WAX SECRETION IN THE CAPE HONEYBEE (*APIS MELLIFERA CAPENSIS* ESCH.) IN RELATION TO JUVENILE HORMONE AND AGE POLYETHISM

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT

Wax secretion in worker honeybees is significantly related to the age of the worker and, while the secretion pattern remains the same, the absolute amount of wax secreted varies seasonally. Comb building festoons, previously thought to be the site of wax secretion, contain only a fraction of the newly-secreted wax in the nest. Festooning behaviour was also found to be seasonal. The amount of wax secreted by workers was significantly affected by hive.

Although age-related changes in behaviour and physiology of worker honeybees appears to be modulated by juvenile hormone (JH), wax secretion is not dependent on JH. Manipulating JH III titres by injecting the hormone and manipulating the only source of the hormone (the corpora allata: CA) did not affect wax secretion. Increasing haemolymph JH titre shortly after eclosion did not affect the amount of wax produced by workers aged 3 to 21 days, nor could a critical period be found during which elevated hormone titres would affect the rate of wax secretion. Allatectomy of newly eclosed workers did not affect wax production. Removing the putative neural feedback inhibition on the CA did not result in a change in wax production. Implanting CA from older workers into younger workers had no significant effect. Methoprene, a widely-used JH analog, caused reduced wax secretion in workers. It is suggested that methoprene poisons worker honeybees.

The results obtained are consistent with an alternative model for wax secretion proposed by Butler (1954). The methodological problems found in this work are present in many other studies. When viewed in this light, the role of JH in polyethism appears dubious and there are alternative models of polyethism that do not have these shortcomings.

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CHAPTER 1

GENERAL INTRODUCTION: WAX SECRETION, AGE POLYETHISM AND JUVENILE HORMONE IN HONEYBEES

The processes of wax secretion and comb building by honeybees, and the composition of wax, are much studied aspects of the honeybee life (Hepburn 1986). Wax secretion takes place in the presence or absence of a queen (Whiffler 1990; Whiffler and Hepburn 1991) and is not necessarily accompanied by comb building (King 1928; Whiffler 1990; Whiffler and Hepburn 1991). Wax secretion was thought to take place only in a festoon (Huber 1792) and that only workers in festoons secreted wax and built comb. It was thought that comb building was a phase in the polyethism of adult honeybee workers between house duties and foraging (Rösch 1925, 1927; Lindauer 1952; Ribbands 1953; Seeley 1982; Winston 1987).

AGE POLYETHISM IN HONEYBEES

Eusocial insect societies such as those of the honeybee display two types of division of labour, or polyethism, one based on physical caste, where "caste" is defined as a set of individuals, morphogenetically distinct from other members of the colony (Villet 1992), and the other on age and the social role of the individual insects in their colony (Oster and Wilson 1978). In honeybee societies there are two female castes: queens and workers. Furthermore, the worker caste displays temporal polyethism related to age, which has captivated researchers for several centuries.

Age polyethism is interpreted as a pattern of changes in the probability of a worker of a given age performing a certain task (Jeanne 1986) in a nest in which the shared environment allows for the co-ordination of individuals to meet colony-level needs (Seeley 1989a). The spatial differentiation of task performance, the associated life-time sequence of task performance, and

the flexibility of age polyethism in workers, are well established (Rösch 1925, 1927, 1930; Nelson 1927; Milojevic 1940; Lindauer 1952, 1954; Ribbands 1953; Sakagami 1953; Hoffman 1961; Wittekindt 1962; Free 1965; Seeley 1982; Kolmes 1990; Dyer and Seeley 1991; Seeley and Kolmes 1991). There is a spatial trend of movement away from the central brood nest as workers age (Free 1960; Seeley 1982), with the lifespan of the worker being limited by the amount of time spent foraging rather than the time spent performing hive duties or with the level of activity of the worker (Sekiguchi and Sakagami 1966; Schmid-Hempel and Wolf 1988). The most risky tasks, e.g. foraging, tend to be isolated demographically and performed exclusively and last in the task sequence (Jeanne 1986).

Although unverified, Ribbands (1952) proposed that rather than workers undergoing a stereotyped age-related behavioural sequence they simply swapped to the most pressing tasks, particularly during colony upheavals. Behavioural responses of individuals and of colonies to changes in their environment have been investigated, especially with respect to the ages at which individuals are most likely to perform various tasks e.g. foraging (Lindauer 1954; Dreischer 1956; Sekiguchi and Sakagami 1966; Seeley 1983, 1989b; Winston and Fergusson 1986; Pham-Delègue *et al.* 1990), dorsoventral abdominal vibrations (Gahl 1975; Schneider 1987), grooming (Kolmes 1989), and defensive behaviour (Szabo and Townsend 1974; Allan *et al.* 1987; Moore *et al.* 1987; Whiffler *et al.* 1988; Breed *et al.* 1990). Workers have been noted to spend a large part of their time resting (Lindauer 1952; Winston 1987) or "hiding" (van der Blom 1991) and these bees may provide a reserve work force able to respond to changes in the labour needs of the hive. Kolmes' (1985c) results suggested a utilisation of work potential, rather than a reallocation of workers to a new set of tasks.

Variation in the behaviour of individual workers, modulated by colony requirements and environmental conditions, results in the frequently observed flexibility in the pattern of worker age polyethism (Winston and Neilson Punnett 1982; Kolmes 1985c; Winston and Fergusson 1985, 1986; Crailsheim 1986; Winston 1987; Kolmes and Winston 1988; Crailsheim and Stolberg 1989; van der Blom 1991), making its stereotyped nature, which was reinforced by Seeley's (1982) model of 5 behavioural roles in honeybees, doubtful. This model was later refined and redefined to only 3 roles (Seeley and Kolmes 1991). Although task specialization has been found, differences between studies have been largely attributed to differences in

sampling methods, the complexity of the behavioural ethograms used and the method of statistical analysis employed (Kolmes 1985a, b, 1986, 1989; Kolmes and Winston 1986; Seeley 1986; Seeley and Kolmes 1991; van der Blom 1991). However, Kolmes (1984) found no difference between scan and focal bee sampling, provided sufficiently large sample sizes are used.

AGE POLYETHISM AND WORKER HONEYBEE PHYSIOLOGY

Physiological, glandular and behavioural changes in *Apis mellifera* workers are age- and season-related. Differences in the summer and winter physiology of the temperate races are well-documented. In winter, workers have higher haemolymph volumes, higher levels of haemolymph protein and vitellogenin, and consistently lower juvenile hormone III titres than in summer (Fluri *et al.* 1977; Fluri *et al.* 1982; Crailsheim 1985). The hypopharyngeal glands (HPG) in "winter" workers remain small, while in "summer" workers the HPG were reduced only in foragers (Fluri *et al.* 1982).

Aspects of physiology have been correlated with colony conditions (Crailsheim 1986), season (Sekiguchi and Sakagami 1966) and age of the workers. Haemolymph volume decreases with age (Bühler *et al.* 1983; Crailsheim 1985); and body weight, both dry and wet, has been related to age (Harrison 1986; Crailsheim and Stolberg 1989). Weight loss is associated with onset of foraging activity e.g. a decrease in weight from the abdomen, which appears to enhance flight capacity (Bounias 1978), associated with an increased storage of glucose and fructose in the thoracic muscles. Further evidence for physiological changes in queenright colonies (Fluri *et al.* 1982) include age-related changes in the production of various proteins (Rutz and Lüscher 1974; Grogan and Hunt 1980; Fluri *et al.* 1982; Crailsheim 1986, 1991; Moritz and Crailsheim 1987; Pabst *et al.* 1988; Severson *et al.* 1991; Crailsheim *et al.* 1992) and in juvenile hormone III titres (Rutz *et al.* 1976; Fluri *et al.* 1982; Sasagawa and Kuwahara 1988). Age-related changes in protein production have been associated with changes in behaviour in adult worker honeybees e.g. production of venom protein and associated defensive behaviour (Allan *et al.* 1987; Whiffler *et al.* 1988).

Age-related changes in size and activity of glands such as the pharyngeal, hypopharyngeal, mandibular, wax and defense glands are well established phenomena and are thought to depend on the social role of workers which, in turn, is subject to colony needs (King 1928; Kratky 1931; Milojevic 1940; Dreischer 1956; Free 1961, 1965; Boch and Shearer 1967; Brouwers 1982, 1983; Fluri *et al.* 1982; Hepburn *et al.* 1984; Allan *et al.* 1987; Fluri and Bogdanov 1987; Moritz and Crailsheim 1987; Whiffler *et al.* 1988; Huang and Otis 1989; Hepburn *et al.* 1991). Although colony needs can influence and direct worker behaviour to a large extent, Sekiguchi and Sakagami (1966) found that individual differences in behaviour are not always correlated with colony conditions. Kerr and Hebling (1964) found that body weight correlated with the probability of workers performing certain tasks. Cause and effect are difficult to separate and many of these changes may be spuriously correlated.

Age polyethism is thought to have arisen as a result of the genetic determination for the tendency to perform different tasks (Breed 1988; Page *et al.* 1989). Assumptions underlying models which predict worker behaviour are that worker behaviour is largely stereotyped, that this behaviour is affected by genotype, and that genotypically based differences in behaviour lead to differences in colony performance (Calderone and Page 1988, 1991; Moritz and Hillesheim 1989; Robinson and Page 1989a, b; Bienefeld and Pirchner 1991).

Opposing schools of thought exist regarding the genetic component of the underlying mechanisms regulating worker honeybee behaviour. Although genotype must play a role in the behaviour of adult worker honeybees (Dewsbury 1991; Moore 1991), it is not the only regulating factor and performance is affected by the demands of the colony (Ribbands 1952; Palmquist Momot and Rothenbuhler 1971). Using genetic lines of workers with high and low learning ability, Brandes (1990) found that genotype did not affect the ages at which tasks were performed, but that workers of a higher learning ability were more likely to participate in tasks.

The opposing point of view is that colony organization is influenced by the probability of performing various tasks, which is affected in turn by the genotype of the honeybee worker. This is supported by several studies in which the patrilines of the colonies are phenotypically identifiable e.g. by using allozyme markers, colour of worker exoskeleton or propensity

towards a particular behaviour, especially hygienic behaviour. The genotypes of workers performing various tasks e.g. queen rearing (Page *et al.* 1989), foraging and hoarding behaviour (Calderone and Page 1988, 1991; Calderone *et al.* 1989; Kolmes *et al.* 1989; Moritz and Hillesheim 1989; Robinson and Page 1989a, b), scouting (Robinson and Page 1989a, b), guarding (Robinson and Page 1988, 1989a, b; Calderone *et al.* 1989; Breed *et al.* 1990), undertaking (Robinson and Page 1988, 1989a, b), grooming and trophallaxis (Frumhoff and Schneider 1987; Frumhoff and Baker 1988) have been examined.

However, data presented in support of an overriding genetic basis for age polyethism is unconvincing for a variety of reasons: significant differences were found only in selected trials; the ages of experimental animals were estimated (not measured) according to the area of the nest in which they were found (following Seeley 1982); data were grouped arbitrarily without justification; the number of workers representing each genotype at the sampling period was unknown; genotypic behavioural differences were found only in a few select tasks; no variance in the data was presented; and differences obtained were not consistent. Several studies support this view point. For example, Kolmes *et al.* (1989), in a repeated experiment, could not find a difference between patrilines in the age of first forage. Calderone and Page (1988) presented results which strongly suggest a colony influence overriding a purely genetic component. Workers were found to specialize in grooming but this was not genetic and it is thought that differences which were found were a result of "genetic noise" (Frumhoff and Baker 1988).

INSECT CORPORA ALLATA, JUVENILE HORMONES AND BEHAVIOUR

The influence of hormones on insect behaviour can be tested in one of several ways (Truman and Riddiford 1974). The first is indirect, correlating behaviour with histological evidence of onset of hormonal secretion. The rest deal with direct manipulation of the inherent hormone titre e.g. by removing the endocrine source, by application of the hormone or the gland producing the hormone, or finally, by removal of the endocrine gland with subsequent hormone application. The idea that juvenile hormone (JH) could modulate responses to received stimuli by affecting the cells of the nervous system directly and thus altering

behaviour has been supported for many years (Haskell and Moorhouse 1963; Riddiford and Truman 1974; Robinson 1987c).

Juvenile hormones are implicated in a range of physiological and behavioural functions in both juvenile and adult insects. The first demonstration of hormonal influence on insect behaviour was in 1938 (Bounhiol 1938, cited in Truman and Riddiford 1974) when precocial pupation was induced by removal of the corpora allata of penultimate instars. JH has morphogenetic or gonadotropic effects (Röller and Dahm 1968; Cassier 1979): it is associated with egg maturation (Wigglesworth 1936; Rankin and Riddiford 1978; Röseler *et al.* 1980; Röseler *et al.* 1981; Couillaud *et al.* 1984), induction and maintenance of vitellogenesis (Tobe and Stay 1977), development of accessory glands in males (Wigglesworth 1936), oviposition (Stay and Tobe 1978; Bellés *et al.* 1987), spermatophore release (Stay and Tobe 1978), physical caste formation in eusocial insects (de Wilde and Beetsma 1982; Strambi *et al.* 1984; Bonetti *et al.* 1990), maintenance of juvenile characters (Wigglesworth 1940), accelerated development (Idriss 1990), changes in behaviour (Haskell and Moorhouse 1963) and migratory flight (Rankin and Riddiford 1978).

Different modes of action for JH on insect physiology and behaviour have been proposed. Puffing patterns on the chromosomes were found by Schneiderman and Gilbert (1964) who then suggested that JH could affect the genome directly by altering such patterns thereby affecting the expression of the genome and, ultimately, physiology and behaviour. Nijhout and Wheeler (1982) hypothesized that juvenile hormones could regulate gene switching or gene expression, especially during sensitive or critical periods in the life cycle of the insect. The mode of action of JH is thought to be similar to that of steroid hormones as they are able to enter the cells and have binding sites on the nuclei (Osir and Riddiford 1988). These hormone-receptor complexes could then conceivably interact with the regulatory regions of DNA to cause changes in gene expression.

Wigglesworth (1935, 1936, 1940) recognised that the corpora allata (CA) secreted a hormone, which was found to be non-specific and blood-borne. Because the presence of the hormone causes the retention of juvenile characters to the next larval, pupal or adult phase (Röller and Dahm 1968), it was termed juvenile hormone. The first direct evidence that JH was secreted

by the CA was provided by Röller and Dahm (1970), after the medium in which CA were cultivated was extracted and analysed. Various methods are employed to identify the inherent JHs in insects, as well as to measure their titres and CA biosynthetic activity. Bioassays for the measuring of JH activity were developed as far back as 1960 (Gilbert and Schneidermann, the development of the *Galleria* wax moth test). Riddiford and Ajami (1973) developed a new method, using the tobacco hornworm, *Manduca sexta*, for elucidating the active JH titre, but could not use the bioassay to identify the JH.

Four forms of juvenile hormone have been identified, based on the length of the carbon chain. The first to be described was JH I (Röller *et al.* 1967) and the most recent, JH 0 (Bergot *et al.* 1980). While traces of JH I were found in fifth instar larvae of honeybees, the only JH homolog present in all stages of development is JH III (Hagenguth and Rembold 1978). The empirical formula of this, the smallest homolog, is $C_{16}H_{26}O_3$ (Judy *et al.* 1973). An intensive study revealed that JH III has a flexible structure but is thought to exist preferentially in one of its smaller conformations (Katagi *et al.* 1989). It is suspected that JH III is hydrophobic and possesses polar regions. Sixteen lower energy conformers of the JH III molecule exist, but the active conformer has not yet been identified. It can be deactivated by three classes of enzymes: epoxide hydrolases, esterases and oxidases (Katagi *et al.* 1989).

