In these measurements, the loading vectors differ greatly from those operating on cell walls. In whole combs there is a small biphasic increase in relative strength with increasing silk content at 23 and 36 % (Figure 5.6.1), a trend mirrored in the relative resistance to deformation (Figure 5.6.3). Breaking strain, conversely, appears almost independent of the percentage contribution of silk to the composite (Figure 5.6.2).

These variations in test results of whole comb are associated with gross differences in the fracture behaviour of the failed specimens. Within each silk percentage class, three modes of failure were observed. Some fractures were

propagated intermurally, others intramurally, while a third comprised a combination of both. These modes of fracture are indicative of the gross structural heterogeneity of whole combs, and are further reflected in the fracture energy characteristics of the specimens (Figure 5.6.4).

In conclusion then, brood comb is a composite material consisting of an elastic element, silk, embedded into a plastic one, wax. The native silk of brood comb is a hygroscopic, anisotropic crystalline fibroin which is mechanically constant between 25 and 45 °C. Native wax is a hydrophobic, isotropic and paracrystalline plastic whose strength and rigidity greatly decrease with increasing temperature. Propolis, often added to comb, is mechanically similar to wax but structurally inferior to it.

Intact brood comb is a planar isotropic silk-wax composite material in which the silk acts as a fibre reinforcement that greatly improves the overall mechanical properties of the comb. With increasing use, the percentage of silk present in the wall increases and so enhances the load-bearing capacity of the comb. The mechanical properties of the composite are extremely sensitive to small changes in temperature that naturally occur. At 40 °C the wax phase is a viscous plastic that contributes virtually nothing to the functional composite, so that the latter is compromised to the point of failure. Inasmuch as honeybees die in droves at 40 °C (Free and Spencer-Booth, 1958) the entire nest is thus likely to fail at this temperature. Further, what honeybees may

gain in improved material performance at lower temperatures (less than 35°C), would cost them minimally twice as much in expenditure on construction because of the temperature dependent workability of their materials. In view of the thermal tolerances operative on maker and material, it would therefore appear that both evolved in a mutualism mediated by thermoregulation.

5.A Appendix

5.A.1 Chloroform-Soxhlet extraction of waxes (Mann and Saunders, 1938)

In brief, this process involves purification by steam distillation. The lipid component of wax was isolated in an uncontaminated form by repeated extraction with hot chloroform. For this purpose, Soxhlet's Extraction Apparatus (Figure 5.A.1) was employed.

The extractor consists of a glass cylinder A, terminating at its base in an open tube B. Fused at the top of A is another tube C. This communicates with A through an orifice, D. A fine glass tube leads from the base of C, rises two-thirds of the height of the cylinder to E, and then returns past F into the open tube B.

The wax was placed in a porous, fat-free thimble (Millipore) G which was positioned within inner tube C as demonstrated. The apparatus was then fitted below to a bolt-head flask H containing chloroform, and above to a reflux water condenser J. The solvent in H was then gently boiled.

The chloroform vapour passed up B, through the annular space between tubes A and C, and so through orifice D into the reflux condenser above. Here it condensed and accumulated in the thimble G, slowly filling the body of C. This solution was further heated by the ascending mantle of hot vapour in the surrounding annular space. When the condensed liquid reached the top of the fine glass tube, at point E, it siphoned down into the flask H, taking with it that portion of the wax which it extracted in G.

This process was repeated until complete extraction of the wax was effected. The extracted component was isolated from the chloroform by simply allowing the solvent to evaporate under nitrogen.

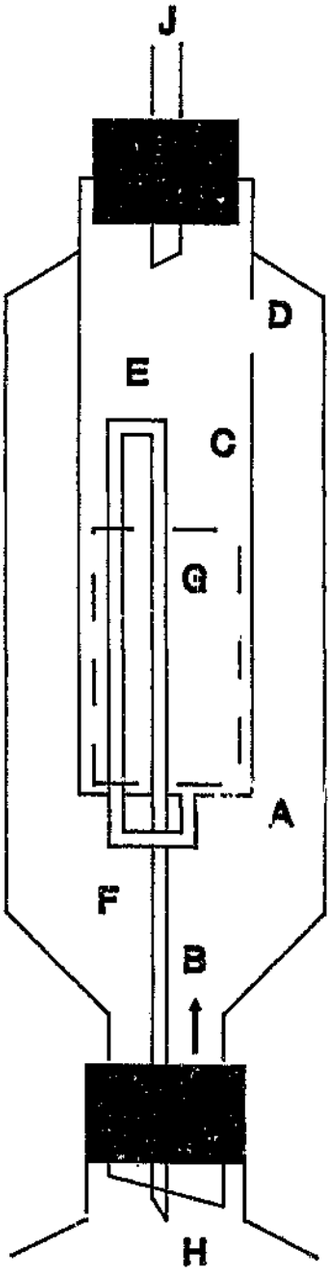


Figure 5.A.1 Soxhlet's extraction apparatus.

5.A.2 Preparation of sheeted wax samples

The sheeted waxes were prepared on a spreader blade coater (Figure 5.A.2). This device is often employed in industry for preliminary investigations in paper coating. Siliconised release paper was used as the substrate. Molten wax was poured from a prewarmed glass pipette into the angle formed by the glass doctor and release paper. The paper was then pulled at a uniform rate in the direction indicated. Constant wax layer thickness was obtained under conditions of maximal paper tension and minimal gape between substrate and glass applicator. The sheeted wax was then fashioned into tensile specimens as described.

