Ultrastructural Development in the Corpus Allatum of the Adult Worker Honey Bee

THESIS

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"Long ago it became evident that the key to every biological problem must finally be sought in the cell, for every living organism is, or at sometime has been, a cell."

Edmund B. Wilson (1925)

"The Cell in Development and Heredity." 3rd Ed. Macmillan, Inc.

ABSTRACT

The ultrastructure of the corpus allatum of the Cape worker honey bee has been examined in a systematic way during the first thirty days of adult life.

Corpus allatum size in the Cape worker honey bee shows the age--dependent increase typical of the European worker honey bee, and in the Cape worker bee, the duration of increase is protracted.

Analysis of ultrastructural development provides three indicators of metabolic status: mean mitochondrial size, "light and dark" cells, and extracted vacuoles.

Significant fluctuations in mean mitochondrial size indicate a cyclical nature of cellular activity. New thought on the nature of "light and dark" cells proves that "dark" cells are almost certainly active in the process of JH biosynthesis, whilst "light" cells are definitely not active in JH biosynthesis. Extracted vacuoles found in corpus allatum cells during this study are thought to be remnants of lipid vacuoles, and the build up in number of these vacuoles is regarded as an indicator of reduced biosynthetic activity.

Since the two indicators of decreased JH production ("light" cells and extracted vacuoles) co-exist with smaller mean mitochondrial size, larger mean mitochondrial size is taken as indicating increased levels of JH biosynthesis. Hence, fluctuations in mean mitochondrial size suggest cycles in the levels of JH production in individual corpora allata of the adult worker honey bee.

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INTRODUCTION

Since the pioneering experiments of Sir Vincent Wigglesworth in the early 1930's the corpora allata have been under the intense scrutiny of very many biologists. By secreting juvenile hormone (JH), the corpora allata play a vital role in the regulation of metamorphosis in almost every insect species. With the progress of time, the scientific riddle posed by this pair of hormone glands has become increasingly intricate. As well as ensuring retention of larval body structure during moulting in the immature insect, JH influences caste formation in social insects and regulates a variety of physiological processes in the adult insect, notably those related to reproductive development (see Schneiderman, 1972).

In adult worker honey bees, JH has complex and far-reaching effects on physiology and behaviour. In a review of the endocrinology of the honey bee, Hardie and Lees (1985) provide the following scenario for adult worker honey bees in a European colony during summer: After adult ecdysis, the corpora allata of the worker bee increase in size. Associated with this size increase is an increase in haemolymph JH titre. The increase in JH titre appears to affect the polyethism of worker bees wherein workers switch from task to task with age, notably from hive-bound activities to field activities. If one raises the haemolymph JH titre of newly ecdysed bees artificially (by implantation of supernumary corpora allata, or by injection of JH or a JH analogue) the bees move from hive-bound activities to guarding and foraging activities at a younger age. Various physiological factors normally associated with this behavioural switch are also found to be affected by corpus allatum or JH manipulation.

For example, the timing of atrophy of the hypopharyngeal feeding glands is brought forward by an injection of JH.

Through its effects on individual worker behaviour, JH plays a central role in determining the division of labor within a colony. Robinson (1987) has demonstrated that the timing of each of the four major categories of behaviour that individual worker bees perform is significantly affected by topical application of JH analogue, in a dose-dependent fashion. Further experiments (Robinson et al., 1989) suggest that a colony's ability to adapt to environmental conditions rests ultimately upon varied and reversible rates of JH production in the corpora allata.

Considering the quantity of research that has been done on the physiology of corpora allata and JH in the honey bee, an exploration of corpus allatum functional morphology makes a pertinent contribution to honey bee endocrinology. Wirtz (1973) and Goewie (1978) have described corpus allatum ultrastructure in honey bee larvae. They have documented morphological development in relation to caste differentiation. General morphology of the corpus allatum of the adult worker honey bee, including a detailed description of the gland wall, nervous innervation, and tracheal supply have been documented by van Laere and Lagasse (1973). Breed (1983) has described structural relationships between the corpora allata and the rest of the retrocerebral endocrine complex of the adult worker bee.

In the present work, dissection of adult worker honey bees of known ages provides observations of corpus allatum ultrastructure at regular intervals in the first thirty days of adult life. Interpret-

ation of changes in morphology during this time period gives insight into the nature of corpus allatum function.