It has been proposed that the four different JHs may have different functions, but this has not yet been tested (Nijhout and Wheeler 1982). As JH III is the most phylogenetically widespread of the JHs (found in a range of insect orders and crustaceans), it has been speculated that the remaining JHs may have arisen as a result of a different synthetic pathway (Cusson *et al.* 1991). The proposed theory is that more hormones imply a more complex control of physiological events, especially in more evolved taxa. Cassier (1979) proposed that JH III is the original hormone and the remaining JHs "evolutionary achievements". JH III has been found in a variety of families in four diverse insect orders and a universal function for JH III has been proposed (Trautmann *et al.* 1974).

The insect CA are connected to the brain via the nervi corporis allati (NCA) I and to the suboesophageal ganglion via the NCA II. By injecting markers into the nerve cells, it was shown that the connections NCA I and NCA II deliver neurosecretions to the CA (Tobe and

Stay 1985). In honeybees, the CA are attached to the oesophagus by striated muscle and connected to the brain via the corpora cardiaca (CC) and nervi corporis cardiaca (NCC) (van Laere 1970) with the axons of the NCA in direct contact with the cell membranes of the cells of the CA (van Laere and Lagasse 1973).

Extensive reviews by Cassier (1979), Tobe and Stay (1985) and Sedlak (1985) revealed the ultrastructural endocrine nature of the corpora allata. The overwhelming consensus is that no general rule relates the structure and volume of the gland to any measure of its activity. Although intracellular changes do occur in CA cells when they are active, the meaning of the changes is not fully understood. A recent study by Piulachs *et al.* (1989) could not conclusively correlate changes in CA ultrastructure with increase in JH biosynthesis. Johnson and Hill (1973) found that changes in CA volume are indicative of insect growth, rather than changes in biosynthetic activity. Although no correlation between CA volume and JH biosynthesis has been established, larger glands do appear to be capable of higher levels of activity than smaller CA (Röseler *et al.* 1981; Khan *et al.* 1982). The larger glands may be a result of larger body size; JH titres and CA volumes need to be correlated for individual insects rather than correlating pooled samples.

The activity of insect CA is regulated by both a neural and a humoral system through which information from the internal and external environments is integrated (de Wilde and Beetsma 1982; Tobe and Stay 1985). The amount of neurosecretory material in the honeybee CA increases within 24 hours of eclosion (Nenadovic *et al.* 1985). Couillaud *et al.* (1984) found that severing the NCA 1 resulted in a change in JH biosynthesis in locusts. Thompson *et al.* (1990) were able to inhibit JH biosynthesis by application of octopamine, a neurotransmitter and neuromodulator found in the CA of locusts and cockroaches, and suggested that octopamine is a natural neuromodulator of JH and acts by regulating the ion channels on the CA cells.

Cassier (1979) suggested that, as there are no obvious structures which could reasonably be associated with JH packaging, the JH is released by diffusion as it is synthesized. Studies by Tobe and Pratt (1974) and Tobe and Stay (1977) showed that JH is released as it is synthesized: CA appear to have a physical limit and do not store JH. Thus CA activity is

limited by the rate of synthesis, rather than the rate of release (Tobe and Stay 1977, 1985). The synthesis and release of JH has been found to be linearly related in several insect species (Pratt *et al.* 1975; Röseler and Röseler 1978; Feyereisen and Tobe 1981; Bellés *et al.* 1987).

JH synthesis may be regulated in one of three ways (Tobe and Stay 1985). The first is by controlling the number of cells in the CA (which has little supporting evidence); the second is by coordination of the enzymes required to produce the JH; and the third by control of carbon flow rate by a multivalent feedback system. It has been suggested that the modulation of CA activity arises from a change in synthesis, rather than release, rate of JH (Cassier 1979). Both positive and negative feedback loops interact to regulate CA activity, e.g. excess JH, too little JH, ovarian cycles, ecdysteroids, feeding, body size, social interaction and environment (Weaver 1984). Alteration of the rate of JH synthesis in response to either external or internal stimuli may be either fast or slow (Cusson *et al.* 1991); a fast response involves the inhibition of allatostatic factors or activation of allatotropic factors which control the enzymes for JH production, and a slow response involves cellular changes in the CA e.g. changes in the cytoplasmic volume, the number of cells in the CA, or the enzyme concentration in the CA. Evidence reviewed by Tobe and Stay (1985) suggests that there is more than one rate-limiting step in JH biosynthesis in the CA, and it is at these steps that humoral or neural input can regulate the activity of the CA.

When unilateral allatectomy was performed on Colorado potato beetles, *Leptinotarsa decemlineata*, (Schooneveld *et al.* 1979), the remaining CA increased its JH biosynthetic rate and the overall titre remained the same as when a pair of CA was present. Tobe (1977) found randomly distributed asymmetry in the production of JH by CA in the desert locust. A denervated CA/corpora cardiaca complex maintained its activity, while implantation of a CA pair resulted in suppression of the host's CA (Schooneveld *et al.* 1979). Similar adaptability of CA was shown by Stay and Tobe (1978). Severed CA continued synthesizing JH, and at times the rate was higher than that of the host's CA, but the rate of synthesis showed a physiological limit. When the host CA was denervated as well, both sets of CA showed similar levels of JH production, indicating that a feedback from the JH titres was not the rate-limiting stage and that the CA are under neural control. Rachinsky and Hartfelder (1991)

suggested that methyl farnesoate epoxidation may be a rate-limiting step in the biosynthesis of JH.

Techniques for the identification and quantification of JHs are being continually developed and refined. The need for a rapid and accurate method for identifying and quantifying JHs (necessary to eliminate inaccurate and time consuming bioassays) was outlined by Schooley (1977). A number of techniques are available for the purification, identification and quantification of JH from both *in vivo* and *in vitro* biological samples (Rembold *et al.* 1980; Strambi *et al.* 1981; Feldlaufer *et al.* 1982; Khan *et al.* 1982; Granger and Goodman 1983, 1988; Rembold and Lackner 1985; Tobe and Stay 1985; Sasagawa and Kuwahara 1988; Kimura *et al.* 1989; Goodman *et al.* 1990). Although numerous techniques are available for the purification, identification and quantification of JH, e.g. RIA, TLC, HPLC with UV monitoring and multiple ion detectors, and (SIM) GC-MS, each has its problems and these are discussed extensively in the literature. The issue of measuring whole-body or haemolymph extracts remains controversial. More tissue is obtained from whole-body extracts, but this is not an accurate reflection of the JH titre because JH stored in body tissue is unavailable to other organs (Goodman *et al.* 1990). On the other hand, pooled samples are usually required to obtain sufficient material for a haemolymph extract analysis, but unless multiple samples of pooled extracts are used, no estimate of variation is available.

CORPORA ALLATA AND JUVENILE HORMONE IN HONEYBEES

Juvenile hormone has been studied repeatedly in honeybees, especially with respect to the regulation of age polyethism. Evidence supporting the modulation of morphogenesis and behaviour by JH titres has been presented in numerous papers: worker honeybee larval development and adult worker behaviour have been changed by the application of juvenile hormone analogs and mimics (Jaycox *et al.* 1974; Beetsma and ten Houten 1975; Jaycox 1976; Robinson 1985, 1987a, b). However, neither racial nor genetic differences in the JH trend or titres have emerged (Robinson *et al.* 1987; Robinson *et al.* 1992).

Haydak (1943) suspected that caste differentiation in honeybees was more than just a question of the amount and quality of food the larvae were fed during their developmental period. This idea was supported by Dietz *et al.* (1979) who concluded, after a series of feeding experiments, that caste determination is regulated by the endocrine system, while food intake may be regulated by the CA. Caste differentiation has been the subject of research for a number of years and as the analytical techniques have become more refined, so the process of caste differentiation has become more defined. JH titre measurements during different larval and prepupal stages, measuring of CA activity during these phases, application of JH homologs and analogs, and measurements of gene expression and DNA content of the CA have revealed the presence of critical periods during which differences in the JH titre of larvae result in queen or worker imagos (Wirtz and Beetsma 1972; Rembold *et al.* 1974; de Kort *et al.* 1977; Mane and Rembold 1977; Copijn *et al.* 1978; Beetsma 1979; Rembold 1987; Severson *et al.* 1989; Hartfelder *et al.* 1990; Rachinsky and Hartfelder 1990; Rachinsky *et al.* 1990; Yaginuma *et al.* 1990; Rachinsky and Hartfelder 1991). There are caste-specific differences in the development of the CA of honeybee queen and worker larvae (Dixon and Moser 1972; Liu and Dixon 1973; Ulrich and Rembold 1983).

The growth of CA in adult worker honeybees was found to be subject to the presence of the queen (Gast 1967), nutrition, and presence of nestmates (Breed 1983) but has been shown, on numerous occasions, to increase in volume with age (Pflugfelder 1948; van Laere 1966; Gast 1967; Breed 1983; Sasagawa *et al.* 1989). Although the CA volume patterns are by no means consistent they have been correlated with worker behaviour (van Laere 1971) by measuring CA volumes of workers of known activity. When whole-body and haemolymph JH III titres are plotted on a similar age axis as the CA data, the trend of increasing JH titre with age can only loosely be correlated with CA volume changes and indicates that CA volume does not reflect biosynthetic activity.

SCOPE AND AIMS

Several weaknesses in JH-related work on honeybees have become apparent and have led to the development of a series of experiments examining the effect of JH and its associated

gland, the CA, on wax production in the honeybee. It has been claimed that wax production is affected by colony requirements (Fergusson and Winston 1988; Naumann and Winston 1990a, b) and as festoons are found on the new building edge of a comb (Huber 1792), the amount of wax production is usually measured as the amount of comb built in wax-deprived colonies. The aim of the study undertaken was to ascertain whether wax secretion, and associated behaviour, was age-related and whether this age-related task could be modulated by JH III, the CA or an analog.

CHAPTER 2

TEMPORAL AND SPATIAL PATTERNS OF WAX SECRETION AND RELATED BEHAVIOUR IN THE DIVISION OF LABOUR IN THE HONEYBEE, *Apis mellifera capensis*

SUMMARY

Current ethograms for honeybee behaviour are hypothesized to consist of age-related cohorts of workers having a high probability of performing a small set of related tasks in a restricted portion of the nest. Wax secretion and wax working related activities were assayed in this light. It is shown that wax secretion is significantly related to worker age and that bees between 3 and 21 days old form such a cohort. Comb-building festoons, previously thought to be the site of wax secretion, represent only a small fraction of newly secreted wax in the nest. Wax secretion remains constant relative to age in the cohort but varies significantly with season as does bee participation in festooning behaviour. Wax secretion and wax working are both definable in terms of time and space in the nest, the relative probability of activity changing with season. Secretion itself is constrained by the cyclical activity of the underlying wax gland complex.

INTRODUCTION

Following the discovery of a dramatic, age-related transition from hive to field activities in honeybees (Dönhoff 1855), more subtly differentiated tasks were found among house bees (Rösch 1925, 1927; King 1928). This led to the concept of an age-related division of labour of tasks among honeybees that was essentially correct (Ribbands 1953) even if early interpretations were too rigid (Winston 1987). Knowledge of polyethism was refined through

the experimental demonstration that worker bees form age-related cohorts that have a high probability of performing only a limited set of tasks, each usually restricted to certain areas of the nest (Seeley 1982). The tasks change as the worker bee becomes older; there is also a shift away from the brood area until she eventually becomes a forager and leaves the nest.

Although research on the activities of house bees has out-paced that on the physiological basis of behaviour (Seeley 1982), the classic works have shown that the activities of some glands (e.g. wax glands) are age-related and might be closely linked to task differentiation (Rösch 1925, 1927; King 1928; Ribbands 1953). For example, wax-working includes festooning behaviour (Huber 1792; King 1928) and cell capping (Lineburg 1923a, b; Lindauer 1952) both of which are spatially separated (Hepburn 1986) and either or both of which may be driven by the secretory cycle of the wax gland complex (Hepburn *et al.* 1991). In view of the above, there is now sufficient information to ask how the probability of a cohort performing given tasks is constrained by underlying physiological characteristics (glandular secretions) and whether the tasks are susceptible to modulating stimuli (queen pheromones, nectar influx etc.). To this end the temporal and spatial characteristics of wax secretion and wax-working behaviour and how they are integrated in colonies of honeybees were investigated.

MATERIALS AND METHODS

Experiments were performed in the field throughout the year with seven queenright colonies of the Cape honeybee, *Apis mellifera capensis*, of eastern Cape origin. Each colony had three frames of drawn comb with brood and food stores and two empty frames as comb building sites. Bees were fed *ad libitum* on a 25 - 50% sucrose solution. Newly emerged adult worker bees were paint-marked for age and introduced into the colonies at the rate of 150 bees/colony/day at three day intervals for 18 days. On the 21st day, festoons were harvested from building frames 3 times. The following day, all remaining marked bees were collected from the colonies. Festoon participation was defined as the number of marked bees, of any particular age group, present in a festoon, expressed as a percentage of the total number of marked bees of that age in the hive. The wax scales were removed from the wax mirrors of all the recaptured bees and the scales of each individual worker bee were weighed separately

on a microbalance. Experimental data were analysed using ANOVA and Scheffe's test (BMDP 1990).

RESULTS

Wax secretion

The secretion of wax by worker bees was analysed with respect to hive, worker age, season of the year and participation in a festoon. Wax secretion was significantly affected by hive ($p < 0.0000$). The relationship between wax secretion and worker age was determined simply by weighing the standing crop of wax scales recovered from individual bees of known age. Comparisons of bees of different ages showed that not all groups differed significantly ($p > 0.05$) in the amount of wax borne; however, the amount of wax borne by any particular bee is significantly affected by its age class (Table 2.1).

It has tacitly been assumed for two centuries (Huber 1792) that the standing crop of raw wax in a colony is associated with festoons - i.e. that only workers in festoons secrete wax. We re-examined this view by dividing our marked bees, on capture, into those present in festoons and those found elsewhere in the hive (mainly on brood and honey capping areas). When the data was analysed in this way (Table 2.1), there were no significant differences in the mean amounts of wax borne by workers of the same age group, whether in festoons or elsewhere in the nest ($p > 0.05$). Similarly, there were no significant differences in the total amount of wax present on bees of the same age cohort whether captured in festoon or non-festoon areas of the nest.

Because honeybee biology varies seasonally (Seeley 1985), we analysed the mean amount of wax borne by different age cohorts and the total standing crop of raw wax in the colony, with respect to season in the quasi-temperate southern Cape (Schulze 1965). The amount of wax borne within any particular age cohort varied significantly with season (Fig. 2.1; $p < 0.0000$): least wax was present in winter, significantly more in summer and spring, and even more in autumn. However, the amount of wax recoverable from any particular cohort relative to age

remained constant among cohorts across the seasons (Fig. 2.1). About half of the standing crop of raw wax was recovered from festoon bees and the other half from bees elsewhere in the nest (Fig. 2.2), except in winter, when non-festoon wax production was significantly higher than festoon wax production.