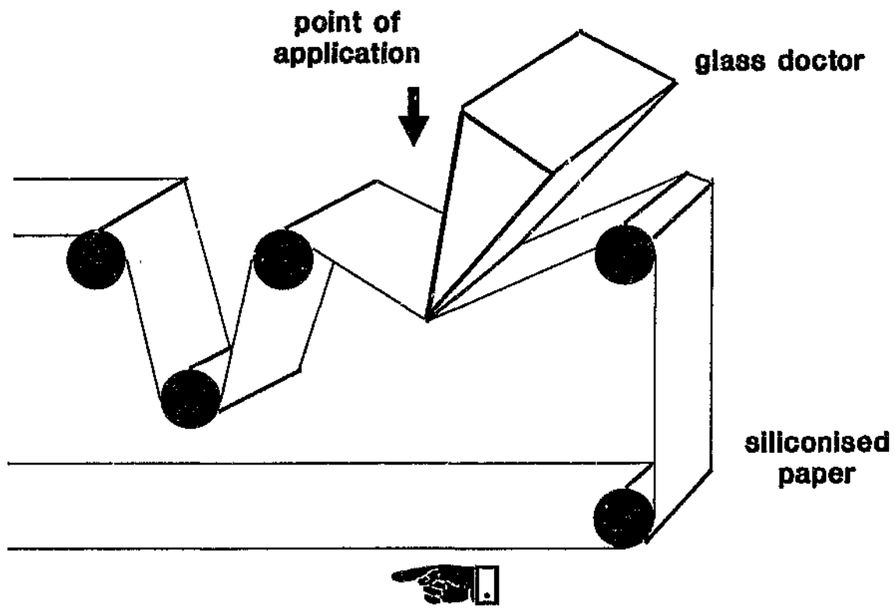


Figure 5.A.2 Spreader blade coater.

5.A.3 The extensometer of Joffe and Hepburn (1974)

This instrument consists of a straight optical bench of triangular cross-section. A steel plate carrying an electrically driven micrometer screw system is attached to one end of the optical bench, and a saddle bearing a force transducer is free to move over the remaining length.

A reversible synchronous motor drives a screw that is firmly supported between two thrust bearings. These allow the screw to turn relatively freely while permitting negligible lateral displacement. The extension of the specimen is produced by the action of this screw on one of the cross-members of a supporting frame. Displacement is measured using a linear variable differential transformer whose plunger is mounted to an adjustable arm clamped to the sliding frame. The body of the transformer is mounted on a carriage attached to a micrometer screw gauge, thus allowing the zero of the system to be set at any point. The rate of extension is adjustable from 7 μm/minute to 3 500 μm/minute and calibration is affected by means of a built in dial gauge with a 1 μm resolution.

The force transducer is rigidly mounted on a brass plate attached perpendicularly to the sliding saddle. The mounting holes in this plate are slotted so that accurate alignment of the system is possible.

The outputs from both force and displacement transducers are fed into coherent amplifiers and a direct current voltage is obtained which is accurately proportional to the property being measured. These, in turn, are displaced on a suitable x-y plotter (Figure 5.A.3).

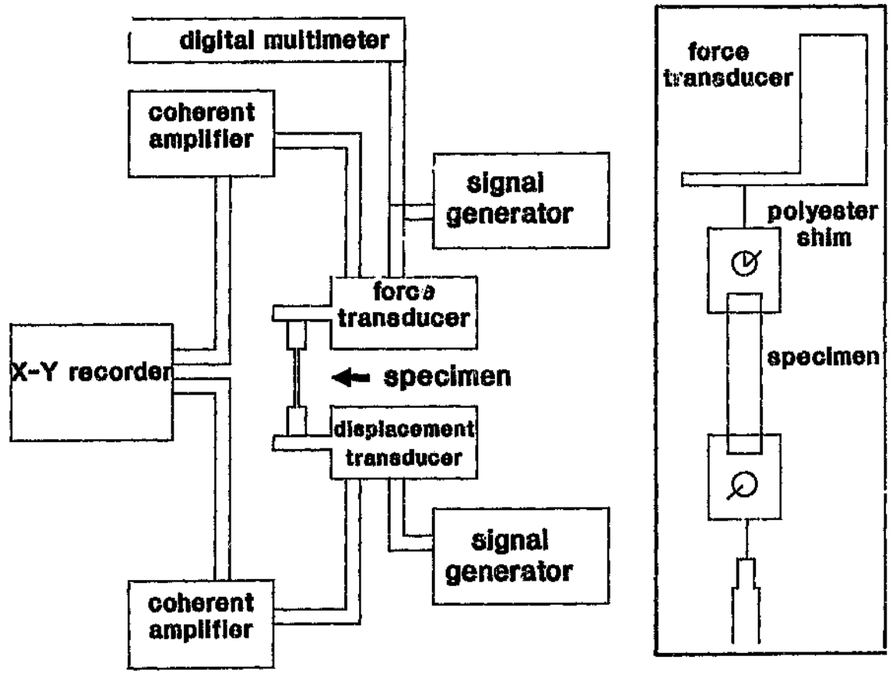


Figure 5.A.3 Schematic of the extensometer of Joffe and Hepburn. Inset: wax specimen mounted on polyester shims.

5.A.4 Conversion of recorded data to engineering values (Hepburn and Chandler, 1980)

When a force is applied to a body, the body usually responds by deforming. Clearly, the degree of deformation is a function of both the size and shape of the body. Force is therefore usually described in terms of the cross-sectional area over which it acts. This is stress (σ). Formally it may be defined as the deforming force (or tensile load) F divided by the cross-sectional area A_o of the body perpendicular to the direction of the applied force:

$$\sigma = F/A_o \quad \text{(units: N mm}^{-2}\text{, or MPa)}$$

Engineering stress differs from true stress in that only the original cross-sectional area is considered in the calculations. Since many materials may undergo large extensions prior to fracture, the cross-sectional area may vary considerably. Ideally then, the area existing during the deformation process should be considered.

The deformation produced in a body due to external loading is expressed as strain (ϵ). This value too is expressed relative to original body proportion.

Thus, the engineering strain is defined by the ratio of change in length (Δl) to original length (l_o):

$$\epsilon = \Delta l / l_o \quad \text{(units: \%, or dimensionless)}$$

Again, the more precise description of strain, true strain, relates the change in length to the current specimen length.