Workers of the Cape honey bee Apis mellifera capensis were the subjects of study. The Cape honey bee is known to differ from other African and European honey bees, especially in reproductive development of the worker caste (Hepburn and Crewe, 1990). For this reason, an initial investigation was performed into the sizes of corpora allata at different ages in the Cape worker bee to confirm the age-dependent increase in size found in the European subspecies. This confirmation permits a greater confidence in extrapolation of the following ultrastructural findings to knowledge of the European honey bee and vice-versa.

MATERIALS AND METHODS

Newly emerged worker bees were individually marked with enamel paint and placed into a hive. Ten bees were taken for immediate dissection. Thereafter, ten marked bees were sampled every three days until the thirtieth day. Bees for dissection were placed in a deep freeze (-20°C) for approximately ten minutes. This usually killed the bees but never resulted in freezing of body tissues (I discovered to my inconvenience that complete freezing of bees resulted in such alteration of body tissues as to render dissection of corpora allata almost impossible.) Corpora allata were dissected out under 2.5% gluteraldehyde in 0.1% phosphate buffer.

In preparation of corpora allata for transmission electron microscopy, double fixation was used: primary fixation in 2.5% gluteraldehyde and secondary fixation in 1% osmium tetroxide (both fixatives in 0.1% phosphate buffer.) The embedding medium was a resin mixture containing Araldite CY212 and Agar 100. Agar 100 was later substituted for TAAB 812. The latter mixture is of tested reliability in a wide range of biological tissues (Cross, 1989.) After infiltration overnight in resin, the corpora allata were embedded in fresh resin in flat moulds and the resulting blocks were polymerized at 60°C for forty hours. Ultramicrotomy was performed on an LKB UM III using 50° glass knives (clearance angle 4 - 6°; cutting speed 2 mm/S.) It was extremely difficult to obtain good sections from the worker bee corpus allatum due to its minuteness. For this reason, standardization was not attempted although I tried in each case to section as close to the centre of the gland as possible. Sections were collected on 200 mesh copper grids and double stained using 5% aqueous uranyl acetate followed by concentrated lead citrate. EM viewing was at 80kV on a JEOL JEM 100 CX II.

Corpus allatum size

Various methods have been employed in the measurement of the size of corpora allata in the adult worker honey bee. Pflugfelder (1948, in van Laere, 1971) cut the corpus allatum into 8µm sections and calculated overall volume from planimetric measurements of plate reconstructions of the sections. Gast (1967) also made use of a planimeter in volume assessment but cut the corpus allatum into thicker sections of 25µm. To avoid the time consuming process of sectioning, van Laere (1971) photographed corpora allata and projected the image of each onto paper. He then measured the area of the image with a planimeter and reasoned that the average of a large number of measurements gives a true reflection of overall volume. Breed (1983) returned to the practice of sectioning of the corpus allatum. However, he assumed mistakenly that the corpus allatum of the worker bee is spherical, and used the radius of one largest section to calculate volume.

Since in developmental studies one is normally only interested in relative corpus allatum size, the determination of absolute volume is not necessary. The method of Sasagawa et al. (1989) for measurement of corpus allatum size avoids the time consuming practice of sectioning, and the expense of photography. They call their size variable "ellipsoidal area". Its measurement is simple and accounts for the tendency of honey bee corpora allata to have one diameter longer than the other. Their method was adapted for the present study as follows: After embedding of corpora allata in resin, two diameters of each were measured under a dissecting microscope with an eye-piece micrometer. Size was calculated according to the

formula:

$$S = \pi \times a/2 \times b/2$$

S : Size in um^2 - a measure of ellipsoidal area
a : longest diameter
b : shortest diameter

Any effects of fixation and embedding on corpus allatum size were assumed to be consistent for all. Szibbo and Tobe (1981) found that both fresh and fixed corpora allata were equally satisfactory for measurement of relative size. They demonstrated a linear relationship between "fresh volume" and "histological volume" for 48 insects. Not only does this indicate that shrinkage caused by tissue preparation is consistent, but also that internal chemical conditions (haemolymph osmolality) do not appear to confound the volume/age-of--insect relationship.

Mitochondrial size

Mitochondrial size was measured with a Jandel Sigma Scan Digitizer. A trace of the boundary of each mitochondrion is made on a graphics tablet and the Digitizer immediately computes the area inside each trace. Micrographs of cytoplasm from each corpus allatum sampling age were made at a standard magnification (x11520) for digitizing.