Distribution of raw wax

The amount of wax borne by bees varies with age (Table 2.1) and season (Fig. 2.1), as does wax production in festoons (Fig. 2.2). Of the total number of marked bees recovered from festoons, approximately 56% were captured in summer, 37% in spring and autumn but only 7% during winter (Fig. 2.3). In summer and autumn, 18% of the marked bees were recovered from festoons, with 8% in spring and only 3% in winter (Fig. 2.3). Yet the age composition of the festoon remained constant. By pooling the data into a year-long assessment, we can see that of the total number of workers caught in festoons (47%, Fig. 2.3) only 53.3% (Table 2.1) carried wax scales; this means that only 25% of workers of wax producing age in a colony will be in festoons. No significant correlation was found between festoon participation and wax secreted by workers ($p > 0.05$); nor was there any more wax/bee in festoons than outside the festoons.

Festoon size also varied seasonally and is reflected in the mean number of marked bees captured in festoons per colony: summer, $n = 276.4 \pm 55.9$; autumn, $n = 140.0 \pm 69.2$; winter, $n = 66.3 \pm 48.8$; spring, 123.3 ± 118.7. Continuous time-lapse video recordings, over 72 h intervals, also showed that during summer, festoons were larger at night, when all of the field force was in the hive, than during day when they foraged. Day-time festoon size varied with the prevailing weather, being larger on cool days than warmer ones. The festoons were not the stable clusters previously envisaged (Huber 1792; Darchen 1962), but had a high turnover rate of bees coming and going. The length of time a bee spent in a festoon varied from 30 minutes to 4 hours. Wax secretion and length of time a bee spends in a festoon are independent activities. It is tempting to suggest that the main function of the festoon is to keep the colony temperature constant by varying the size of the festoon and length of time spent in a festoon.

DISCUSSION

Age-related shifts in the tasks performed by house bees have been grouped in different, usually functional ways (Rösch 1925; Ribbands 1953; Seeley 1982, 1985; Kolmes 1985a, b, c; Winston 1987). However, several tasks are wholly dependent upon cycles of glandular activity (Rösch 1925, 1927; King 1928; Ribbands 1953) that predetermine their temporal limits. This basic concept was clearly established in the classical work of Lindauer (1952). Wax secretion is reflected in cyclical changes in the tissues of the wax gland (Dreyling 1903; Boehm 1965) and is correlated with worker age for bees between 3 and 21 days old (Hepburn *et al.* 1984; Hepburn *et al.* 1991; Table 2.1 and Fig. 2.1, this chapter). The fundamental rise-fall characteristics of wax secretion with age remain intact through the seasons (Fig. 2.1); however total wax production per colony is seasonal and is independent of queen quality (Whiffler and Hepburn 1991) but may vary with nutrition (Goetze and Bessling 1959; Freudenstein 1960) and swarming (Turrell 1972; Hepburn and Whiffler 1988).

Perhaps most importantly for a temporal and spatial characterization of this polyethism, wax secretion is obviously essential to comb building, but comb building does not of necessity follow wax secretion (Lindauer 1952; Hepburn 1986). Indeed, comb construction only reaches parity with other wax works during the height of nest growth in the summer and autumn under Cape conditions, where the Cape honeybee occurs naturally. This leads to considerations of where the raw wax and the tasks requiring wax can be found in the nest: "major" building operations (= comb construction) and several "minor" ones associated with brood and honey capping, and repairs and refinements to existing combs (Meyer 1952, 1954; Lau 1959). The former is conducted by many individuals working in concert (comb-building festoons) and the latter by unco-ordinated individuals (Lindauer 1952; Smith 1959). In terms of wax work done, the word "minor" is a serious malapropism because in a thriving colony, given a good nectar flow, the wax required for capping work alone easily matches, and may exceed, that needed for new comb construction (Lineburg 1923a, b).

Changing ratios of major to minor works have not previously been documented, but can now be inferred from a consideration of where the raw wax in a colony may be found. That the

relative percentages of wax bees present in festoons varies with the seasons (Fig. 2.3), while age composition remains relatively constant, suggests a "wax-need" relationship with season. Indirectly, season determines what construction is to take place i.e. major or minor works. By measuring the precise whereabouts of any age cohort in a colony and adjusting for season, one can calculate where the bulk of the standing crop of wax is likely to be and, more importantly, what type of wax construction can occur in any particular place.

Over a whole year almost as much wax will be found in festoons as elsewhere in the nest (Table 2.1), but the seasonal picture is quite different (Figs. 2.1 and 2.2). It suggests that wax bees are in the "right" places at the "right" times. Thus, for wax secretion, and probably wax-working as well, temporal limits are well defined. We know which bees of what ages produce how much wax and during which season. It must be remembered that different seasonal nectar flows in different regions will produce a different seasonal distribution of wax production. Numerical shifts of wax bees from one area of the nest to another, where different tasks are performed, ensure a close correspondence in time and place for the execution of a suite of wax-related behaviours by a cohort of house bees.

Only a few of the several exocrine glands of the honeybee worker have been analysed for the temporal coincidence of synthetic and secretory performance and appropriate behavioural activity. These requirements coincide in the case of colony defense (da Cruz Landim 1963; Whiffler *et al.* 1988; Breed *et al.* 1990) and brood care (Seeley 1982; Crailsheim and Stolberg 1989; Liu 1989), and both are spatially and temporally predictable. The cohort involved in wax secretion and wax-working can now be added to a growing list of glandular-based and age-related polyethisms in honeybees.

Table 2.1: Percentage bees with wax (%), sample sizes (n) and the mean amount of wax (mg/bee ± s.d.) produced by festoon and non-festoon worker honeybees for each age group

age	FESTOON			NON-FESTOON		
(days)	n	%	mg/bee	n	%	mg/bee
3	307	22.2	0.06 ± 0.14	1053	36.7	0.11 ± 0.18
6	682	60.3	0.21 ± 0.26	861	42.5	0.16 ± 0.25
9	676	71.0	0.32 ± 0.35	959	56.4	0.23 ± 0.30
12	717	62.5	0.28 ± 0.37	1286	40.5	0.15 ± 0.27
15	694	54.9	0.27 ± 0.45	1279	28.9	0.10 ± 0.23
18	515	39.2	0.17 ± 0.31	1037	23.9	0.08 ± 0.20
21	390	28.2	0.10 ± 0.24	840	13.7	0.04 ± 0.15
	3981	X=53.3	X=0.22 ± 0.34	7315	X=35.5	X=0.13 ± 0.24

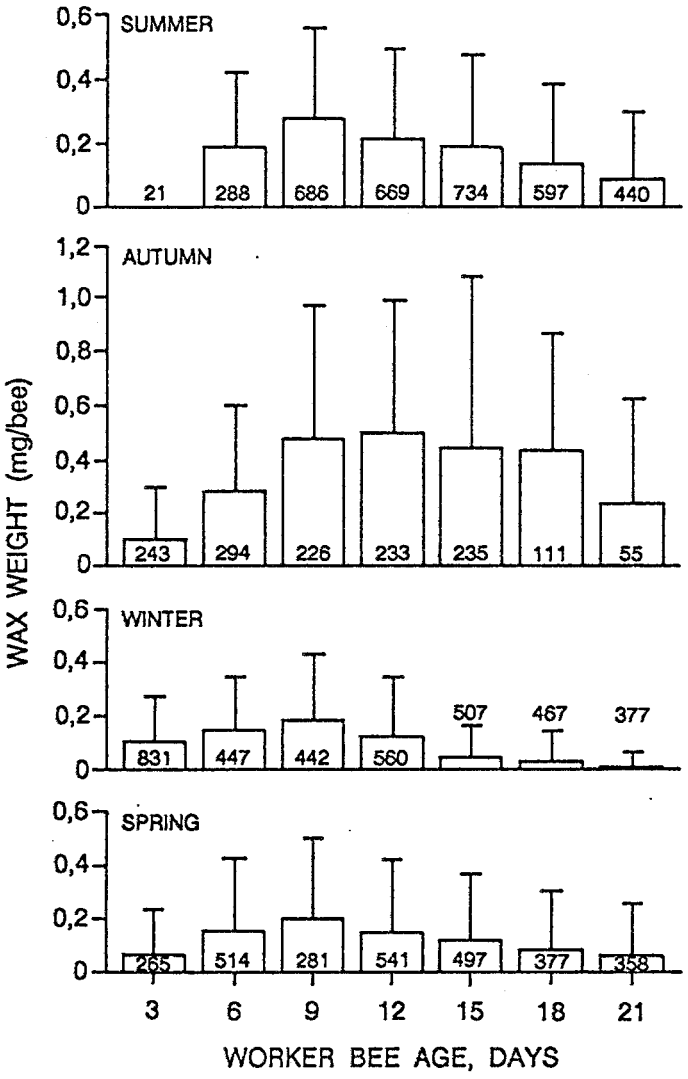

Figure 2.1 Seasonal wax production in honeybees (means ± SD). Sample sizes are indicated in the bars

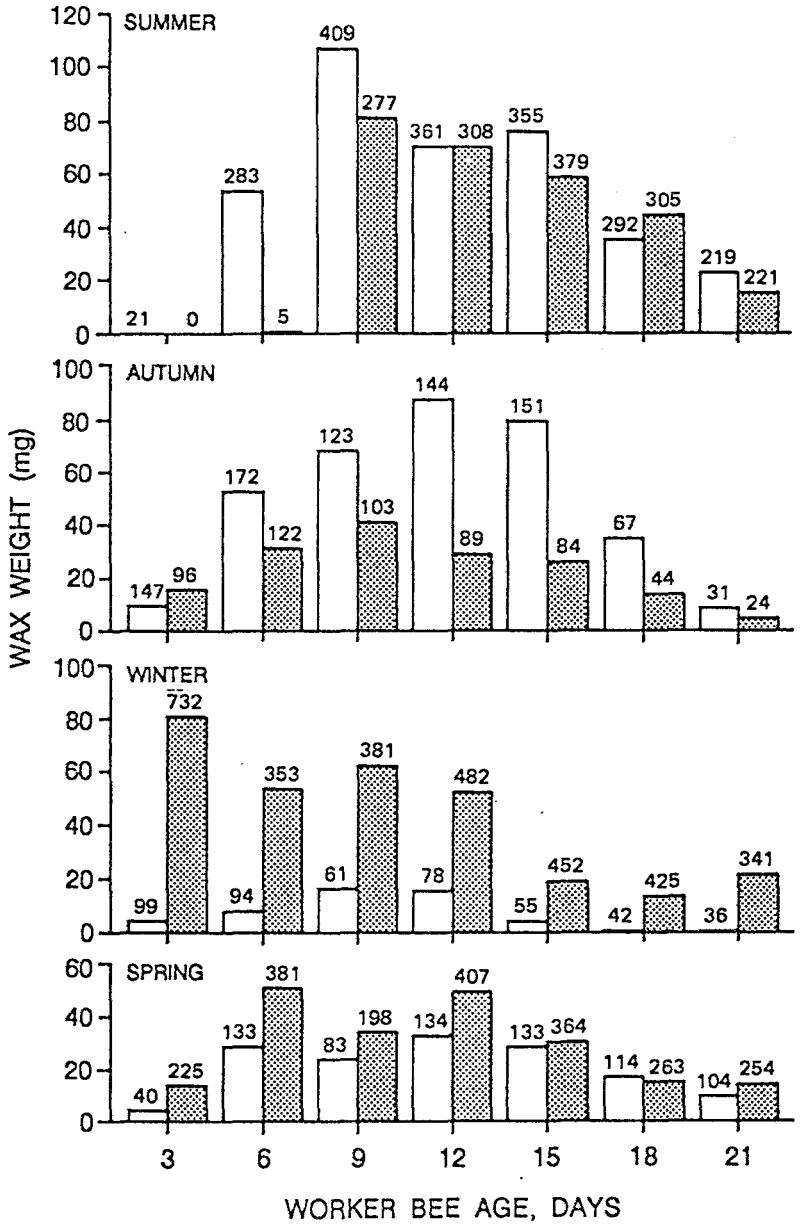

Figure 2.2 Seasonal total wax production (mg) by festoon and non-festoon worker honeybees. Open bars represent festoon workers and stippled bars represent non-festoon workers. Sample sizes are indicated in the bars

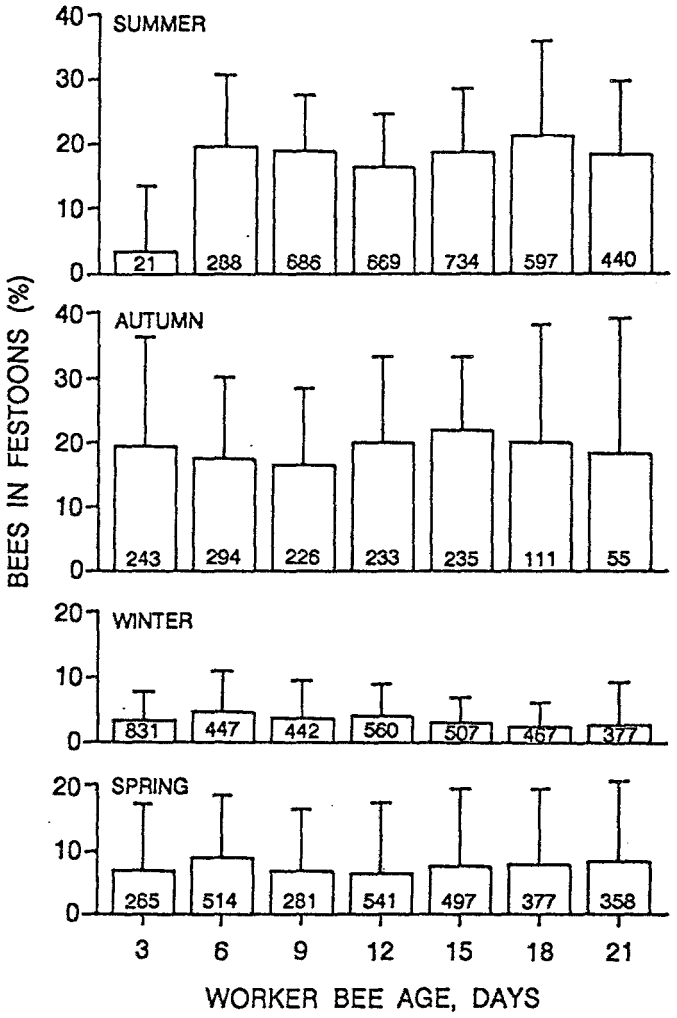

Figure 2.3 Seasonal festoon participation by honeybee workers (mean ± SD). Total sample sizes for each age category are indicated in the bars; the bar represents the percentage of bees of that age recaptured in a festoon

CHAPTER 3

THE EFFECT OF JH III ON WAX SECRETION IN THE HONEYBEE, *Apis mellifera capensis*

SUMMARY

Single large injected doses of JH III did not significantly modulate wax secretion. An increased JH III haemolymph titre shortly after eclosion did not affect the mean amount of wax produced by workers aged 3 to 21 days. A critical period during which an elevated JH III titre, in 0-8-day-old workers, would affect the rate of wax secretion could not be established. These results cast doubt on the role of JH III in regulating the age-related physiology of wax secretion in adult worker honeybees.