For most materials, the application of a small stress produces a correspondingly small strain which reverses itself exactly when the applied force is removed. Such behaviour is termed elastic. In materials exhibiting linear elastic behaviour, stress is proportional to strain:

$$\sigma = E.\epsilon \quad \text{or} \quad E = \sigma / \epsilon$$

The proportionality constant E is called the elastic modulus, or Young's modulus, and has the units of stress. For specimens of uniform dimension, modulus may be related to stiffness, which is the force required to produce unit extension ($F/\Delta l$).

The capacity of a material to absorb and release energy is the elastic resilience of that material and is simply the area under the linear portion of

the stress-strain curve. For materials which are substantially elastic to the point of failure, the energy (work) to fracture (U) is given by:

$$U = \tfrac{1}{2}\,\sigma.\epsilon \qquad \text{(units: } MJm^{-3}\text{)}$$

Other materials, however, exhibit plastic deformation. Here, after removal of the applied tensile stress, a residual strain remains in the component. The stress beyond which the body fails to return to its original dimensions is the yield stress, and it marks the point at which irrecoverable flow occurs and the material fails. Workability may thus alternatively be described as either relative, that area of the stress-strain curve bounded by the origin and the yield co-ordinates, or absolute, the area under the curve from the origin to the point of fracture.

Table 5.A.1 Tensile behaviour related to temperature.

Material	Temperature (°C)		Yield stress (MPa)	Yield strain (%)	Stiffness (MPa)	Work to yield (MJm^{-3})
Comb wax	25		1.13	30.0	3.77	1.20
		±	0.08	4.28	0.56	0.18
	30		0.83	14.0	5.93	0.50
		±	0.06	2.86	1.04	0.15
	35		0.54	12.0	4.50	0.23
		±	0.08	1.79	0.94	0.09
	40		0.21	4.2	5.0	0.04
		±	0.03	0.72	0.45	0.02
	45		0.07	4.0	1.75	0.01
		±	0.01	0.36	0.49	0.02
Scale wax	25		1.50	100.0	1.88	0.59
		±	0.04	4.3	0.28	0.09
	30		1.80	71.5	2.54	0.58
		±	0.17	3.0	0.29	0.10
	35		1.80	70.6	2.52	0.52
		±	0.06	1.8	0.15	0.01
	40		1.21	87.8	1.39	0.73
		±	0.05	3.0	0.08	0.05
	45		0.93	67.0	1.44	0.52
		±	0.05	7.0	0.15	0.05

For each value, n = 6.

Table 5.A.2 Tensile properties of cocoon silk.

Temperature (°C)		Relative Strength (Nmm^{-1})	Breaking Strain (%)	Relative Stiffness (Nmm^{-1})	Work (MJm^{-3})
25		32.0	97.5	32.9	28.8
	±	5.1	11.1	4.3	6.6
30		32.4	81.3	39.6	28.0
	±	5.6	9.9	3.9	7.2
35		25.7	85.0	31.4	22.0
	±	3.12	10.1	2.7	4.4
40		38.9	105.3	36.5	38.1
	±	5.6	6.3	4.5	7.2
45		42.9	105.9	40.7	48.3
	±	6.3	9.4	4.6	9.5

For each value, n = 6.

Table 5.A.3 Tensile properties as a function of temperature.

Material	Temperature (°C)		Yield stress (MPa)	Yield strain (%)	Stiffness (MPa)	Work to yield (MJm^{-3})
White Comb wax	25		1.13	30.0	3.77	1.20
		±	0.08	4.28	0.56	0.18
	30		0.83	14.0	5.93	0.50
		±	0.06	2.86	1.04	0.15
	35		0.54	12.0	4.50	0.23
		±	0.08	1.79	0.94	0.09
	40		0.21	4.2	5.0	0.04
		±	0.03	0.72	0.45	0.02
	45		0.07	4.0	1.75	0.01
		±	0.01	0.36	0.49	0.02
Brown Comb wax	25		4.26	16.5	26.5	17.8
		±	0.19	1.7	2.3	1.68
	30		4.44	15.0	30.5	16.0
		±	0.17	0.5	4.1	1.80
	35		4.56	15.7	29.6	16.7
		±	0.06	1.1	2.9	1.77
	40		3.00	13.8	22.9	10.0
		±	0.05	1.1	3.2	0.90
	45		2.10	13.0	16.7	6.90
		±	0.14	0.5	1.5	0.50
Propolis	25		0.26	38.0	0.76	0.07
		±	0.07	4.8	0.01	0.01
	30		0.16	23.0	0.72	0.03
		±	0.03	2.5	0.04	0.01
	35		0.12	17.0	0.69	0.02
		±	0.02	2.3	0.02	0.003
	40		0.([illegible]	16.0	0.49	0.01
		±	0. 2	1.5	0.01	0.003
	45		0.03	14.0	0.26	0.004
		±	0.01	1.2	0.02	0.0005

For each value, n = 6

Table 5.A.4 Properties of comb wax of differing silk content.

Silk content (%)		Strength (MPa)	Strain (%)	Stiffness (MPa)	Work (MJm^{-3})
17		3.24	13.5	25.2	1.24
	±	0.28	1.3	4.2	0.3
20		8.22	52.6	15.8	15.9
	±	1.10	6.6	1.7	2.7
21		6.80	33.5	20.8	6.7
	±	0.67	4.2	1.1	1.3
22		6.38	37.2	18.0	11.4
	±	0.95	3.6	3.5	2.2
23		8.33	46.8	20.9	14.4
	±	1.26	8.4	5.2	2.8
24		6.61	35.9	19.1	8.5
	±	0.65	4.0	1.9	1.1
26		6.10	23.8	26.1	4.3
	±	1.00	4.7	1.2	1.2
29		2.50	12.0	21.6	1.5
	±	0.14	1.2	2.2	0.3
35		9.59	36.3	26.4	11.2
	±	1.5	5.3	0.8	2.3
36		9.01	49.0	22.8	21.6
	±	1.47	8.3	5.8	4.9
37		8.44	38.9	22.9	15.7
	±	1.2	7.4	2.9	4.8

For each value, n = 6.

Table 5.A.5 Properties of whole comb of differing silk content.