Altogether the corpora allata from fifteen bees were examined under EM. Approximately twenty sections were cut from each corpus allatum. In one section one would find $12^{\pm}$ cells. All cells were closely inspected before micrographs were taken. It was noticed at a glance

that mitochondrial size range was consistent throughout all cells in any one section. Consequently, a micrograph was chosen that would include as many mitochondria as possible to enhance statistical evaluation of mean mitochondrial size. I considered it an unnecessary expense to take more than two micrographs per section. All micrograph reprints were printed at the same time.

Hive conditions

Altogether three hives were used for sampling. All were queenright. Sampling began on 18th February 1991 from the first hive. However, this hive absconded between sampling days 12 and 15. Corpora allata from these bees were measured and sectioned but the data was kept separate from that reported in this thesis. They provided practice at sectioning and enabled me to familiarize myself with observation in the EM. The second hive absconded before any painted bees could be sampled. The third hive provided a fully successful sampling schedule, beginning on 28th March and ending 48 days later. All data in this thesis comes from the third hive.

RESULTS AND DISCUSSION

CORPUS ALLATUM SIZE

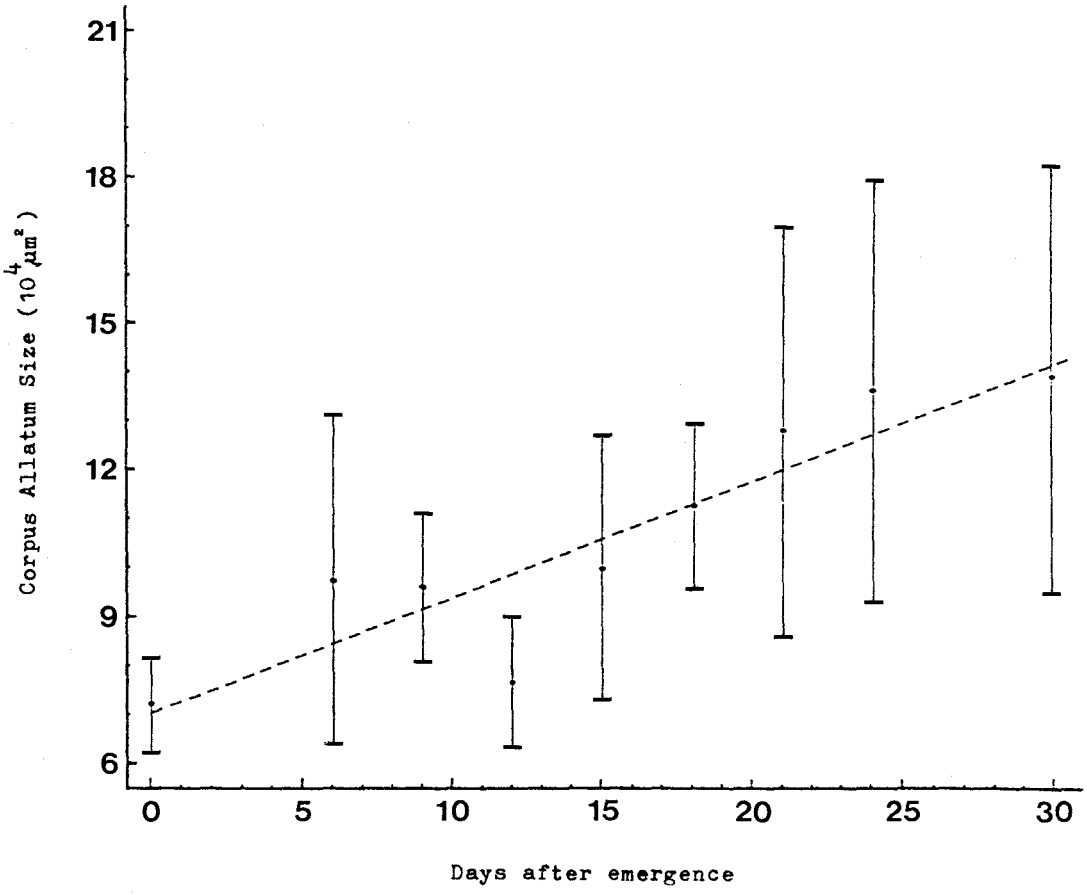
An age-dependent increase in size of the corpus allatum was found in the worker bees studied (fig. 1).