INTRODUCTION

Application of JH homologs, JH analogs and JH mimics have been reported to modulate both the behaviour and physiology of adult honeybee workers, primarily inducing guard and forager behaviour and physiology prematurely (Jaycox *et al.* 1974; Jaycox 1976; Robinson 1985, 1987a, b). Reported physiological changes induced by application of JH have included modulation of the haemolymph protein and vitellogenin titres (Imboden *et al.* 1976; Rutz *et al.* 1976) and histological changes in the hypopharyngeal glands and corpora allata (Rutz *et al.* 1974; Rutz *et al.* 1976; Brouwers 1983; Liu 1989; Sasagawa *et al.* 1989). Application of JH analogs elicited histological changes in gland structure more readily than did application of JH III, while responses to the same doses of hormone showed variation and were not consistent. Associated with the age-related nature of development and degeneration of glands are the secretory products of these glands. Robinson (1985) induced the premature production of alarm pheromone in adult worker honeybees.

The postulated (Robinson 1987c, 1992) central role of JH in co-ordinating the behavioural and physiological changes associated with honeybee polyethism was directly tested by examining the effect of increased JH III titres on wax secretion.

MATERIALS AND METHODS

All experiments were carried out at the Department of Zoology and Entomology, Rhodes University. In each experiment, three queenright colonies, housed in 5-framed nucleus hives, were manipulated such that each had 3 central brood and food frames with an empty frame on either side of the broodnest. As there was a nectar dearth during the experimental period, bees were fed a 25-50% sucrose solution *ad libitum*.

Experiment A: Increased JH III titres in newly emerged workers

Newly emerged adult workers, eclosed overnight in an incubator at 32°C - 34°C, were divided into 4 treatment groups with 150 workers in each, per colony. Each bee was subjected to one of the following treatments: control (no further treatment); control injection (1 µl sweetoil); and two experimental groups (1 µg JH III/1 µl sweetoil injected and 10 µg JH III/1 µl sweetoil injected); marked according to treatment and released into a colony. This was repeated every 3 days until there were 7 experimental age groups in each hive. On day 21 of the experiment, festoons were collected from the end frames, without disturbing the central brood nest, and killed by anaesthetizing with CO_2 and freezing immediately. The following day, the remaining marked bees were recaptured and killed. Wax scales were removed from individual workers, counted and weighed on a Cahn C-31 microbalance to measure the amount of wax secreted by individuals. Data were analysed using ANOVA and Scheffe's test (BMDP 1990).

Experiment B: Increased JH III titres in workers aged 0-8 days

Newly emerged adult workers (eclosed overnight in an incubator at 32°C - 34°C) were marked and released into each of the three experimental colonies. Each day, 150 marked bees were recaptured from each colony and subjected to 1 of 3 treatments: control (no further treatment); control injection (1 µl sweetoil); and 1 µg JH III/1 µl sweetoil injection. Workers were anaesthetized on ice for 5 - 10 minutes so that treatment could be carried out. Once immobile, workers were re-marked, treated; any wax scales were removed; and once fully recovered, they were returned to their original hives. Workers treated on days 0 and 1 were not stripped because there was so little wax that removing the scales would have damaged the bees. When the bees were 9 days old (age of maximum wax production, Fig. 2.1), all the marked bees were recaptured and killed, and their wax scales removed, counted and weighed on a Cahn C-31 microbalance. Data were analysed using ANOVA and Scheffe's test (BMDP 1990).

Experiment C: Modifications of Experiments A and B

The short half-life of JH (for JH I in honeybee larvae at 30°C it is 3½ hours: de Kort *et al.* 1977), along with the long period between experimental age groups and the unknown effect of removing the wax scales, necessitated a modified repetition of Experiments A and B. On day 0 of the experiment, 1200 newly eclosed adult worker honeybees were marked and released into the colonies. Experiment A was repeated, but workers were treated each day instead of every 3 days. Experiment B proceeded as before, but the wax scales were not removed from the wax mirrors. The treatment groups were as before: control (no treatment apart from marking); control injection (1 µl sweetoil); and 1 µg JH III/1 µl sweetoil injection. On day 6 of the experiment, all marked bees from the experimental colonies were recaptured and killed. Consequently, there were 6-day old workers which had been treated when they were 0 - 5 days old; and there were workers aged 1 - 6 days, which had all been treated shortly after eclosion. Wax scales were removed, counted and weighed. Data were analysed using ANOVA and Scheffe's test (BMDP 1990).

In each of the experiments, injection rather than topical application was used because JH treatment is more likely to elicit a sustained response when applied internally as the JH leaks out of the carrier (Wigglesworth 1969; Schwieter-Peyer 1973; Riddiford and Ajami 1973; Jaycox 1976; Brouwers 1983).

RESULTS

Experiment A: Increased JH III titres in newly emerged workers

The mean amount of wax produced by festoon and non-festoon workers did not differ significantly ($p = 0.8392$), confirming earlier results (chapter 2). The amount of wax produced by workers from different hives varied significantly ($p < 0.0000$). The age of the worker significantly affected the amount of wax found on the wax mirrors ($p < 0.0000$). There was a significant treatment effect ($p < 0.0000$), with the treated workers (control injection and both 1 and 10 µg JH III/ 1 µl sweetoil) showing reduced wax production. However, there was no difference in the mean amount of wax produced by control-injected and JH III-injected workers ($p > 0.05$) and in particular, there was no dosage effect. Although the mean amount of wax produced was significantly affected by both age and treatment (Fig. 3.1) and an interaction between these factors significantly affected the mean amount of wax produced ($p = 0.0052$), a Scheffe's test (BMDP 1990) revealed the differences to exist between, rather than within, age cohorts. Including hive as a further level showed the same result. The number of wax scales found on the workers showed similar results, with 3 day old workers possessing fewer wax scales and the untreated controls more wax scales.

Experiment B: Increased JH III titres in workers aged 0-8 days

Since no obvious dose-dependent effects of JH III application on day 0 emerged from Experiment A, 1 µg JH III/ 1 µl sweetoil was used to determine whether there was a critical period during which elevated JH III titres could modulate wax secretion in adult worker honeybees. There was a significant hive effect ($p = 0.0022$) in that the mean amount of wax

found on the workers was significantly affected by the hive from which they were removed. The age at which the workers were treated significantly affected the mean amount of wax produced ($p < 0.0000$), with the workers whose wax scales were not removed during treatment producing more wax.

The treatment effect was also significant: untreated workers produced significantly more wax than either of the treatment groups ($p = 0.0005$) while there was no significant difference between the control-injected and 1 µg JH III/ 1 µl sweetoil-injected cohorts ($p > 0.05$). An interaction between the age at which the workers were treated and the treatment they received did not significantly affect the amount of wax the workers were able to secrete by 9 days (Fig. 3.2, $p = 0.6439$) and introducing hive as a third interacting factor did not alter this result. Bearing in mind that the 0 and 1 day old workers were not stripped of wax scales when they were treated, it appears (Fig. 3.2) as though it takes 7-9 days to secrete the amount of wax found on 9 day old workers. The number of wax scales found on the 9 day old workers revealed similar results with a strong treatment effect apparent.

Experiment C: Modifications of Experiments A and B

The repeat of Experiment A and B revealed that the mean amount of wax produced was significantly affected by the hive from which the workers were removed ($p = 0.0050$). In the repeat of Experiment A, where workers were treated shortly after eclosion, there were significant differences in the mean amount of wax secreted by workers of different ages ($p < 0.0000$) where the young workers secreted significantly less wax than the older workers. Treatment significantly reduced the mean amount of wax found on the workers ($p = 0.0001$) but JH III treatment did not affect the wax production significantly. An interaction between age and treatment significantly affects wax production (Fig. 3.3, $p < 0.0000$), but a Scheffe's test (BMDP 1990) revealed the differences to exist between, rather than within, age cohorts. An interaction including hive as a factor was also found to be highly significant ($p < 0.0000$), emphasizing hive effects in wax production. The number of wax scales found on the workers revealed similar trends.

In Experiment B, the age at which workers were treated significantly affected the mean wax production ($p = 0.0117$) while JH III treatment significantly reduced wax production in 6 day old workers ($p < 0.05$). An interaction between age and treatment proved to be non-significant (Fig. 3.4, $p = 0.9754$). Introducing hive as a further factor significantly affected wax production ($p < 0.0000$) but the differences were between, rather than within, levels. The number of wax scales found on 6-day-old workers followed similar trends to the mean weight of wax found on the workers.

DISCUSSION

Age-related behavioural, physiological and glandular changes take place in worker honeybees and have been correlated with an inherent rise in JH III titre (chapter 1). Similarly, wax glands develop and degenerate with age (Hepburn *et al.* 1984; Hepburn *et al.* 1991), a trend reflected in the amount of wax which can be found on workers of various ages (chapter 2; Muller and Hepburn 1992). The correlation between the intrinsic rise in JH III levels and the state of development of the wax glands gave rise to experiments reported here in which JH III titres were increased by injection in an attempt to modulate wax production. By increasing haemolymph JH III titres it was thought that peak wax production could be induced at a younger age. However, wax production was significantly affected by the application method, rather than by application of JH III *per se* (Experiments A, B and C) and a significant hive effect was apparent in all experiments. Neither the onset of wax secretion (Figs. 3.1 and 3.3) nor the rate of wax secretion (Figs. 3.2 and 3.4) could be altered by increasing haemolymph JH III titres .

It was assumed that a single large dose of JH III would be sufficient to initiate a change in wax gland activity and maintain the altered wax glands at the new level of activity. However, a series of applications with a relatively low dose have been found to be more effective than a single large dose (Patterson 1973; Staal 1975; Tobe and Stay 1977). De Loof and de Wilde (1970) were able to initiate vitellogenesis in the Colorado potato beetle (*Leptinotarsa decemlineata*) by single application of a JH analog, but could not maintain it, which suggests either a pharmacological action by the JH analog, or that elevated JH titres are not required

for the maintenance of vitellogenesis. The inability of a single large dose to elicit and maintain a response may result from the short half-life of JH (Metzler *et al.* 1972; Ajami and Riddiford 1973; Erley *et al.* 1975; Staal 1975; Copijn *et al.* 1978; Edwards and Rowlands 1978; Tobe and Stay 1985) along with feedback-inhibition of JH biosynthesis induced by application of JH (Schooneveld *et al.* 1979). The latter inhibits the production of JH until its circulating levels have receded sufficiently to remove the negative feedback-inhibition.

There was no dose-dependent response to JH III (Fig. 3.1) although the doses used to affect wax secretion were sufficiently high to have done so, as the injected doses should have raised haemolymph titres to 10^6-10^7 times above the circulating haemolymph JH III titre of newly emerged workers (Rutz *et al.* 1976; Fluri *et al.* 1977; Fluri *et al.* 1982; Bühler *et al.* 1983; Robinson *et al.* 1987; Sasagawa and Kuwahara 1988; Robinson *et al.* 1989, 1992; Huang *et al.* 1991).

As the activity of applied JH is dependent on time of application (Wigglesworth 1940; Staal 1975), and wax secretion peaks at 9 days (Fig. 2.1; Muller and Hepburn 1992), the critical period during which applied JH could have been expected to modulate the trend of wax production was shortly after emergence. However, results from these experiments (Figs. 3.1 and 3.3) suggest that this is not the critical period for the modulation of wax production. An attempt to uncover a hypothetical JH-sensitive period during which elevated JH titres could modulate the rate of wax secretion also proved unsuccessful (Figs. 3.2 and 3.4). When considering the inherent rise of JH III titres in the haemolymph of the adult workers (Rutz *et al.* 1976; Fluri *et al.* 1982; Sasagawa and Kuwahara 1988), there are no obvious titre changes which could be associated with wax gland development and secretion of wax. These correlations, or lack of, contradict a role for JH III in initiating or modulating wax secretion in honeybees. The ability of the wax glands to synthesize and secrete wax even though not fully developed (Hepburn *et al.* 1991), as well as the inability to change the pattern of wax secretion, suggests that the wax glands are not under JH III control.

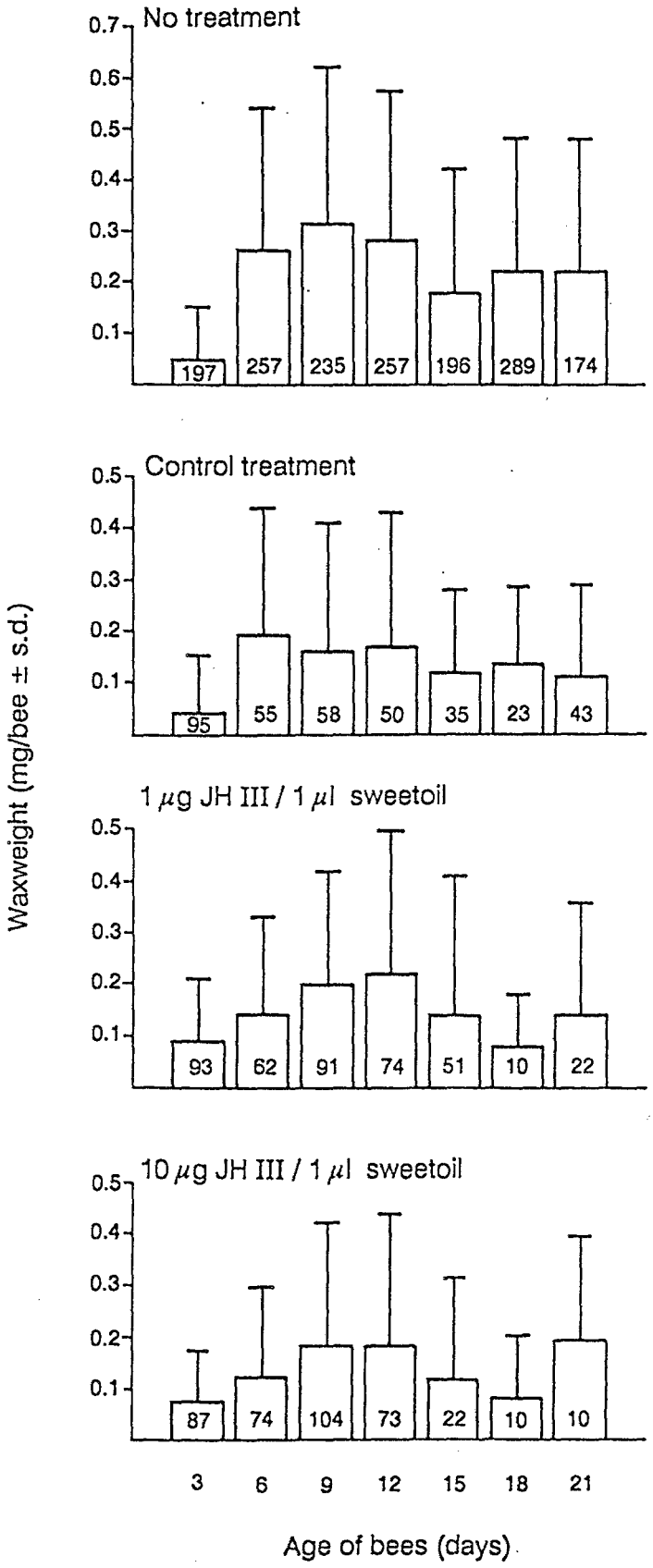

Figure 3.1 Mean amount of wax produced by workers, aged 3-21 days old, subjected to different JH III treatments (mg/bee ± SD). Sample sizes are indicated in the bars