Silk content (%)		Relative Strength ($kNmm^{-1}$)	Strain (%)	Relative Stiffness ($kNmm^{-1}$)	Fracture Energy (kN)
17		0.25	0.20	1.3	2.60
	±	0.035	0.02	0.17	0.48
20		0.26	0.15	1.8	1.94
	±	0.01	0.02	0.26	0.15
21		0.25	0.14	1.8	1.77
	±	0.02	0.01	0.18	0.25
22		0.23	0.13	1.7	1.70
	±	0.05	0.02	0.19	0.6
23		0.39	0.16	2.5	3.15
	±	0.03	0.01	0.15	0.44
24		0.26	0.15	1.8	1.91
	±	0.03	0.01	0.16	0.27
26		0.29	0.20	1.5	2.90
	±	0.02	0.01	0.13	0.22
29		0.26	0.15	1.8	1.92
	±	0.03	0.02	0.16	0.38
35		0.27	0.18	1.5	2.45
	±	0.03	0.02	0.04	0.50
36		0.39	0.15	3.0	2.90
	±	0.02	0.02	0.43	0.45
37		0.22	0.11	2.2	1.20
	±	0.04	0.01	0.42	0.26

For each value, n = 6.

Chapter 6

6. Conclusions

The honeybee colony inhabits a suspended nest comprised predominately of wax. Bees covet the comb face, involved in a myriad of tasks from thermoregulation to communication. Every comb consists of a regular array of horizontal hexagonal cells, each precisely fashioned. These cells are used for brood rearing and the storage of nectar, honey and pollen. The colouration of the wax often reflects the cells' function, age and composition. When new, cells consist almost entirely of white wax. After brood have been reared in them, they are lined with cocoons and are light brown, which following repeated use becomes darkened or even black.

In this highly social and domicile dependent society, the construction and maintenance of the honeycomb is crucial. When swarms arrive at a chosen nest site, comb building begins immediately, while in established colonies, festoons of bees are often involved in routine restoration usually by reworking old wax. Amid the bustle of the hive, these chains of wax workers exemplify evolutionary adaptation by their ability to meet the high architectural demands of the nest within the constraints of honeybee physiology. In this thesis, the structural significance of the metamorphosis of wax, as demonstrated by a complex series of modifications from scale to comb wax, was investigated.

Beeswax is secreted by the wax gland complex of oenocyte fat body and epidermal cells. The newly formed wax appears as thin translucent flakes on

the paired wax mirrors of the ventral aspect of the insec' Bees actively involved in construction transfer the scales to their mouthparts, where they are partially or completely masticated. Here a salivary addition is included. The emulsion is then moulded into comb. In mandibulation, the wax undergoes significant textural and chemical modifications.

In Chapter 2, a comparison was made between the crystallite arrangement of scale and comb wax. X-ray transmission photographs revealed the molecular c-axis of the aliphatic components of virgin wax to be approximately perpendi. r to the plane of the scales. Comb wax, on the other hand, exhibited no diffraction texture, implying a random crystallographic arrangement. These results are indicative of a significant textural rearrangement following mastication, and hence mechanical disruption, of the wax.

In Chapters 3 and 4, the chemical differences between comb and scale wax were investigated. The lipid profiles of the two waxes were shown to differ by thin layer chromatography, enzymatic analyses and gas-liquid chromatography (Chapter 3). In comb construction there is a reduction in the scale diacylglycerol pool, with a concomitant increase in the comb monoacylglycerol fraction, particularly in the saturated fatty acid component. These finding strongly suggest the presence of a lipolytic enzyme in honeybee saliva.

Proteins from virgin scales and comb wax were isolated and compared in Chapter 4. This electrophoretic study revealed polypeptides unique to both scale and comb as well as a common group. It is speculated that the common protein species represent transport molecules essential in the secretion of wax, as suggested by their similarity in size to apolipophorin II, one of the associated hemolymph lipid transport molecules. The differences in wax protein profiles may be explained either on the basis of enzymatic reduction of proteins or the inclusion of species, such as the diacylglycerol lipase, following mastication.

The structural significance of these differences between virgin and comb wax were considered both individually and collectively in Chapter 5. The consequence of the change in texture was revealed in a comparison of the tensile properties of nascent scale wax and the sheeted equivalent. The random crystallographic arrangement developed in the course of sheeting resulted in at least a proportion of the molecules with their axis aligned in the direction of loading. The loading in native scales, conversely, was normal to the c-axes of the crystallites. This explains the greater strength and resistance to deformation observed in the sheeted wax.

The contribution made by altering the lipid composition was divulged by comparing sheeted preparations of Soxhlet-extracted comb and scale wax. Here the scale wax was shown to be stronger and more distensible, though not

as stiff, as the comparable comb wax sample. These results suggest that although the strength of the wax is compromised through mastication by the reduction in the diacylglycerol pool, the corresponding increase in saturated monoacylglycerols preserves the resistance to deformation.

The effect of proteinaceous material on the tensile behaviour of wax was assessed in a comparison of Soxhletted and sheeted waxes. In both comb and scale wax preparations, the protein additives markedly improved the stiffness of the wax.

The overall result of these alterations in texture and chemical composition is to convert a significantly distensible, and thus easily malleable material to one which, due to its greater resistance to deformation, is structurally superior. The relevance of this conversion process was emphasized by comparing the tensile properties of native scale and comb wax over the range of temperatures likely to impinge on the honeybee nest. At 35 °C, the optimal nest temperature, scales are more distensible and less stiff than comb. At temperatures in excess of 40 °C, although the wax is easier to manipulate, the structural competence of the nest is compromised, while at lower temperatures, the cost of construction is energetically exorbitant.

Alterations in the mechanical integrity of honeybee waxes are not confined to those instituted during mastication. As the comb matures, numerous adjuvant

materials are incorporated into the nest. Propolis, the resinous substance collected from plants, and cocoon silk, usually accompanied by a faeculent paste in brood comb, are the most prominent additives.