One-way ANOVA reveals significant changes in corpus allatum size with time (F-ratio 6.720; $P < 0.0001$; df 8,87). Scheffé tests (95%) show that the drop in mean size on Day 12 is not significant with respect to mean sizes on Days 0, 6, 9, 15 and 18. However, mean sizes on Day 21, 24 and 30 are significantly higher than on Days 0 and 12. A linear model ($y = 6964 + 241x$) draws the line of best fit ($R^2 = 30.86\%$).

This confirms for the Cape honey bee the well known age-dependent size increase in the corpus allatum of the European adult worker honey bee (Gast, 1967; van Laere, 1971; Breed, 1983a; Sasagawa et al., 1989). In fact, this is the first statistically verified evidence of a relationship between corpus allatum size and time, and of an increase in size with time. No previous author made use of analysis of variance and multiple range analysis of corpus allatum size data. This is also the first report of the increase in mean size continuing for as long as 30 days.

No two authors have provided identical traces of change in corpus allatum size with time. Gast (1967) claimed that he found two phases of corpus allatum volume increase in adult European worker honey bees during the first 20 days after ecdysis, and that the presence

Fig. 1 Development in corpus allatum size (mean ± SD) with age after adult emergence. (Size is a measure of ellipsoidal area calculated from two diameters.)
Sample sizes (n): 12 (Day 0); 9 (Day 6); 8 (Day 9); 11 (Day 12); 14 (Day 15); 11 (Day 18); 15 (Day 21); 9 (Day 24); 7 (Day 30).



of a queen suppressed the first phase of volume increase and promoted the second. During the first 21 days of worker bee adult life, Van Laere (1971) reported two phases of corpus allatum size increase with a period of size decrease in between. However, if one replots the raw data which he has provided, all that one can conclude with statistical verification is that there is a steady and gradual increase in size between the time of adult emergence and 21 days later (Professor H.R. Hepburn, pers. comm.) Breed (1983) reported a gradual increase in corpus allatum volume for the first five days of adult worker bee life, followed by a rapid increase up to day 15. Sasagawa et al.(1989) reported a rapid increase in corpus allatum ellipsoidal area during the first twelve days of adult worker bee life, followed by constancy in size for the rest of adult life. The inconsistency in the above-mentioned developmental trends are possibly attributable to seasonal influence (see Fluri et al., 1982 re. influence of season on honey bee corpus allatum activity.) The above-mentioned authors sampled bees during different months of the year, ranging from the end of March (van Laere) to August (Gast). Unfortunately, the validity of any of these developmental trends has not been verified by appropiate statistical tests.

Nevertheless, the present finding of continued corpus allatum size increase in the adult Cape worker honey bee for as long as 30 days of investigation appears to be out of the ordinary, and deserves further attention. On sampling Day 30, the average corpus allatum size had increased by 93% of its original size at adult emergence. One can calculate such percentages from the charts and data of van Laere (1971) and Sasagawa et al., and one obtains far lower percentage size increases at the point of maximum development: 42% and 28%

respectively. It appears that corpus allatum size development in the Cape worker honey bee is not only prolonged in comparison with the European honey bee, it is also of greater dimension. (Unfortunately, volume figures provided by other authors mentioned above cannot be compared with present findings because measures of volume increase by a cubed factor whilst measures of area increase only by a squared factor for any given corpus allatum.)

The greater range of corpus allatum size increase in the adult Cape worker bee is probably explained by the fact that corpora allata are smaller in the emerging adult Cape worker bee than in the emerging adult European worker bee. Sasagawa et al. found corpus allatum ellipsoidal area of the emerging European worker to be approximately 1.4×10^4 μm^2. In the emerging Cape worker average ellipsoidal area is 0.7×10^4 μm^2. Sasagawa et al. measured freshly dissected corpora allata. In the present study, fixation undoubtedly caused some shrinkage. However, shrinkage of the corpus allatum does not explain the greater range of corpus allatum size increase in the Cape worker bee and the final size in the latter is not considerably smaller than in the European worker bee (ca. 1.4×10^4 μm^2 in the Cape worker bee; ca. 1.8×10^4 μm^2 in the European worker bee.)