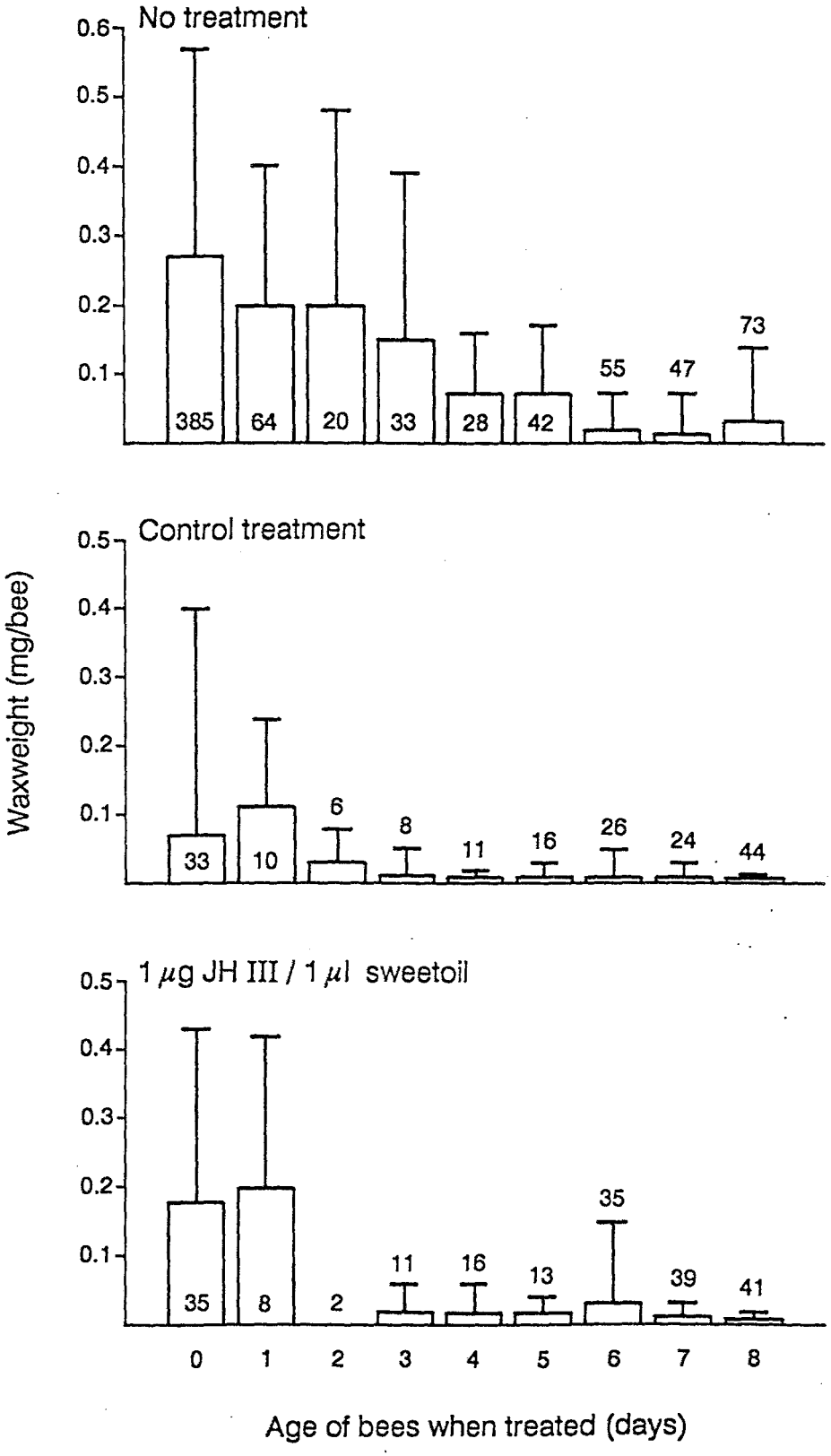

Figure 3.2 Mean amount of wax produced by 9-day-old workers, treated at different ages (mg/bee ± SD). Sample sizes are indicated in the bars

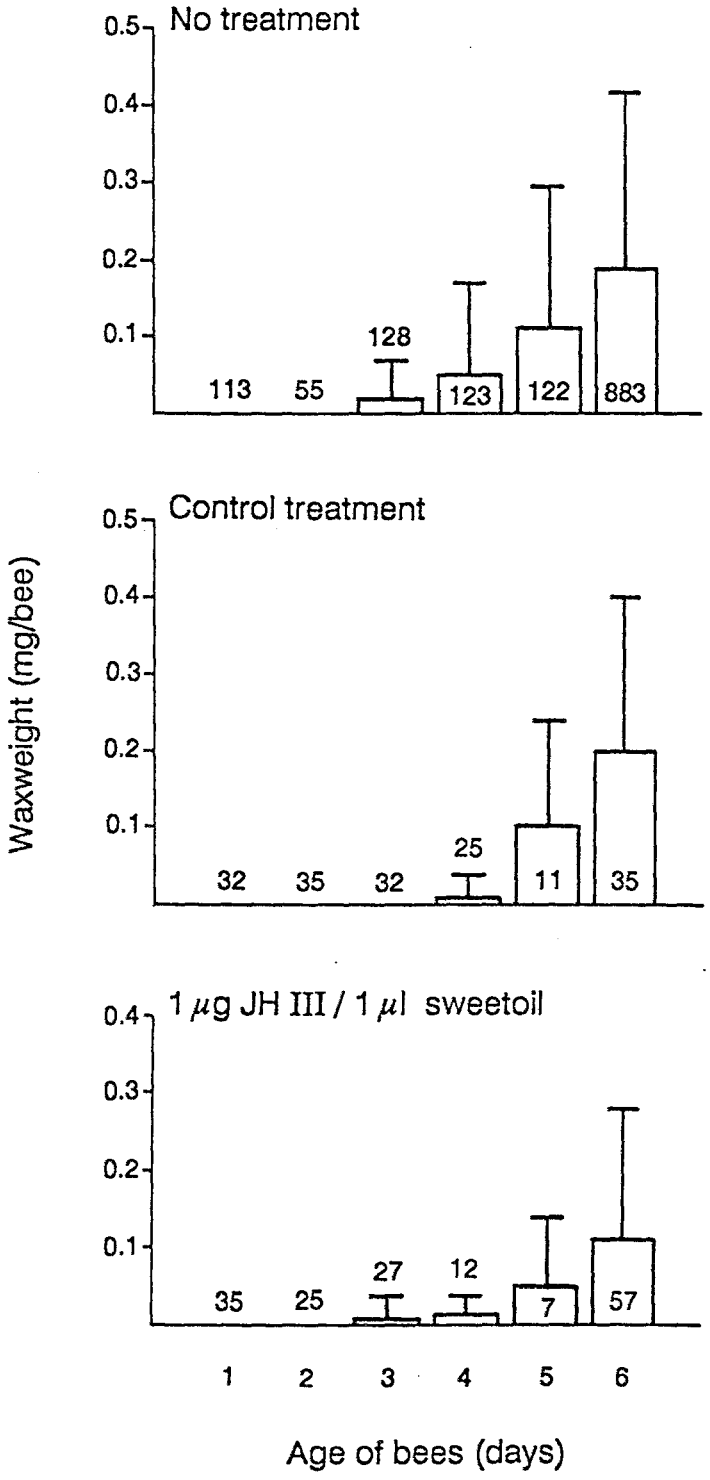

Figure 3.3 Mean amount of wax produced by workers, aged 1-6 days old, subjected to JH III treatments (mg/bee ± SD). Samples sizes are indicated in the bars

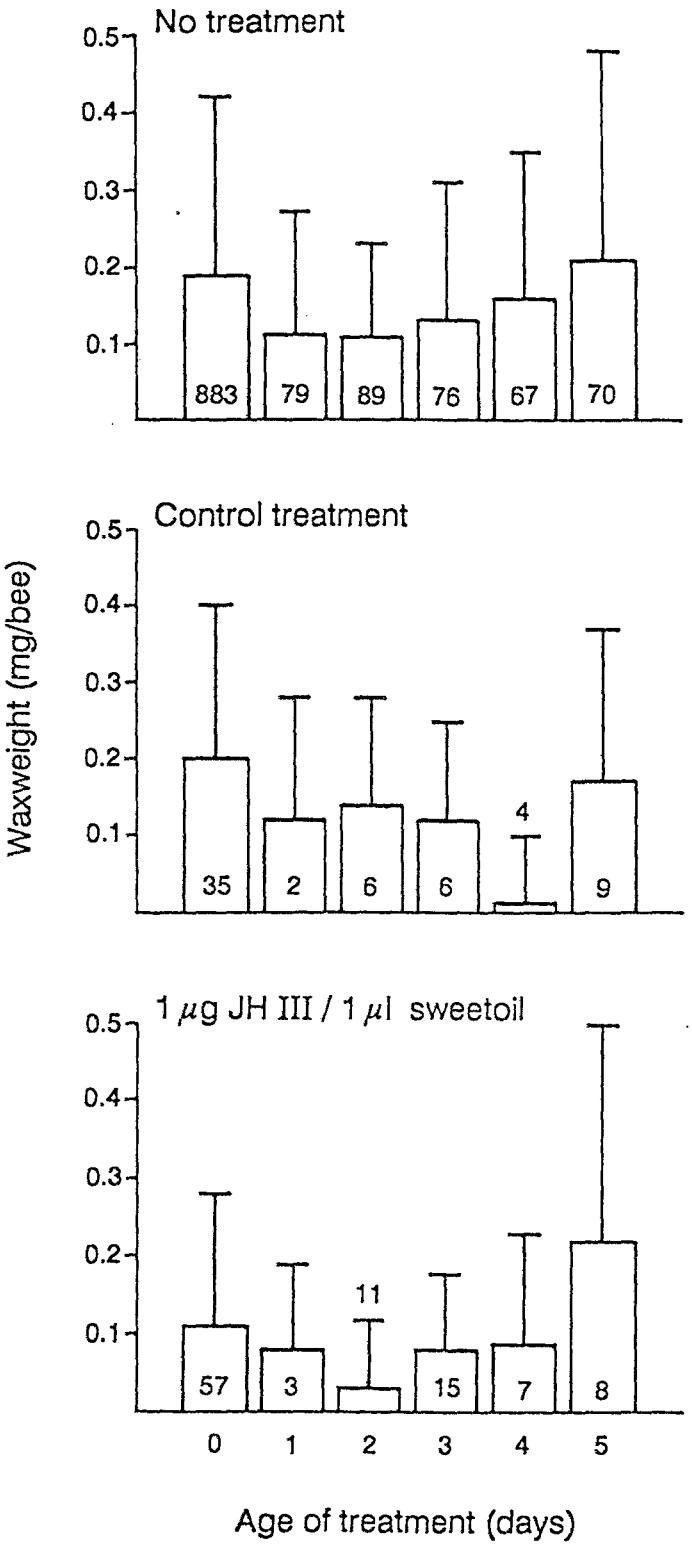

Figure 3.4 Mean amount of wax produced by 6-day old workers, treated at different ages (mg/bee ± SD). Sample sizes are indicated in the bars

CHAPTER 4

THE INFLUENCE OF THE CORPORA ALLATA ON WAX SECRETION IN HONEYBEES, *Apis mellifera capensis*

SUMMARY

The activity of the corpora allata does not play a role in the onset of wax production in honeybees. Allatectomy of newly eclosed workers did not affect wax production in adult worker honeybees. Removing the putative neural feedback inhibition on the CA by severing the NCA did not result in a premature onset of wax production. Implanting corpora allata from older workers into younger workers had no significant effect on the onset of wax production.

INTRODUCTION

Examining possible hormonal influences on the physiology and behaviour of insects requires the manipulation of circulating endogenous juvenile hormone titres (Truman and Riddiford 1974). Increasing the inherent JH III titres 10^6 times did not affect the age of the onset of wax secretion, nor did it significantly affect the rate of wax secretion (chapter 3) in worker honeybees. This, however, does not provide conclusive evidence that JH III does not affect wax secretion. In particular the massive doses may have had pharmacological rather than physiological effects. To overcome this, physiological titres should be tested e.g. by CA implantation. The opposite effect can be expected from allatectomy. The only source of JH thus far found in honeybees is the corpora allata (Truman and Riddiford 1974), and may provide evidence of the effect of JH on wax secretion in honeybees. Anti-JH compounds were not used because the full effects of these substances, much less their physiological

mechanisms, are not clearly understood. In some experiments they induced cytotoxic effects, while in others they left the JH titre unaffected (Fluri 1983; Piulachs *et al.* 1989).

Hypopharyngeal gland degeneration, an age-related process, could be slowed down by allatectomising adult workers (Imboden and Lüscher 1975). Because of this, the effect of CA on wax secretion in adult honeybee workers was examined, independently of it being a source of JH III.

MATERIALS AND METHODS

All experiments were carried out at the Department of Zoology and Entomology, Rhodes University. Three queenright colonies, housed in 5-frame nucleus hives, were used in each of the experiments. Each had a central brood nest of 3 frames, containing brood and food, and an empty frame on either side of the central nest where additional comb building could take place. The allatectomy method used was that described by Imboden and Lüscher (1975), rather than the method described by van Laere (1974), as the former resulted in a lower mortality of adult honeybee workers (pers. obs.).

Experiment A: Allatectomy of newly emerged workers

Allatectomies were performed on newly eclosed workers to remove the source of circulating juvenile hormone from the honeybee as soon as possible after eclosion, thus minimizing possible effects of juvenile hormone. Only complete allatectomies were performed as CA are capable of asymmetrical JH biosynthesis (Tobe 1977; Schooneveld *et al.* 1979) and studies have shown that, in unilateral allatectomy studies, the remaining CA is capable of increasing its JH biosynthetic rate enough that the haemolymph JH titre remains the same as when both CA were present (Schooneveld *et al.* 1979).

One hundred and twenty newly emerged adult workers (eclosed overnight in an incubator at 32°C - 34°C) were evenly divided into 1 of 3 treatment groups: control workers (no further

treatment); control incision or sham-operated workers (workers were cut and sealed); and allatectomised workers (both CA were removed). Workers were anaesthetized on ice until immobile; surgically treated; marked according to age and treatment; and released once they had fully recovered. This was repeated every 3 days, until there were 7 age groups. On day 21 of the experiment, all surviving marked bees were recaptured and killed, and their wax scales removed and counted. The total amount of wax produced by individual workers was weighed on a Cahn C-31 microbalance. Data were analysed using ANOVA and Scheffe's test (BMDP 1990).

Experiment B: Severing of NCA I and II of newly emerged workers

The CA are regulated by a feedback system which appears to function even when the nerves to the CA/CC complex have been severed (Schooneveld *et al.* 1979). An experiment, to test whether removing the neural feedback from the CA would modulate wax secretion, was designed.

One hundred and twenty newly emerged adult workers (eclosed overnight in an incubator at 32°C - 34°C) were evenly divided into 3 treatment groups. The groups were: untreated control workers; incision control (or sham-operated) workers; and surgically treated workers. The same surgical procedure as in Experiment A was used except that the severed CA were not removed. This was repeated every 3 days, until the oldest treatment group was 12 days old. All surviving marked bees were then recaptured and killed, and the total number of wax scales produced by each worker counted and weighed on a Cahn C-31 microbalance. Data were analysed using ANOVA and Scheffe's test (BMDP 1990).