The tensile properties of both honeybee silk and propolis were contrasted to those of nascent honeycomb (Chapter 5). The temperature dependent indices for strength, strain, stiffness and workability of comb wax and propolis demonstrated a decrease in mechanical integrity with increasing temperature. Silk, on the other hand, possess temperature independent characteristics. The combined effects of these plastic (wax and propolis) and elastic (silk) elements were observed in the tensile profiles of mature brood comb. In this two-phase composite, strength and stiffness are significantly enhanced, implying that fibroin contributes greatly to the structural stability of the comb. This conclusion was further corroborated in a study of the tensile performance of silk-bearing comb cell walls of varying ratios. These results suggested that the greater the silk to wax ratio, the stronger the cell wall.

In the final analyses, the conversion of virgin scales to comb wax is a highly complex process. It incorporates alterations in basic chemical composition as well as molecular orientation. While these modifications are essential in maintaining the structural integrity of the nest, with aging the addition of supplementary materials, in the form of resins and silks, is of comparable significance.

References

Alfonsus EC. Some sources of propolis. Methods of gathering and conditions under which this work is done. Glean Bee Cult 1933; 61: 92.

Ambronn H. Anleitung zur benutzung des polarisationsmikroskops bei histologischen untersuchungen. 1892 (Cited from Schmidt, 1941).

Arnhart L. Das puppenhäuschen der honigbiene. Z Ang Entomol 1919; 5: 231-251.

Atkins EDT. A four-stranded coil-coiled model for some insect fibrous proteins. J Mol Biol 1967; 24: 139-141.

Beament JWL. Wax secretion in the cockroach. J Exp Biol 1955; 32: 514-538.

Blomquist GJ, Soliday CL, Byers BA, Brakke JW, Jackson LL. Cuticular lipids of insects. V. Cuticular wax esters of secondary alcohols from the grasshoppers Melaplus packardi and Melaplus sanguinipes. Lipids 1972; 7: 356-362.

Blomquist GJ, Howard RW, McDaniel CA. Structure of the cuticular hydrocarbons of the termite Zootermopsis angusticollis (Hagen). Insect Biochem 1979; 9: 365-370.

Blomquist GJ, Ries MK. The enzymatic synthesis of wax esters by a microsomal preparation from the honeybee, Apis melifera L. Insect Biochem 1979; 9: 183-188.

Blomquist GJ, Chu AJ, Ramaley S. Biosynthesis of wax in the honeybee, Apis mellifera L. Insect Biochem 1980; 10: 313-321.

Boehm B. Beziehungen zwisschen fettkörper, oenocyten und wachsdrüsenentwicklung bei Apis mellifica L. Z Zellforsch Mikrosk Anat 1965; 65: 74-115.

Bradford MM. A rapid and sensitive method the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.

Brewster D. Experiments on the depolarisation of light as exhibited by various mineral, animal and vegetable bodies, with a reference to the general principle of polarisation. Philos Trans R Soc London B 1815; 104: 187-211.

Brooks GT. Penetration and distribution of insecticides. In: Wilkinson CF. ed. Insecticide Biochemistry and Physiology, New York and London: Plenum Press, 1976; 3-58.

Butler C. The feminine monarchie. Oxford: Barnes, 1609.

Casteel DB. The manipulation of the wax scales of the honeybee. Circ US Bur Entomol 1912; 161: 1-13.

Chauvin R. Sur le ne...assement des vieilles cires. Ann Abielle 1962; 5: 59-63.

Chibnall AC, Piper SH, Pollard A, Williams EF, Sahai PN. The constitution of the primary alcohols, fatty acids and paraffins present in plant and insect waxes. Biochem J 1934; 28: 2189-2208.

Chino H, Gilbert LI. Diglyceride release from insect fat body. Science 1964; 143: 359-361.

Chino H, Maya Y, Sato S. Fu...r characterization of lepidopteran vitellogenin from haemolymph and mature eggs. Insect Biochem 1977; 7: 125-131.

Chow M, Newman JFE, Filman D, Hogle JM, Rowlands DJ, Brown F. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature 1987; 327: 482-486.

Chrambach A, Reisfeld RA, Wyckoff M, Zaccari J. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresi . Anal Biochem 1967; 20: 150-154.

Coudron TA, Nelson DR. Hydrocarbons in the surface lipids of pupal tobacco budworms, Heliothis virescens. Insect Biochem 1978; 8: 59-66.

Crewe RM. Compositional variability: the key to the social sig produced by the honeybee mandibular glands. In: Breed MD, Michener CD, Evans HE. ed. The biology of social insects. Westview: Boulder, 1982.

Cruz Landim C da. Evaluation of the wax and scent glands in the Apinae (Hymenoptera: Apidae). J NY Entomol Soc 1963; 21: 2-13.

Darchen R. Queques régulations sociales dans la construction chez les abielle. Insectes Soc 1954; 1: 219-228.

Darchen R. La reine d'Apis mellifica, les ouvrières pondeuses et les constructions cirières. Insectes Soc 1957; 4: 321-325.

Darchen R. Observation directe du dévelopment d'un rayon de cire. Le rôle des chaînes d'abielles. Insectes Soc 1962; 9: 103-120.

Darchen R. La cire, son recyclage et son rôle probable à l'intérieur d'une colonie d'Apis mellifica. Apidologie 1980; 11: 193-202.

Davis BJ. Disc electrophoresis-II. Method and application to human serum proteins. Ann NY Acad Sci 1964; 121: 404-427.

Dean RL, Locke M, Collins JV. Structure of the fat body. In: Kerkut GA, Gilbert LI. ed. Comprehensive insect physiology biochemistry and pharmacology. Vol 3. Oxford: Permagon, 1985.

Denham WS, Lonsdale T. The tensile properties of silk filaments. Trans Faraday Soc 1933; 29: 305-316.

De Réaumur RAF. Memoires pour servir à l'histoire des insectes, vol 5. Paris: L'Imprimeries Royale, 1740.

Diehl PA. Paraffin synthesis in the oenocytes of the desert locust. Nature 1973; 243: 468-470.