There is a relationship between corpus allatum development and oocyte development in insects (Tobe and Stay, 1985) which has been demonstrated in the worker honey bee (Gast, 1967). Cape worker bees have on average more oocytes per ovary than workers of other African bees, a characteristic particularly useful in differentiating the subspecies (Hepburn and Crewe, 1990). Rutz et al. (1976) have demonstrated that vitellogenin (oocyte protein) synthesis in adult worker bees is at its maximum when haemolymph JH titre is low, a

condition which is found during the first two weeks of adult life when corpora allata are still developing. A protracted period of smaller corpus allatum size (and hence of low JH production) in the Cape worker bee would allow protracted oocyte development, and hence a greater final ovary size.

Since the method of corpus allatum size determination of Sasagawa et al. (1989) is simple and elegant, it should be employed as a standard method for comparing size development in freshly dissected corpora allata of the Cape honey bee with that of other subspecies.

The corpora allata of the honey bee are of the large-cell type found in Hymenoptera (van Laere and Lagasse, 1973). No cell division has been reported in these corpora allata although Ulrich and Rembold (1983) discovered that endomitosis (nuclear duplication without cell division) occurs in the corpus allatum cells of the honey bee larva. Therefore, overall size of the gland relates to cell size.

CORPUS ALLATUM ULTRASTRUCTURE

In the following report, attention is given to ultrastructural features that have indicated cellular activity in corpora allata of several insect species. Such features are assessed here in terms of their value as indicators of cellular activity in the corpora allata of the adult worker honey bee.

Endoplasmic reticulum

In the corpus allatum cells of the adult worker Cape honey bee there was no clear evidence of smooth endoplasmic reticulum (SER). In the corpus allatum cells of a newly ecdysed bee there were many irregularly shaped vesicles which had ribosomes attached to their outer surfaces (fig. 2 and 3). The attachment of ribosomes indicates rough endoplasmic reticulum (RER), although it does not usually take on such irregular appearance in corpora allata. Ribosome-studded vesicles were present in the corpora allata of worker bees on sampling Days 3, 6, 12, 18, 21 and 30, but never as extensively as in the emerging bee, where large concentrations occurred near the cell nuclei. As RER is always attached to the nuclear envelope (Alberts et al., 1983), the perinuclear location of ribosome--studded vesicles lends support to the conclusion that they constitute RER. Possibly this is a declining RER, as RER is well developed in the honey bee larva, consisting of elongated cisternae in whorls and spider-like formations (Wirtz, 1973).

In the only documented report of general EM ultrastructure of the corpus allatum of adult worker honey bees, van Laere and Lagasse (1973) state that they found some strands of "granular" ER and no Golgi apparatus, but they make no mention of the presence or absence of SER. In the present study, I found groups of small vesicles scattered amongst the ribosome-studded vesicles (fig. 2). There is a possibility that these smooth vesicles constitute SER.

The obscurity of SER in adult worker bee corpora allata was unexpected. SER has been implicated in the production of juvenile hor-