Experiment C: Implanting 9-day-old CA into newly emerged workers

One hundred newly emerged adult workers were marked and released into each experimental colony every 3 days. When the first cohort of marked bees was 9 days old, 20 of these workers were removed from each colony, immobilized on ice and their corpora allata

surgically removed. These fresh CA were immediately transplanted into the heads of newly eclosed adult workers. Both a treated control and an untreated control were created and marked accordingly. After all the workers had been treated and had fully recovered, they were returned to their hives. This was repeated every 3 days, until there were 7 age cohorts. On day 21 of the experiment, all surviving marked workers were recaptured and killed. Wax scales produced by each worker were removed, counted and weighed on a Cahn C-31 microbalance. Data were analysed using ANOVA and Scheffe's test (BMDP 1990).

RESULTS

Experiment A: Allatectomy of newly emerged workers

The hive from which the workers were removed significantly affected the wax production of the workers ($p = 0.0019$) with workers from one of the hives producing significantly less wax than the others. The mean amount of wax produced by the youngest and oldest workers was significantly different from that of the remaining age cohorts ($p < 0.0000$). The untreated control workers produced significantly more wax than either of the treated cohorts ($p = 0.0042$), while there was no significant difference between control treatment and allatectomised workers. Despite there being significant age and treatment effects, an interaction between these factors proved to be non-significant (Fig. 4.1, $p = 0.7675$); the hive effect was also found to be non-significant in an interaction. Similar results were shown by the number of wax scales found on workers of different ages and subjected to different treatments.

Experiment B: Severing of NCA I and II of newly emerged workers

The mean amount of wax produced was unaffected by the hive from which the workers were removed ($p = 0.1765$). The age significantly affects the amount of wax produced, with young workers producing significantly less wax than old workers ($p < 0.0000$). The control-treated workers produced significantly more wax than either the untreated control or NCA-severed

workers ($p = 0.0011$) but there was no difference between the untreated controls and NCA-severed workers. An interaction between age and treatment revealed there was no significant difference in the mean amount of wax produced (Fig. 4.2, $p = 0.2711$) while an interaction including hive also proved to be non-significant ($p = 0.3250$).

The number of wax scales produced by workers from the different hives was significantly different ($p = 0.0001$) with one of the hives producing significantly more wax scales. Age and treatment affected the number of wax scales in a similar manner to the mean amount of wax produced. When hive was included in an ANOVA (BMDP 1990), however, there was no significant difference ($p = 0.8722$) in the number of wax scales produced.

Experiment C: Implanting 9-day-old CA into newly emerged workers

The hive from which the workers were removed did not significantly affect the mean amount of wax found on the workers ($p = 0.1061$). Three- and 6-day-old workers produced significantly less wax than older workers ($p < 0.0000$). A significant treatment effect revealed that workers with implanted CA produced less wax than the untreated controls ($p < 0.0000$). There was no significant difference in the mean amount of wax produced between the untreated and treated control workers nor was there a significant difference between the treatment control and the workers who had received CA ($p > 0.05$). Although an interaction between age and treatment was found to significantly affect mean wax production (Fig. 4.3, $p = 0.0121$), Scheffe's test (BMDP 1990) revealed the differences to exist between, rather than within, discrete age groups.

The number of wax scales produced was significantly affected by hive (one of the hives produced significantly fewer wax scales, $p = 0.0056$), age (3 day old workers produced significantly fewer wax scales, $p < 0.0000$) and treatment (workers with implanted CA produced fewer wax scales, $p = 0.0006$). However, interactions between these factors revealed that differences existed between, rather than within, levels.

DISCUSSION

That the amount of wax produced was largely influenced by the hives from which the workers were recaptured, although not in each of the experiments, suggests that wax production is stimulated by colony need. A strong but inconsistent treatment effect was present in each of the experiments, resulting in reduced wax production in Experiments A and C, but increased wax production in Experiment B. However, further CA treatment did not significantly affect wax production.

Removing the CA from young workers did not affect the age-related pattern of wax production (Fig. 4.1), measured either as the mean amount of wax produced or as the number of wax scales found on the workers of various ages. These results contrast with those obtained for HPG (Imboden and Lüscher 1975) and vitellogenin synthesis (Imboden *et al.* 1976) where allatectomy resulted in effects which were opposite to those resulting from application of JH III to the workers. However, neither study analysed treatment effects using treatment controls.

The biosynthetic activity of the CA is altered, but not inhibited, by denervation (Couillaud *et al.* 1984; Weaver 1984). Activity of the CA is regulated by both neural and humoral systems (Stay and Tobe 1978; de Wilde and Beetsma 1982; Tobe and Stay 1985). Denervated CA/CC complexes, of both a cockroach and the Colorado potato beetle, maintained their activity, while implantation of CA suppressed the host CA activity (Stay and Tobe 1978; Schooneveld *et al.* 1979). However, removal of the neural feedback system of the CA in adult worker honeybees did not result in a change in wax production (Fig. 4.2).

Wax secretion remained unaffected by implanting CA (presumed active) from older bees into younger workers (Fig. 4.3). Implanted CA can continue producing JH and can suppress the activity of the host CA (Stay and Tobe 1978; Schooneveld *et al.* 1979). Müssbichler (1952) was unable to alter the ovary size of workers, thought to be under JH regulation, by implantation of CA. Implanting 9-day-old CA into newly eclosed workers was thought to have a similar effect to raising the circulating JH titres, but to be of a longer duration as there is no half-life effect of the single-dose injected JH III. Although it has since been shown that

cohorts of house bees of different ages do not differ in their mean JH biosynthetic rate (Huang *et al.* 1991), the ability of implanted CA to continue synthesizing JH, often at an elevated level within physiologically normal concentrations (Stay and Tobe 1978), without neural inhibition, would result in prematurely elevated JH III titres in workers.

The age-related increase in adult worker honeybee CA size has been loosely correlated with age-related behaviour, physiology and glandular development (chapter 1). As wax secretion is age-related (chapters 1-4) it was thought that this could be under CA, and JH III, regulation. The experiments reported here have shown that wax secretion is not affected by corpora allata whether they are absent, or their neural feedback is removed, or their number in an experimental host increased.

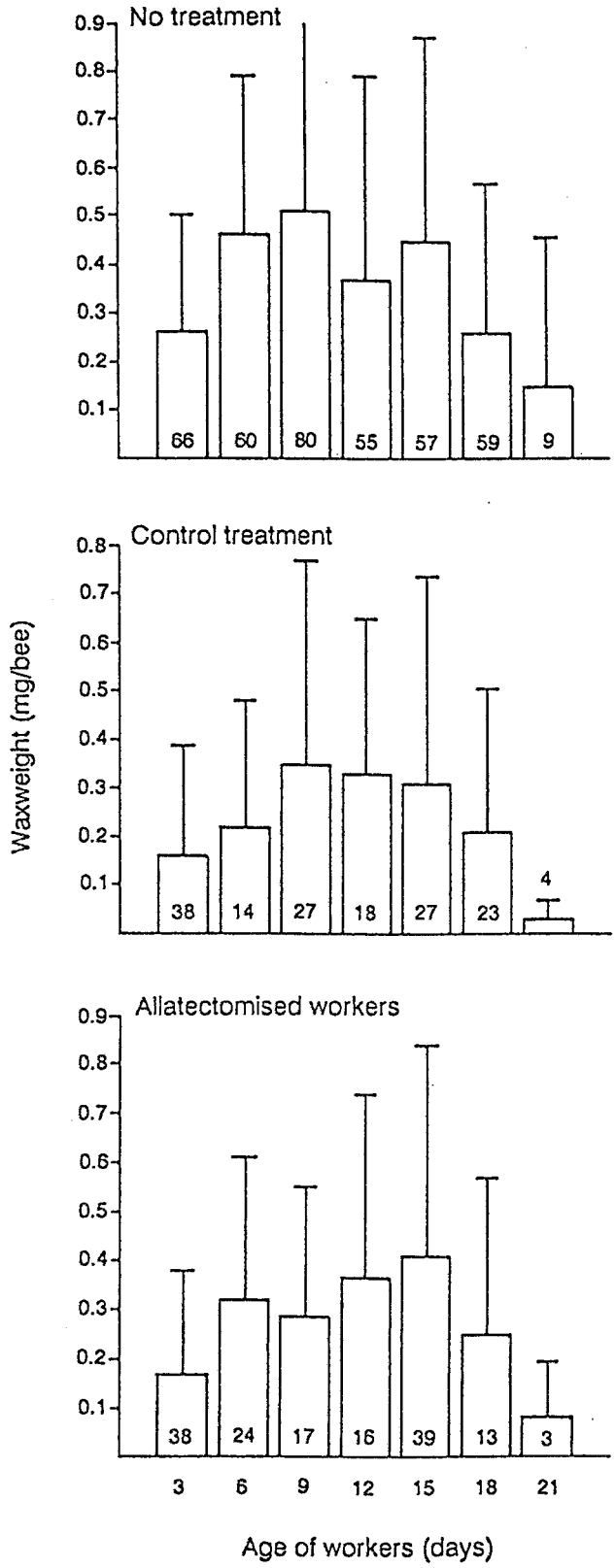

Figure 4.1 Mean amount of wax produced by workers allatectomised within 24 hours of eclosion (mg/bee ± SD). Sample sizes are indicated in the bars

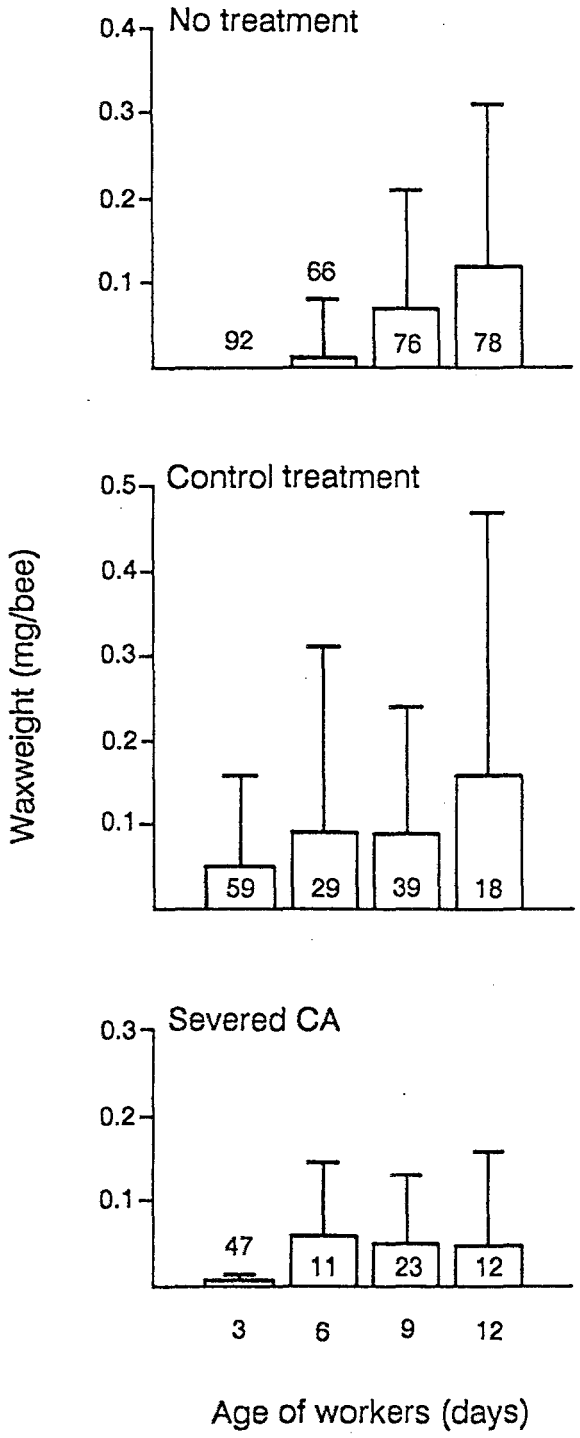

Figure 4.2 Mean amount of wax produced by workers whose CA were severed within 24 hours of eclosion (mg/bee ± SD). Sample sizes are indicated in the bars

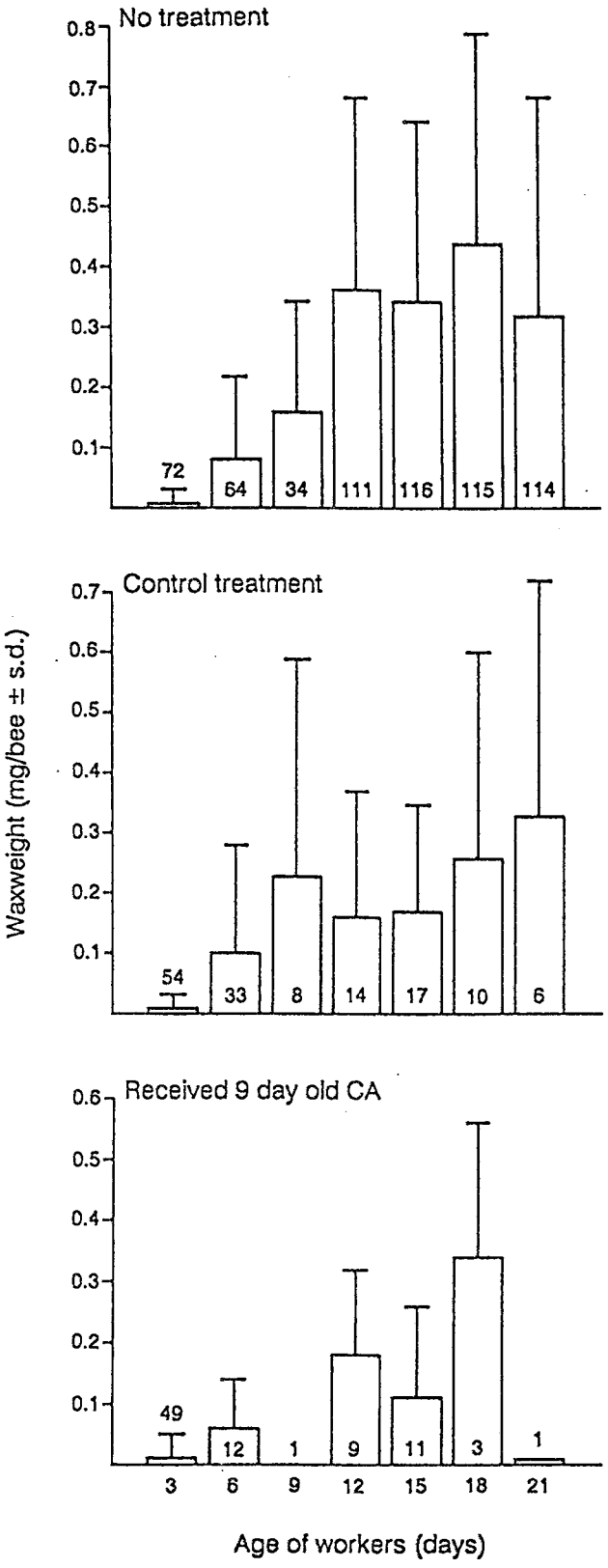

Figure 4.3 Mean amount of wax produced by workers with implanted 9-day-old CA (mg/bee ± SD). Sample sizes are indicated in the bars

CHAPTER 5

A COMPARISON OF THE EFFECT OF METHOPRENE AND JH III ON WAX PRODUCTION IN THE HONEYBEE, *Apis mellifera capensis*

SUMMARY

Reduced amounts of wax were found on workers treated with methoprene. Because large doses of methoprene apparently poison worker honeybees, the validity of experiments in which methoprene was used to alter the behaviour and physiology of adult worker honeybees is questioned. Under these conditions the use of a treated control and an untreated control in the same experiment becomes vital. Application method did not significantly affect wax production. The amount of wax found on the workers was significantly affected by hive and age.