Diehl PA. Synthesis and release of hydrocarbons by the oenocytes of the desert locust, Schistocerca gregaria. J Insect Physiol 1975; 21: 1237-1246.

Dobbs A. A letter from Authur Dobbs Esq; to Charles Stanhope Esq; F.R.S. concerning bees, and their method of gathering wax and honey. Philos Trans R Soc 1750; 46: 536-549.

Dorset DL, Ghiradella H. Insect wax secretion. The growth of tubular crystals. Biochem Biophys Acta 1983; 760: 136-142.

Downing DT, Kranz ZH, Lamberton JA, Murray KE, Redcliffe AH. Studies in waxes XVIII. Beeswax: a spectroscopic and gas chromatographic examination. Aust J Chem 1961; 14: 253-263.

Dreischer H. Untersuchungen über die arbeitstätigkeit ünd drüsenentwicklung alterbestimmter bienen im weisellosen volk. Zool Jahrb Abt Allg Zool Physiol Tiere 1956; 66: 429-472.

Dujardin F. Sur l'étude microscopique de la cire appliquée à la recherche de cette substance chez animaux et végétaux. C R Hebd Seances Acad Sci Ser D Sci Nat Paris 1850; 30: 1-8.

Ehrenberg F. Mitteilungen über resultate bei anwendung des chromatisch polarisierten lichtes für mikroskopische verhältnisse. Ber Verh Preuss Akad Berlin. 1849 (Cited from Schmidt, 1941).

Faurot-Bouchet E, Michel G. Composition of insect waxes. I. Waxes of exotic coccidae: Gascardia madagascariensis, Coccus ceriferus and Tachardia lacca. J Am Oil Chem Soc 1964; 41: 418-421.

Faurot-Bouchet E, Michel G. Composition of insect waxes. II. Waxes of the coccids: Ceroplastes rusci, Icerya purchasi, Pluvinaria flocifera and Quadraspidiotus perniciosus. Bull Soc Chim Biol 1965; 47: 93-97, CA 62 : 16664 g.

Fraser HM. Beekeeping in antiquity. London: University of London Press, 1931.

Free JB, Spencer-Booth HY. Observations on the temperature regulation and food consumption of the honeybee (Apis mellifica). Entomol Exp Appl 1958; 3: 222-230.

Free JB. The social orginisation of honeybees. London: Arnold, 1977.

Freudenstein H. Einfluss der pollennahrung auf des bauvermögen, die wachsdrüsen undden fettkörperder honigbiene (Apis mellifera L). Zool Jahrb Allg Zool Physiol Tiere 1960; 69: 95-124.

Folch J, Lees M, Sloane Stanely GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957; 266: 497-509.

Foldi I. Ultrastructure of the wax gland system in subterranean scale insects (Homoptera, Coccidae, Margarodidae). J Morphol 1981; 168: 159-170.

Gaubert P. Sur les cristaux mous. Bull Soc Fr Mineral Cristallogr 1910a; 33: 326-335.

Gaubert P. Sur les cristaux mous et sur la mesure de leurs indices de réfraction. CR Hebd Séances Acad Sci Ser D Sci Nat Paris 1910b; 151: 532-534.

Ghisalberti EL. Propolis: a review. Bee World 1979; 60:59-84.

Gilby AR. Absence of natural volatile solvents in cockroach grease. Nature 1962, 195: 729.

Goetze G, Bessling BK. Die wirkung verschiedener fütterung der honigbiene auf wachserzeugung und bautätigkeit. Z Bienenforsch 1959; 4: 202-209.

Gould JL. Why do honeybees have dialets? Behav Ecol Soc 1982; 10: 53-56.

Graber V. Vorläufiger bericht über den propulsatorischen apparat der insekten. Sber Akad Wiss Wien 1872; 65: 189-204.

Grange JM, Davey RW. Antibacterial properties of propolis (bee glue). J Royl Soc Med 1990; 83: 159-160.

Gwin CM. Further developments concerning wax production by the honeybee colony. 1. A study of the production of wax scales and comb building. J Econ Entomol 1936; 29: 318-321.

Hadley NF. Epicuticular lipids of the desert tenebrionid beetle, Eleodes armata: seasonal and acclimatory effects on composition. Insect Biochem 1977; 7: 277-283.

Hadley NF. Wax secretion and color phases of the desert tenebrionid beetle Cryptoglossa verrucosa (LeConte). Science 1979; 203: 367-369.

Hadley NF. Surface waxes and integumentary permeability. Amer Sci 1980; 68: 546-553.

Hadley NF. Cuticular lipids of terrestrial plants and arthropods: a comparison of their structure, composition and waterproofing function. Biol Rev 1981; 56: 23-47.

Hadley NF. The Adaptive Role of Lipids in Biological Systems. New York: Wiley-Interscience, 1985.

Hanrahan SA, McClain E, Gernecke D. Dermal glands concerned with production of wax blooms in desert tenebrionid beetles. S Afr J Sci 1984; 80: 176-181.

Hanrahan SA, McClain E, Warner SJC. Protein component of the surface "wax" bloom of a desert tenebrionid, Zophosis testudinaria. S Afr J Sci 1987; 83: 495-497.

Hepburn HR, Chandler HD, Davidoff MR. Extensomatric properties of insect fibroins: the green lacewing cross-beta, honeybee alpha-helical and greater waxmoth parallel-beta conformations. Insect Biochem 1979; 9: 69-77.

Hepburn HR, Chandler HD. Material testing of arthropod cuticle preparations. In: Miller TA. ed..Cuticle techniques in arthropods. New York, Heidelberg, Berlin: Springer, 1980.

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

Hepburn HR. Honeybees and Wax. An Experimental Natural History. Heidelberg and Berlin: Springer-Verlag, 1986.

Herbert PN, Bausserman LL, Henderson LO, Heinen RJ, LaPiana MJ, Church EC, Schulman RS. In: Peeters J. ed. The Lipoprotein Molecule. New York: Plenum Press, 1977; 35-56.