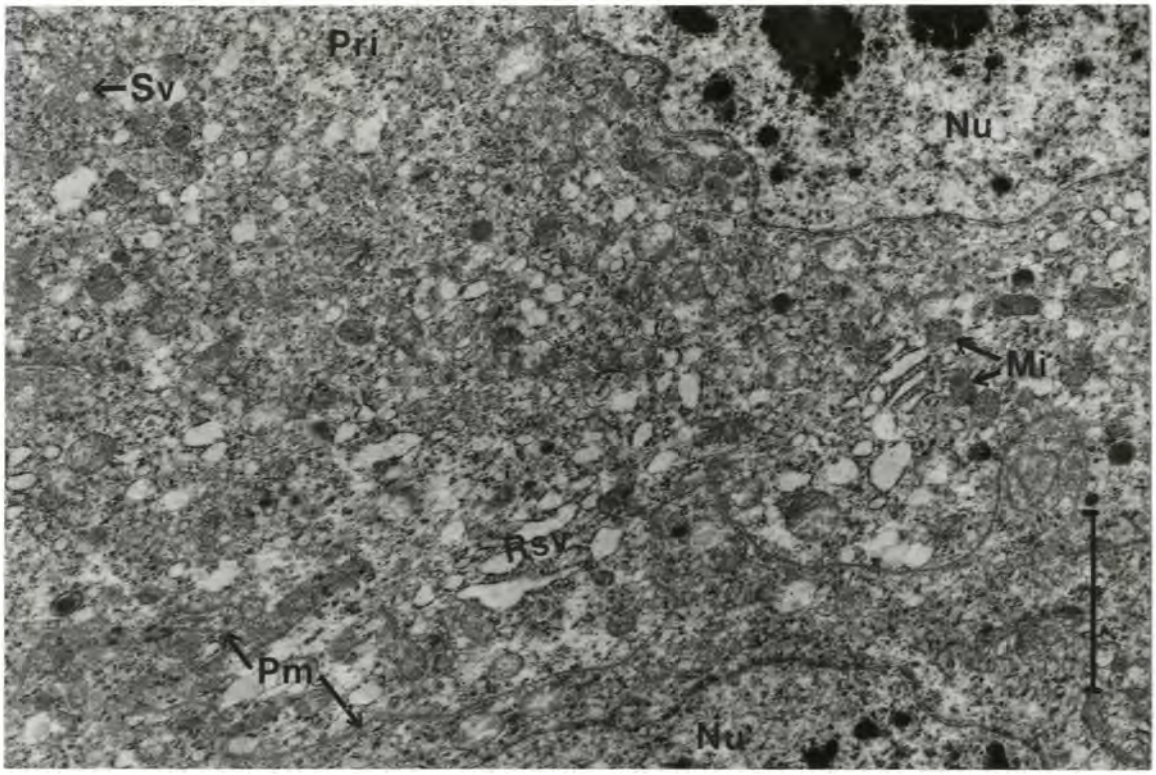


Fig. 2. The cytoplasm of corpus allatum cells in a newly emerged worker honey bee contains many irregularly shaped ribosome-studded vesicles (Rsv). (Mi) mitochondrion; (Nu) nucleus; (Pri) polyribosomes; (Pm) plasma membrane; (Sv) smooth vesicles.

x11520

scale bar = 2 µm

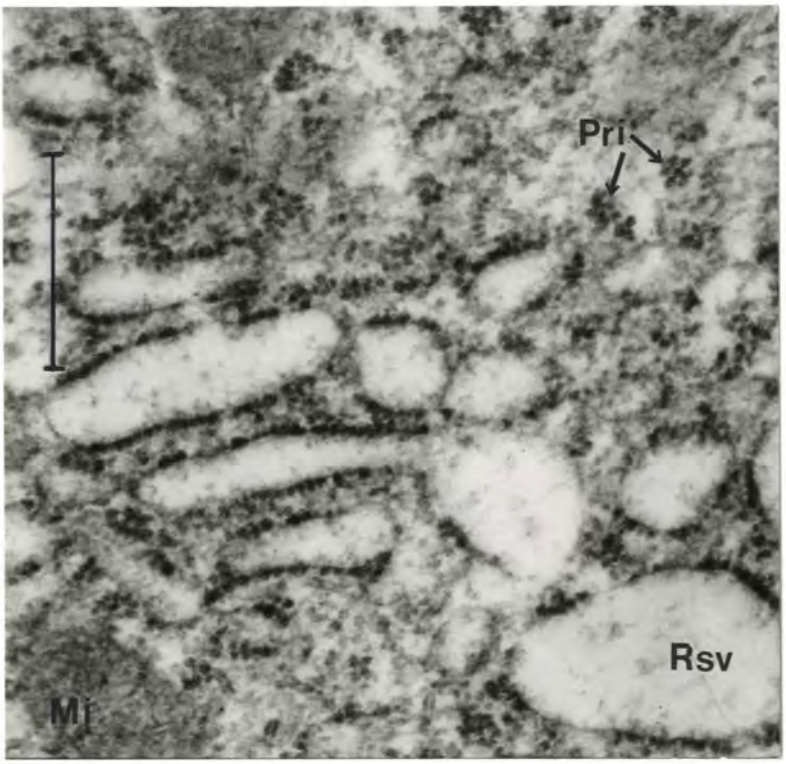


Fig. 3. Ribosome-studded vesicles (Rsv).