INTRODUCTION

Juvenile hormone homologs are chemical compounds which are similar in structure (Dorland's Illustrated Medical Dictionary 1974). A juvenile hormone analog is an hormonally active chemical compound which is similar to JH in its effects, but differs from the JH homologs with respect to certain structural components. Its action may be metabolically similar, or opposite, to that of the homolog (Dorland's Illustrated Medical Dictionary 1974; Chen and Mayer 1985). JH analogs resemble homologs functionally but are not necessarily similar in structure (Retnakaran *et al.* 1985). In many instances, the effects of the analogs and the natural hormones cannot be differentiated (Retnakaran *et al.* 1985). There are many substances with recognised JH activity, some of which are natural and others are synthesized (Schneiderman and Gilbert 1964; Wigglesworth 1969; Staal 1975; Chen and Mayer 1985;

Retnakaran *et al.* 1985). There are over 500 analogs of JH, with varying insecticidal activity (Retnakaran *et al.* 1985) e.g. methoprene and hydroprene.

Manipulating honeybee physiology and behaviour by using analogs has yielded interesting results. Methoprene is an analog of JH that is frequently used as a substitute in studies of honeybee polyethism and physiology (Wirtz and Beetsma 1972; Jaycox *et al.* 1974; Rembold *et al.* 1974; Beetsma and ten Houten 1975; Jaycox 1976; Copijn *et al.* 1978; Robinson 1985, 1987a, b; Sasagawa *et al.* 1986, 1989; Robinson and Ratnieks 1987; Robinson *et al.* 1989, 1992; Sasagawa *et al.* 1990). Robinson and Ratnieks (1987) found that hydroprene was more likely to elicit a response than methoprene, with topical application more efficient than oral application.

The failure of JH III titre manipulation, by application of JH III or CA removal or implantation (chapters 3 and 4), to alter wax production in worker honeybees raised the question of whether results obtained in previous studies, in which behaviour and physiology were altered, may have arisen as a consequence of the treatment or hormone used and were thus experimental artifacts. An experiment was designed in which the effects of methoprene were compared with JH III. The dosages used were those which had elicited responses in previous studies where "positive" responses were reported (Robinson 1985, 1987a, b; Robinson and Ratnieks 1987; Robinson *et al.* 1992). Topical and injection application methods were compared.

MATERIALS AND METHODS

Three queenright colonies were maintained at the Department of Zoology and Entomology, Rhodes University. Each colony had 6 frames, containing the broodnest in 3 central frames with food stores surrounding it. The bees were not fed as ample forage was available for the duration of the experiment.

Newly emerged workers (eclosed overnight in an incubator maintained at 32°C-34°C) were obtained from a non-experimental colony. Each day, the newly emerged workers were divided

into 3 treatment groups as follows: control group (untreated workers); a topical application experimental group and an injected application experimental group. The experimental groups were further treated as follows: 50 µg methoprene/ 1 µl acetone (topical application); 1 µg JH III/ 1 µl acetone (topical application); 1 µl acetone (topical application); 50 µg methoprene/ 1 µl sweetoil (injected); 1 µg JH III/ µl sweetoil (injected); and 1 µl sweetoil (injected). Workers were treated every 3 days and marked according to the treatment received, until there were 4 age cohorts, aged 3 - 12 days. On day 12 of the experiment, all surviving marked and treated workers were recaptured and killed. Wax scales of each worker were removed and weighed, on a Cahn C-31 microbalance. Data were analysed using ANOVA and Scheffe's test (BMDP 1990).

RESULTS

The mean amount of wax found on the workers was significantly affected by the hive from which the workers were recaptured ($p < 0.0000$) with workers from each of the hives producing significantly different amounts of wax. Age significantly affected the wax production ($p = 0.0047$) with 3-day-old workers producing significantly less wax than older workers.

The treatment workers received significantly affected wax production ($p < 0.0000$). There was no significant difference between the three control groups ($p > 0.1$). The injected workers produced less wax than the topically treated workers, but within the cohort of injected workers there was no difference between the methoprene and JH III-treated workers ($p > 0.1$), although the methoprene-injected workers did show relatively reduced wax production. Topical methoprene, injected methoprene and injected JH III-treated workers produced significantly less wax than the control workers ($p < 0.01$); workers treated topically with JH III produced significantly more wax than their injected counterparts ($p < 0.01$) but the reduction in wax shown by the injected workers were possibly as a result of the method of administration.

An interaction between age and treatment did not significantly affect the amount of wax found on the workers (Fig. 5.1, $p = 0.1138$). When the hives were individually examined for an interaction between age and treatment, wax production was significantly affected by the interaction in 2 of the hives but not in the third. However, when hive was included in the analysis as a further factor, the mean amount of wax found on the workers was significantly affected by hive, age and treatment the worker received ($p = 0.0001$). The number of wax scales found on the workers showed the same trends, although the exact p-values differed somewhat.

DISCUSSION

Hive effect

Hive significantly affected the amount of wax produced by workers; the full significance of this was realised only when hive, as an independent interacting factor, was able to significantly affect wax production. It becomes apparent that wax secretion may be a response to colony need (chapter 2) or the nutritional state of the colony (Goetze and Bessling 1959; Freudenstein 1960), rather than purely a product of genes because the treated workers used in the experiment originated from the same parent hive. The influence of the hive environment on a worker's behaviour was also recently demonstrated by Calderone and Page (1992).

Treatment and application method effects

The application method did not affect wax production as there was no significant difference between the control groups, although injecting workers did result in a slight reduction in the mean wax production. The mean amount of wax produced by workers treated topically with methoprene was significantly less than either the topical control group or topical JH III group (Fig. 5.1), yet it was not significantly different from the mean amount of wax produced by injected workers. This suggests that, because the injected workers may show reduced wax

production as a result of application injury, the workers topically treated with methoprene may be exhibiting a pharmacological, rather than physiological, response resulting in a lowered wax output. The mean wax output of the methoprene-injected workers showed a lowered mean wax output which was not significantly different from the JH III-injected group.

Hormone effects

It is important to differentiate between pharmacological and physiological responses to applied drugs; drugs are defined as chemical compounds administered in order to control or improve a physiological condition (Dorland's Illustrated Medical Dictionary 1974). A physiological response is one in which the applied drug enhances a physiological parameter under investigation; a pharmacological reaction suppresses or enhances the physiological parameter to non-normal levels, often resulting in a toxicological response i.e. illness or death. Although the use of an analog in itself might not have resulted in a pharmacological reaction, the dose at which it was applied may have been the cause. The normal JH III titres of newly emerged worker honeybees is approximately 10^6-10^7 times less than the applied doses of analogs and homologs used here and in other studies. While the massive doses of JH III did not affect wax production, the massive doses of methoprene resulted in a decrease in wax production, suggesting that results obtained in previous studies may have been artifacts of treatment and that changes in honeybee behaviour and physiology may have been the product of the workers being ill from poisoning. Methoprene has been registered for pest control (Chen and Mayer 1985) and it must be remembered that analogs were developed as pesticides because they are insect growth regulators which interfere with the normal development of insects (Retnakaran *et al.* 1985).

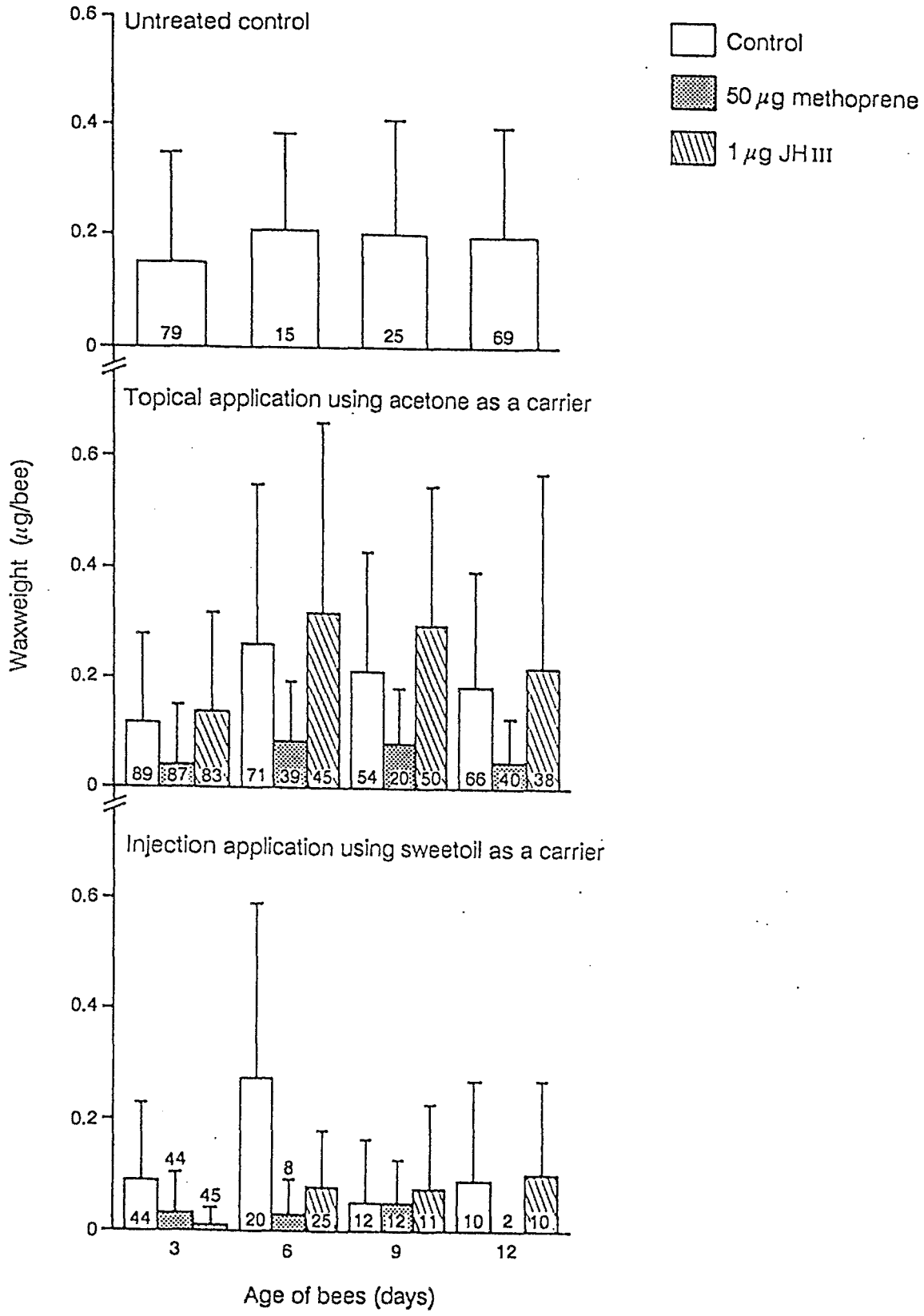

Figure 5.1 Mean amount of wax produced by workers treated topically or internally with 50 µg methoprene or 1 µg JH III (mg/bee ± SD). Sample sizes are indicated in the bars

CHAPTER 6

FINAL DISCUSSION AND CONCLUSIONS

The results of the foregoing chapters have implications for our understanding of both the regulation of wax secretion and the regulation of age polyethism in worker honeybees.

IMPLICATIONS FOR THE REGULATION OF WAX SECRETION

JH and wax secretion

Wax secretion by worker honeybees is related to age (chapters 2-5; Hepburn *et al.* 1984; Hepburn *et al.* 1991; Muller and Hepburn 1992) and is associated with the cycle of development and degeneration of the ultrastructure of the wax glands (Rösch 1927; King 1928; Dreischer 1956; Hepburn *et al.* 1991). It is an integral part of the pattern of age polyethism, which was thought to be controlled by JH III (Robinson 1987c, 1992). However, the results of the previous chapters clearly show that wax secretion is not affected by JH III. Manipulating JH levels, either by injecting workers with JH III doses greatly in excess of the endogenous haemolymph titre, or by using physiological levels achieved through implantation of more mature CA, did not affect wax secretion (chapters 3, 4 and 5). Allatectomising workers, thus removing the only source of JH III known in honeybees, also had no effect on wax production.

Methoprene and wax secretion

Wax production was clearly and significantly reduced by a JH analog (chapter 5) and by the method used to treat the honeybees (chapters 3, 4 and 5). The reduction in wax production

shown by workers treated with an analog appeared to be an abnormal, pharmacological response (chapter 5) comparable to poisoning.

ALTERNATIVE MECHANISM REGULATING WAX SECRETION

An alternative model for the regulation of wax secretion is available. Butler (1954: 93-94) suggested that wax secretion and comb building were a result of the colony's requirements for empty comb in the face of an excess of incoming nectar:

> "If there is insufficient comb space available for the storage of this [incoming] nectar, the household bees are compelled to retain it temporarily within their own honey-stomachs. Now it seems that when a bee has to function as a reservoir for nectar for hours on end a fairly high proportion of the contained sugar is assimilated, and that this results in the wax glands secreting wax scales abundantly...The whole thing is brought about automatically and most effectively...The greater the quantity of surplus sugar that she [the worker] assimilates, the greater the amount of wax she produces, and thus of the raw material for comb construction."

This hypothesis is supported by results from this and other studies. The amount of wax produced is affected by season (chapter 2), by differences between colonies (chapters 2-5) and by colony nutrition (Goetze and Bessling 1959; Freudenstein 1960). It has also been correlated with engorgement of the honey-stomach (Hepburn and Magnuson 1988). These findings imply that adult honeybee workers secrete wax in response to colony needs for comb building or capping of honey or brood, and that levels of wax secretion are independent of endogenous JH III titres. Butler's model needs refining, but more rigorous support of this alternative hypothesis is beyond the scope of this work.

IMPLICATIONS FOR AGE POLYETHISM

Review of experimental work on honeybee age polyethism

Obtaining "positive" responses to exogenously applied JH homologs and analogs depends on: (i) species of experimental insect used and its developmental stage (Wigglesworth 1940; Krishnakumaran and Schneiderman 1965; Staal 1975; Prestwich 1987; Robinson *et al.* 1992);

(ii) the exogenous hormone chosen and the dose in which it is applied (Beetsma and ten Houten 1975; Staal 1975; Sasagawa *et al.* 1986; Prestwich 1987; Robinson and Ratnieks 1987; Hurd *et al.* 1990; Robinson *et al.* 1992); **(iii)** the method of application and the carrier used (Krishnakumaran and Schneiderman 1965; Wigglesworth 1969; Metzler *et al.* 1972; Ajami and Riddiford 1973; Patterson 1973; Riddiford and Ajami 1973; Schwieter-Peyer 1973; Erley *et al.* 1975; Staal 1975; Jaycox 1976; Copijn *et al.* 1978; Edwards and Rowlands 1978; Brouwers 1983; Tobe and Stay 1985); and perhaps **(iv)** on the endogenous JH titre (Varjas *et al.* 1976; Edwards and Rowlands 1978; Hurd *et al.* 1990).

Of the thirteen JH-related behavioural studies in worker honeybees, all revealed at least one of the following methodological shortcomings, discussed in order of their impact on the current study.

Drug effects

Hormones are frequently applied experimentally to insects without consideration of the identity of the endogenous JH or even its titre; even JH homologs show differences in their ability to elicit a response (Riddiford and Ajami 1973; Schwieter-Peyer 1973; Staal 1975).