Holz H. Das organ der Wachsbilding. Bienen-Ztg 1878; 34: 183-184.

Horn HDS, Kranz ZH, Lamberton JA. The composition of Eucalyptus and some other leaf waxes. Aust J Chem 1964; 17: 464-476.

Hornbostel HC. Neue entdeckung wie das wachs von den bienen kommt. Hamburger vermischte Bibliothek 1744; 2: 45-62.

Huber F. Nouvelles observations sur les abeilles. Geneve: Barde, Manget, 1792.

Huber F. Nouvelles observations sur les abeilles, 2. 1814. translated. (1926) Dadant, Hamilton.

Hunter J. Observations on bees. Phil Trans Roy Soc 1792; 82: 128-196.

Jackson LL, Blomquist GJ. Insect Waxes. In: Kolattukudy, P.E. ed. Chemistry and Biochemistry of Natural Waxes. Amsterdam: Elsevier, 1976; 201-233.

Jay SC. The longitudinal orientation of the larval honeybees (Apis mellifera) in their cells. Can J Zool 1963; 41: 717-723.

Jay SC. The cocoon of the honeybee, Apis mellifera L. Can Entomol 1964; 96: 784-792.

Joffe I, Hepburn HR. A simple low-cost tensometer for biomaterials testing. Experentia 1974; 30: 113-114.

Jordan R. Anomale wachsplättchen. Bienenvater 1962; 63: 299-301.

Katase H, Chino H. Transport of hydrocarbons by the lipophorin of insect haemolymph. Biochim Biophys Acta 1982; 710: 341-348.

Koch HG. Der baubeginn der beinenvölker als phänologisches ereignis. Z Angew Met 1961; 4: 69-82.

Koehler A. Weist die biene in ihrem körper reservestoffe für die winterruhe auf? Schweiz Bienen-Ztg 1921; 44: 224-228.

Koeniger N. Über die fähigkeit der bienenkönigin (Apis mellifica L.) zwischen arbeiterinnen - und drohnenzellen zu unterscheiden. Apidologie 1970; 1: 115-142.

Kohlhaas R. Röntgenographische untersuchung on definierten einkristallen des palmitinsäure-cetylesters. Z Krist 1938; 98: 418-438.

Koidsumi K. Antifungal action of cuticular lipids in insects. J Insect Physiol 1957; 1: 40-51.

Kolattukudy PE. Biosynthesis of surface lipids. Science 1968; 159: 498-505.

Kolattukudy PE, Walton TJ. The biochemistry of plant cuticular lipids. Prog Chem Fats Other Lipids 1972: 13: 121-175.

Kolattukudy PE. Biochemistry of cutin, suberin and waxes, the lipid barriers of plants. In: Gallard T, Mercer EI. ed. Recent Advances in Chemistry and Biochemistry of Plant Lipids. London: Academic Press, 1975; 203-246.

Kreger DR. In: Bouman J. ed. X-ray crystallography. Amsterdam: North Holland, 1951; 299-339.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.

Lambremont EN, Wykle RL. Wax synthesis by an enzyme system from the honey bee. Comp Biochem Physiol 1979; 63B: 131-135.

Lees AD, Beament, JWL. An egg-waxing organ in ticks. Quart J Micro Sci 1948; 89: 291-333.

Lewis WJ, Jones RL, Gross HR Jr., Nordlund DA. The role of kairomones and other behavioral chemicals in host finding by parasite insects. Behav Biol 1976; 16: 267-287.

Lindauer M. Schwarmbieuen auf Wohnungssuche. Z Vgl Physiol 1955; 34: 299-235.

Lindauer M. Communication among social bees. Cambridge: Harvard University Press, 1961.

Lineburg B. Comb building. Am Bee J 1924; 64: 271-272.

Locke M. Secretion of wax through the cuticle of insects. Nature 1959; 184: 1967.

Locke M. The cuticle and wax secretion in Calpodes ethlius (Lepidoptera, Hesperidae). Q J Microsc Sci 1961a; 101: 333-338.

Locke M. Pore canals and related structures in insect cuticle. J Biophys Biochem Cytol 1961b; 10: 589-618.

Locke M. The cuticular pattern in an insect - the intersegmental membranes. J Exp Biol 1968; 37: 398-406.

Locke M. The structure and the formation of the integument of insects. In: Rockstein M. ed. The Physiology of Insecta, vol. 6, 2nd ed., New York: Academic press, 1974; 123-313.

Lucas F, Rudall KM. Extracellular fibrous proteins : the silks. In: Florkin M, Stotz EH. ed. Comprehensive Biochemistry vol 26B, Amsterdam: Elsevier, 1968; 475-558.

Mann FG, Saunders BC. Practical Organic Chemistry. 2nd ed. London, New York, Toronto: Longman, Green and Co, 1938; 33-36.

Martin H, Lindauer M. Sinnesphysiologische leistungen beim wabenbau der honigbiene. Z Vgl Physiol 1966; 53: 372-404.

Martin JT, Juniper BE. The Cuticles of Plants. New York: St Martin's Press, 1970.

Merril CR, Goldman D, Sedman SA, Ebert MH. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Nature 1981; 211: 1437-1438.

Meyer W, Ulrich W. Zur analyse der bauinstinkte unserer honigbiene. Untersuchungen über die "kleinbauarbeiten". Naturwissenschaften 1952; 42: 350.

Mody NV, Hedin PA, Neel WW, Miles DH. Hydrocarbons of males, females and larvae of pecan weevil Curculio caryae. Lipids 1975; 10: 117-119.

Nelson DR, Dillwith JW, Blomquist GJ. Cuticular hydrocarbons in the housefly, Musca domestic. Insect Biochem 1981;11: 187-197.

Noirot C, Quennedy A. Fine structure of insect epidermal glands. Annu Rev Entomol 1974; 19: 61-80.

Olofsson S, Bostrom K, Svanberg U, Bondjers G. Isolation and partial characterisation of a polypeptide belonging to apolipoprotein B from low-density lipoproteins of human plasma. Biochem 1980; 19: 1059-1064.