(Mi) mitochondrion; (Pri) polyribosomes; x57600

scale bar = 0.5 µm

mone in several insect species. Odhiambo (1966) found an abundance of SER in the corpora allata of sexually mature male Schistocerca gregaria, and since JH was known to be active in the promotion of sexual maturation, he concluded that SER abundance indicated JH production. Scharrer (1978) found that gonadectomy of adult female Leucophaea maderae led to an appearance of hyperactivity in the corpora allata and that this hyperactivity was reflected in an increase of SER. Deleurance and Charpin (1978) reported cycles in the quantity of SER in the corpus allatum cells of Choleva angustata that were related to moulting cycles.

By 1975, SER had been found in the corpora allata of 17 out of 19 insect species (Melnikova and Panov, 1975). Further reports of the association between SER abundance and corpus allatum activity led Tobe and Stay (1985) to conclude that SER is an integral part of the cellular machinery of JH biosynthesis. This conclusion also rested heavily on the finding that a tritiated JH precursor was found in the area of SER after its uptake by incubated corpora allata (Tobe and Saleuddin, 1977). In a more comprehensive review of the literature on corpus allatum ultrastructure, Sedlak (1985) does not arrive at a definite conclusion that SER is essential in the biosynthesis of JH. There is some interspecific inconsistency in the association between SER quantity and known periods of corpus allatum secretory activity.

The obscurity of SER in adult worker bee corpora allata is particularly surprising as it contrasts sharply with the well developed SER in the larva. In the corpora allata of larval worker honey bees Wirtz (1973) reported that SER occurs in three forms: as isolated vesicles, as parallel vesicles in whorls, and as densely packed elongated vesicles. He also found abundant RER and Golgi apparatus.

Goewie (1978) found RER and Golgi apparatus of such regular and clearly defined appearance in larval honey bee corpora allata that he was able to quantify them as a measure of corpus allatum activity. No Golgi apparatus was found in corpus allatum cells in the adult worker bee in the present study. Palade (1975) asserts that all eukaryotic cells have an endoplasmic reticulum and a Golgi apparatus. Therefore, one is only permitted to conclude that Golgi apparatus is scarce in the corpora allata of the adult worker bee.

Fluctuations in abundance of RER, SER and Golgi apparatus led Wirtz (1973) to speculate that these membrane organelles are indicators of JH biosynthesis in larval honey bee corpora allata. However, hormonal manipulation of 66 hour old worker larvae with topical application of JH-I had no detectable effect on corpus allatum ER or Golgi apparatus, whilst it did result in an increase of granule secretion by the median neurosecretory cells, which is known to inhibit corpus allatum activity (Goewie, 1978).

One would expect ER and Golgi apparatus of similar nature and abundance in both larval and adult worker bee corpora allata if they are sites of JH biosynthesis. The obscurity of RER and SER, and the scarcity of Golgi apparatus in the corpus allatum of the adult worker honey bee leads to the conclusion that they are not necessary sites of JH biosynthesis, and that in the larval corpus allatum, these organelles are involved in a cellular activity other than the production of JH.

Mitochondria

In the worker honey bees under present investigation, mitochondria were abundant in corpus allatum cells through most of adult life. Numbers of mitochondria gave the impression of peaking on Day 15, and then declining so that on Day 30 only three very small mitochondria (diameter: 0.65μm) could be found in an entire section of the central area of a gland.

Mitochondria counts would seem to be good candidates as a way of quantifying indications of cellular activity, and since mitochondria are involved in the process of lipid hormone biosynthesis, including testosterone (Christensen, 1975) and juvenile hormone (Tobe and Stay, 1985), one might expect their numbers to reflect levels of JH biosynthesis in corpora allata. However, as Goewie (1978) points out, mitochondria counts (and organelle counts in general) are subject to influence by swelling (and shrinking) of the corpus allatum. Another problem with the interpretation of mitochondria counts is that mitochondria are known to be mobile. Microcinematography of living cells has revealed that mitochondria move in association with networks of cytoplasmic microtubules (see fig. 4) and become clustered at different regions of the cell at different times (Alberts et al., 1983). The probability of obtaining mitochondria counts that truly reflect the total number of mitochondria in the corpus allatum is low.