Insect JH titre is a balance between synthesis and release on the one hand, and degradation and excretion on the other (Tobe and Stay 1985) and the physiological significance of JH titre is currently being debated. Species-specific differences in the rate of biosynthesis and release of JH from the CA cause differences in titre (Pratt *et al.* 1975). The presence or absence of JH during a critical period is thought to be more important than the actual JH titre at the target organ (Nijhout and Wheeler 1982; Couillaud *et al.* 1984; Strambi *et al.* 1984). This argument is based on the substantial variation which exists in JH titres between conspecific individuals (Lanzrein *et al.* 1978).

On the other hand, the effective JH titre may be the concentration observed in the direct vicinity of the target organ during a critical phase and the physiological responses may be due to high and low titres of JH (de Wilde *et al.* 1971). Caste-specific trends in JH titre have been

noted, lending support to this model (de Wilde *et al.* 1968; Pratt *et al.* 1975; Hartfelder 1987). However, JH titres are usually determined from pooled insect haemolymph samples. They therefore represent an average of the JH titre of several individuals and do not indicate the active titre at the target organ (Varjas *et al.* 1976). Insufficiently sensitive measuring methods make this argument difficult to test at present.

Notwithstanding these problems, measurements of endogenous honeybee JH titres range from 0.7-10 × 10^{-3} µg/g for whole-body extracts that include the CA (Trautmann *et al.* 1974; Hagenguth and Rembold 1978; Rembold and Lackner 1985; Rembold 1987), and 1-120 × 10^{-6} µg/µl for CA-free pooled haemolymph extracts (Rutz *et al.* 1976; Fluri *et al.* 1977; Fluri *et al.* 1982; Bühler *et al.* 1983; Robinson *et al.* 1987; Robinson *et al.* 1989, 1992; Huang *et al.* 1991). Individual titres measured by HPLC (Sasagawa and Kuwahara 1988) revealed titres from 0-7600 × 10^{-6} µg/µl haemolymph. Because of the large variation in CA volume (Pflugfelder 1948; van Laere 1966; Gast 1967; van Laere 1971; Breed 1983) and JH III titres between individuals of the same age, large sample sizes must be used before significant and meaningful trends emerge conclusively. To date, measurements of JH titres and model extrapolation have been inadequate, with too few measurements being made and sample sizes being too small (Rutz *et al.* 1976; Fluri *et al.* 1982; Robinson *et al.* 1987; Robinson *et al.* 1989).

As the doses of applied JH and JH analog used in polyethism studies exceed the JH titre of worker honeybees by about a million times, it is highly probable that the responses obtained are entirely pharmacological and probably toxic. The results of JH studies must therefore be interpreted cautiously, with the endogenous JH titre in mind.

Analog artifacts

Analogs have frequently been used in studies in which the control of age polyethism in adult honeybee workers was investigated (Jaycox *et al.* 1974; Rutz *et al.* 1974, 1976; Jaycox 1976; Robinson 1985, 1987a, b; Sasagawa *et al.* 1986, 1989; Robinson and Ratnieks 1987; Robinson *et al.* 1989, 1992; Sasagawa *et al.* 1990). Responses of workers to analogs were found to be

dependent **(i)** on the drug chosen (Beetsma and ten Houten 1975; Sasagawa *et al.* 1986; Robinson and Ratnieks 1987); **(ii)** on application method (Robinson and Ratnieks 1987); **(iii)** on timing of application (Robinson *et al.* 1992); and **(iv)** on the dose (Jaycox *et al.* 1974; Imboden and Lüscher 1975; Imboden *et al.* 1976; Rutz *et al.* 1976; Copijn *et al.* 1978; Dietz *et al.* 1979; Breed 1983; Robinson 1985, 1987a, b; Sasagawa *et al.* 1986, 1989; Robinson and Ratnieks 1987).

It must be remembered that JH analogs adversely interfere with the normal growth and development of insects by influencing the metamorphic changes in the insect (Retnakaran *et al.* 1985) and were developed as pesticides specifically because of this property. Manipulating honeybee physiology and behaviour with analogs has yielded interesting results but these must be treated as equivocal because of the likelihood that they are toxicological artifacts.

Methoprene, a registered pesticide, is used most commonly in honeybee studies to change behaviour. Methoprene is a 19-Carbon chain molecule and structurally unlike any of the naturally occurring juvenile hormones (Staal 1975) and is therefore a JH analog. For methoprene to be biologically active, it needs the JH receptor sites to recognize its conformation. Katagi *et al.* (1989) predicted the shape of the reactive sites of JH: its size was estimated and thought to consist mainly of hydrophobic regions with both ends of the site possessing opposite charges to the JH. An important consideration when using analogs or mimics is whether the homolog receptor sites can recognize the substitute. To date, there has been no evidence that this has been established for JH analogs. The mode of action of methoprene is not yet fully understood e.g. the effects of methoprene on the genome are not known (Retnakaran *et al.* 1985).

Osir and Riddiford (1988) reported that JH III and methoprene do not compete for binding sites. This suggests that there are either two distinct binding sites on one receptor, or two distinct receptors. In the first case, the same physiological reaction can still occur. If, however, there are separate receptors, the physiological responses elicited by the two could be different. This result implies that, although methoprene may elicit behavioural responses, these may be pharmacological rather than physiological. Wyatt *et al.* (1987) were able to

elicit a response using methoprene, but not by using the endogenous JH - this definitely implies a pharmacological response and that methoprene is not a valid JH substitute.

Krishnakumaran and Schneiderman (1965) stated that a physiological reaction was one in which only nanograms of the applied JH or JH analog are required to elicit a response. Using this as the basis for determining whether a reaction is pharmacological or physiological, all the hormone studies performed on honeybees so far resulted in pharmacological reactions as they all used micrograms of JH, or its analogs, to elicit responses (Jaycox *et al.* 1974; Rutz *et al.* 1974; Imboden and Lüscher 1975; Imboden *et al.* 1976; Jaycox 1976; Rutz *et al.* 1976; Breed 1983; Robinson 1985; Sasagawa *et al.* 1986; Robinson 1987a, b; Robinson and Ratnieks 1987; Liu 1989; Sasagawa *et al.* 1989; Sasagawa *et al.* 1990).

What then is a toxicological response? If methoprene inhibits CA activity (Sasagawa *et al.* 1986), does this constitute poisoning? The application of JH III in physiological doses should not result in the degeneration of the CA. In some non-honeybee studies methoprene elicited responses not shown under normal physiological conditions (Wyatt *et al.* 1987; Cameron and Robinson 1990). Wang and Moeller (1970) found that ill honeybees began foraging at an earlier age. Analogs, especially methoprene (purely because it is the most commonly used analog in honeybee research), have resulted in premature guarding and foraging - the same response obtained from ill workers. This implies that the analog treated workers were ill, possibly poisoned by the high doses, and that the analog is, in effect, toxic to the honeybee workers.

The results of age polyethism studies done with methoprene can, therefore, be discounted.

Half-life effects

Reactions to exogenous hormones are not always sustainable and may even show an extremely localized effect (de Loof and de Wilde 1970). The half-life of exogenous hormones affects the quality of response, as the shorter the half-life, the less time the drug can exert an effect. Half-lives of JHs (particularly JH I) are between 2 and 12 hours, depending on whether

injected or applied topically (Metzler *et al.* 1972; Ajami and Riddiford 1973; Erley *et al.* 1975; Staal 1975; Copijn *et al.* 1978; Edwards and Rowlands 1978; Tobe and Stay 1985); a million-fold overdose of a drug with a 4-hour half-life will be physiologically inactive in five days. Half-lives have been found to be temperature-dependent (de Kort *et al.* 1977). The problem of half-life of the applied hormone can be overcome by injecting in a slow-release carrier but, this can have treatment effects.

Treatment effects

There is often a strong but unpredictable treatment effect caused by the application method, and the carrier used (chapters 3-5), although chapter 5 revealed no significant differences between injection and topical administrations. Because the effect can be non-significant (Breed 1983; Robinson 1985; Sasagawa *et al.* 1989) or significant (Sasagawa *et al.* 1986), it is not predictable and therefore must be tested for. However, the effect of treating workers have largely been ignored in honeybee JH research (Jaycox *et al.* 1974; Rutz *et al.* 1974; Imboden *et al.* 1976; Robinson 1987a, b; Robinson and Ratnieks 1987; Liu 1989; Robinson *et al.* 1989). When they were found, treatment effects were sometimes ignored (Imboden and Lüscher 1975). These treatment effects confound the interpretation of these polyethism experiments.

Hive effects

Whenever it was tested for, a strong hive effect was found (chapters 2-5). In other studies hive effects were not apparent because only 1 colony was used (Jaycox *et al.* 1974; Imboden and Lüscher 1975; Imboden *et al.* 1976; Jaycox 1976; Rutz *et al.* 1976; Robinson and Ratnieks 1987; Robinson *et al.* 1989), or cohorts of bees were used (Rutz *et al.* 1974; Breed 1983), or hive effects were simply not tested for (Robinson 1985, 1987b; Sasagawa *et al.* 1986, 1989; Liu 1989; Robinson *et al.* 1992). The significance of hive effect was demonstrated by Calderone and Page (1992) who suggested that a worker's behaviour was affected largely by her environment, including the genotypes of her nestmates; this was also

clearly demonstrated in chapter 5, where genetically related workers (obtained from the same parent hive) were placed into different hives where they produced significantly different amounts of wax. The hive effect was stronger than the genetic component on wax secretion (chapter 5).

The results of many studies were presented without an indication of how much variation was shown by individual workers (Jaycox *et al.* 1974; Rutz *et al.* 1974; Imboden *et al.* 1976; Jaycox 1976; Sasagawa *et al.* 1986; Robinson 1987b; Robinson and Ratnieks 1987; Liu 1989); in very few studies is the amount of variation indicated (Breed 1983; Robinson 1985, 1987a; Sasagawa *et al.* 1989).

JH and age polyethism

JH titre has been suggested to rise according to a genetically regulated pattern but is subject to modifications by extrinsic factors, leading to plasticity in age polyethism and individual variation in responses to work stimuli (Robinson 1987c, 1991, 1992; Robinson *et al.* 1990). Results obtained by Robinson *et al.* (1989) suggest that behavioural changes are a result of genotypic variation between individuals that affects their responses to extrinsic stimuli by modulating JH titres. Even if all thirteen papers on the effects of JH on age polyethism did not show the serious methodological flaws that they do, could a case be made that JH III titre modulates age-related behaviour in the honeybee worker?

Causation and correlation

Studies in which behaviour was correlated with CA volume (van Laere 1971) and JH III titre (Robinson *et al.* 1989; Huang *et al.* 1991) are difficult to interpret because the direction of the implied cause and effect relation is unclear. Given that JH titre is not modulated by JH esterase activity (de Kort *et al.* 1977, Bühler *et al.* 1983) and that haemolymph volume decreases with age (Crailsheim 1985), then if the CA have a constant rate of synthesis, there would be an increase in JH III titre with age. Similarly, if JH quantity is measured in absolute

terms rather than relative to haemolymph volume, then the exact same amount of JH III will appear to increase as haemolymph volume decreases. However, the rate of JH biosynthesis is age-related, with that of the foragers being significantly higher than that of nurse bees (Huang *et al.* 1991, Robinson *et al.* 1992).

Thus, the causation may flow the opposite way; rather than being age-related, the rate of biosynthesis may be behaviourally mediated since young workers are capable of becoming foragers and foragers can revert to nursing (Rösch 1930; Ribbands 1953; Winston and Fergusson 1985; Robinson *et al.* 1989). This is supported by data from Huang *et al.* (1991), who found a decrease in the biosynthetic rate after a maximum was reached in foragers. The only obvious differences in JH titre were those between the foragers and the house bees; there were no differences between house bees of different ages. With so many co-varying variables, the chances of finding spurious correlations is good. Correlation does not imply causation.

JH and environment

Is age polyethism, then, regulated by the environment, through JH III titre, to bring about the changes in behaviour with age? Bühler *et al.* (1983) were unable to show conclusively that CO_2 and temperature modulated JH III titre, hypopharyngeal gland weight or haemolymph volume. They could not satisfactorily explain changes in behaviour which are normally associated with CO_2 and temperature changes with different brood or hive conditions, in terms of JH titre. Displays of aggression and defensive behaviour could not be conclusively shown to be under JH III modulation (Breed 1983, Robinson *et al.* 1987). Huang *et al.* (1991) found that rates of JH biosynthesis only differed between house and field workers and that there was no difference between age cohorts of house workers. The roles of the CA, and JH, in the development of honeybee ovaries, oogenesis and vitellogenesis are negligible (Müssbichler 1952, Gast 1967, Engels and Rammamurty 1976).

On the other hand, it has been possible to change the schedule of honeybee worker age polyethism and physiology by using pathogenic non-JH substances and methods: CO_2 (Ribbands 1950; Simpson 1954); *Nosema* infection (Wang and Moeller 1970); OAG (a protein

kinase C activator, Sasagawa *et al.* 1990). These appear to have toxic effects, resulting in premature ageing.

In summary, there seems little solid support for a role for JH in causing polyethism in bees.

ALTERNATIVE MECHANISMS REGULATING AGE POLYETHISM

There is no doubt that there is a pattern of changes in the behaviour of adult worker honeybees. However, the evidence discussed in the previous section of this chapter undermines the role of JH III as the mechanism underlying age polyethism. Alternative mechanisms for age polyethism need to be developed. Two distinct, but not entirely separate, models are being developed.

Biogenic amines in the honeybee brain act as neurotransmitters, neuromodulators and neurohormones, and can cause changes in behaviour (Evans 1980), although it is not yet clear whether the changes are due to the amines, metabolites or substances released by the amines. Modulatory chemical processes in the bee brain affect the neural processing of sensory information (Erber *et al.* 1991). Age-related changes in some honeybee brain neuroactive compounds, or biogenic amines, have been reported (Fuchs *et al.* 1989; Taylor *et al.* 1992) and these changes could offer a more likely explanation for the pattern of polyethism found in honeybee workers than do changing JH III titres. Environmental stimuli may result in changes in the neuroactive compounds in the brain which may explain changes in behaviour, through a process of learning or experience, perhaps with a genetic component.

Using a system of regulatory processes, such as those known to exist in honeybees (Seeley *et al.* 1991), biological systems in general can produce different patterns of behaviour based on the information being received (Dickinson 1988), rather than their behaviour being a direct product of hard-wired regulation such as hormone secretions. It has been demonstrated that workers experienced in tasks are more likely to perform the tasks again and recruit novice workers to the task (Seeley 1983; Moore *et al.* 1987). Brandes (1990) found that workers with a higher learning ability were more likely to participate in tasks. Polyethism and other highly

organized behaviours emerge spontaneously from models of self-organization and learning in eusocial insects e.g. ants (Deneubourg *et al.* 1987), wasps (Theraulaz *et al.* in press) and bumble bees (Hogeweg and Hesper 1983). The colony-level pattern of brood, pollen and honey storage on honeybee combs, and the colony-level regulation of nectar foraging, are apparently a product of self-organization (Camazine *et al.* 1990; Camazine 1991; Seeley *et al.* 1991) and similar learning-based feedback models may explain behaviour in honeybees. Age polyethism in honeybees is better understood as the product of a self-organization process based on an interaction between learning and colony need, rather than worker genotype alone, as genes merely supply the material needed but expression is dependent on their environment (Nijhout 1990).

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