Philipp PW. Neues vom wachsschüppchen und der wachszange. Rhein Bienen-Ztg 1935; 8: 241-244.

Piek T. Synthesis of wax in the honeybee (Apis mellifera L.). Proc K Ned Akad Wet Ser C Biol Med Sci 1961; 64: 648-654.

Piek T. Synthesis of wax in the honeybee (Apis mellifera L.). J Insect Physiol 1964; 10: 563-572.

Rajashekharappa BJ, Channabasavanna GP. Importance of mated queen in comb expansion of the honeybee Apis cerena. Mysore J Agric Sci 1979; 13: 51-54.

Reimann K. Neue untersuchungen über die wachsdrüse honigbiene. Zool Jahrb Abt Anat Ont 1952; 72: 147-188.

Rösch GA. Über die bautätigkeit im beinenvolk und das alter der baubienen. Weiterer beitrag zur frage nach der arbeitsteilung im bienenstaat. Z Vgl Physiol 1927; 6: 265-298.

Rösch GA. Untersuchungen über die arbeitsteilung im bienenstaat. 2. Tätigkeiter der arbeitsbienen unter experimentell veränderten bedingungen. Z Vgl Physiol 1930; 12: 1-71.

Rudall KM. Silk and other cocoon proteins. In: Florkin M, Mansin, HS. ed. Comparitive Biochemistry vol 4. New York: Academic Press, 1962; 297-435.

Ryan RO, Schmidt JO, Law JH. Chemical and immunological properties of lipophorins from seven insect orders. Arch Insect Biochem Physiol 1984; 1: 375-383.

Sanford MT, Dietz A. The fine structure of the wax gland of the honeybee (Apis mellifera, L). Apidologie 1976; 7: 197-207.

Scanu AM, Edelstein C. Solubility in aqueous solutions of the small molecular weight peptides of the serum very low density and high density lipoproteins: relevance to the recovery problem during delipidation of serum lipoproteins. Anal Biochem 1971; 44: 576-588.

Schmidt WJ. Die bausteine des tierkörpers in polarisierten lichte. 1924 (Cited from Schmidt, 1941).

Schmidt WJ. Polarisationoptische versuche mit bienenwachs. Kolloid Z 1941; 100: 140-151.

Schoening FRL. The x-ray diffraction pattern and deformation texture of beeswax. S Afri J Sci 1980; 76: 262-265.

Seeley TD, Morse RA. The nest of the honeybee (Apis mellifera, L.). Insectes Soc 1976; 23: 495-512.

Shapiro AL, Vinuela D, Maizel JV. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res Commun 1967; 28: 815.

Shearer DA, Bosch R. 2-Heptanone in the mandibular gland secretion of the honeybee. Nature 1965; 206: 530.

Storry J, Tuckley B. Thin-layer chromatography of plasma lipids by single development. Lipids 1967; 2: 501-502.

Stràsky K, Striebel M, Kubelka V. On natural waxes XIX. Complex esters of the wax of the honeybee (Apis mellifera L.). Colln Czech chem Commum 1971; 36(6): 2281-2297.

Stràsky K, Ubik K, Striebl M. On natural waxes XXIII. Hydroxy esters and free alcohols in the wax of the honeybee (Apis mellifera L.). Colln Czech chem Commum 1972; 37(12): 4099-4105.

Toolson EC, Hadley NF. Cuticular permeability and epicuticlar lipid composition in two Arizona vejovid scorpions. Physiol Zool 1977; 50: 323-330.

Tulloch AP. The composition of beeswax and other waxes secreted by insects. Lipid 1970; 5: 247-258.

Tulloch AP. Beeswax: structure of the esters and their component hydroxy acids and diols. Chem Phys Lipids 1971; 6: 235-265.

Tulloch AP. Chemistry of waxes of higher plants. In: Kolattukudy, PE. ed. Chemistry and Biochemistry of Natural Waxes. Amsterdam: Elsevier, 1976; 235-277.

Tulloch AP. Beeswax - composition and analysis. Bee World 1980; 61: 47-62.

Vanderberg JD, Massie DR, Shimanuki H, Peterson JR, Poskevich, DM. Survival behavior and comb construction by honeybees, Apis mellifera, in zero gravity aboard NASA shuttle mission STS-13. Apidologie 1985; 16: 369-384.

Verlich AV. Entwicklungsmechanische studien an bienenlarven. Z Wiss Zool 1930; 136: 210-222.

Waku Y, Foldi I. The fine structure of insect glands secreting waxy substances. In: King RC, Akai H. ed. Insect Ultrastructure vol 2. New York, London: Plenum Publishing Company, 1984; 303-322.

Warnick GR, Mayfield C, Albers JJ, Hazzard WR Gel isoelectric focusing method for specific diagnosis of familial hyperlipoproteinemia type 3. Clin Chem 1979; 25/2: 279-284.

Warth AH. The chemistry and technology of waxes. 2nd ed. New York: Reinhold, 1956.

Warwicker JO. Comparitive studies of fibroins-II. The crystal structure of various fibroins. J Mol Biol 1960; 2: 350-362.

Weiss K. Über den zuckerverbrauch und die beanspruchung der bienen bei der wachserzeugung. Z Bienenforsch 1965; 8: 106-124.

Wigglesworth VB. The Principles of Insect Physiology. 7th ed, New York: Halsted Press, 1972.

Woog P, Yannaquis N. Sur l'orientation des molécules de la cire d'abeille. CR Hebd Séances Acad Sci Ser D Sci Nat Paris 1935; 201: 1400-1402.

Woog P, Yannaquis N. Orientation des molécules de la cire d'abeille et répercussion sur la solidité des rayons. CR Hebd Séances Acad Sci Ser D Sci Nat Paris 1936a; 202: 76-78.

Woog P, Yannaquis N. Orientation des molécules de la cire d'abeille. Répercussion sur la construction des rayons. Arch Phys Biol 1936b; 13: 134-149.

Young RG. The biosynthesis of beeswax. Life Sci 1963; 2: 676-679.