An alternative form of quantification for mitochondria is a measure of mitochondrial size. Mitochondria have been seen to be larger in cells regarded as active in the corpora allata of L. maderae

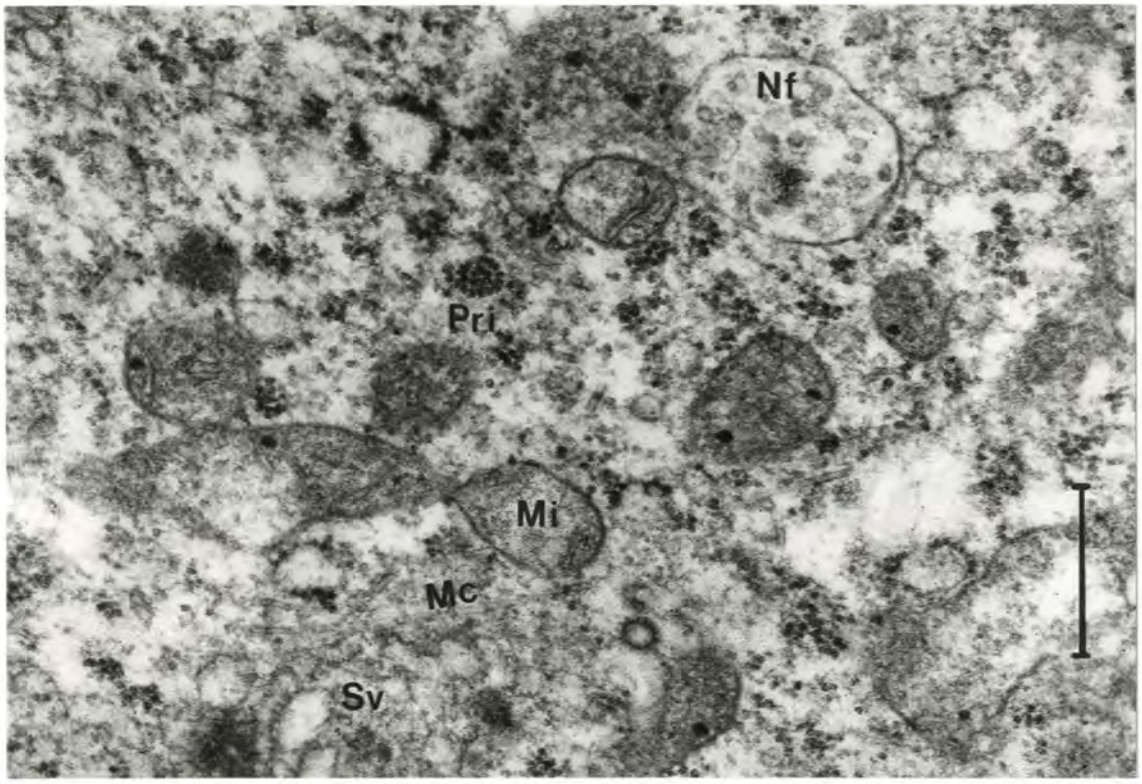


Fig. 4. Mitochondria (Mi), microtubules (Mc) and polyribosomes (Pri). (Nf) nerve fibre; (Sv) smooth vesicles. x46400
scale bar = 0.5 µm

(Scharrer, 1973) and Hyphantria cunea (Melnikova and Panov, 1975). Deleurance and Charpin (1978) report fluctuations in size, shape and electron density of corpus allatum mitochondria during the larval moulting cycles of C. angustata. Manipulation of corpus allatum activity appears to affect mitochondrial size. When Scharrer (1978) removed the ovaries of L. maderae and found indications of hyperactivity in the corpus allatum, one of these indications was an increase in mitochondrial size. Goewie (1978) mentions that after treating honey bee larvae with topical applications of JH-I, mitochondria in the corpora allata had become smaller. A coincident decrease in JH production would have been expected with this treatment.

There is recently a renewed interest in the mitochondria of corpus allatum cells. Liu (1986) noticed that mitochondria were smaller, more elongated and more electron dense in corpora allata of worker honey bees that were infected with the parasite Nosema apis than in healthy honey bees. Further research (Liu, 1990) demonstrated that treatment of infected bees with an anti-Nosema drug restores corpus allatum mitochondria almost to the size and electron density of those of healthy honey bees. Liu noticed that the hypopharyngeal glands of infected worker bees were atrophied, a condition associated with elevated haemolymph JH titre. The possibility exists that Nosema apis in the infected bees is producing a JH - like substance. Alternatively, smaller darker mitochondria indicate an elevated level of JH production in the corpora allata.

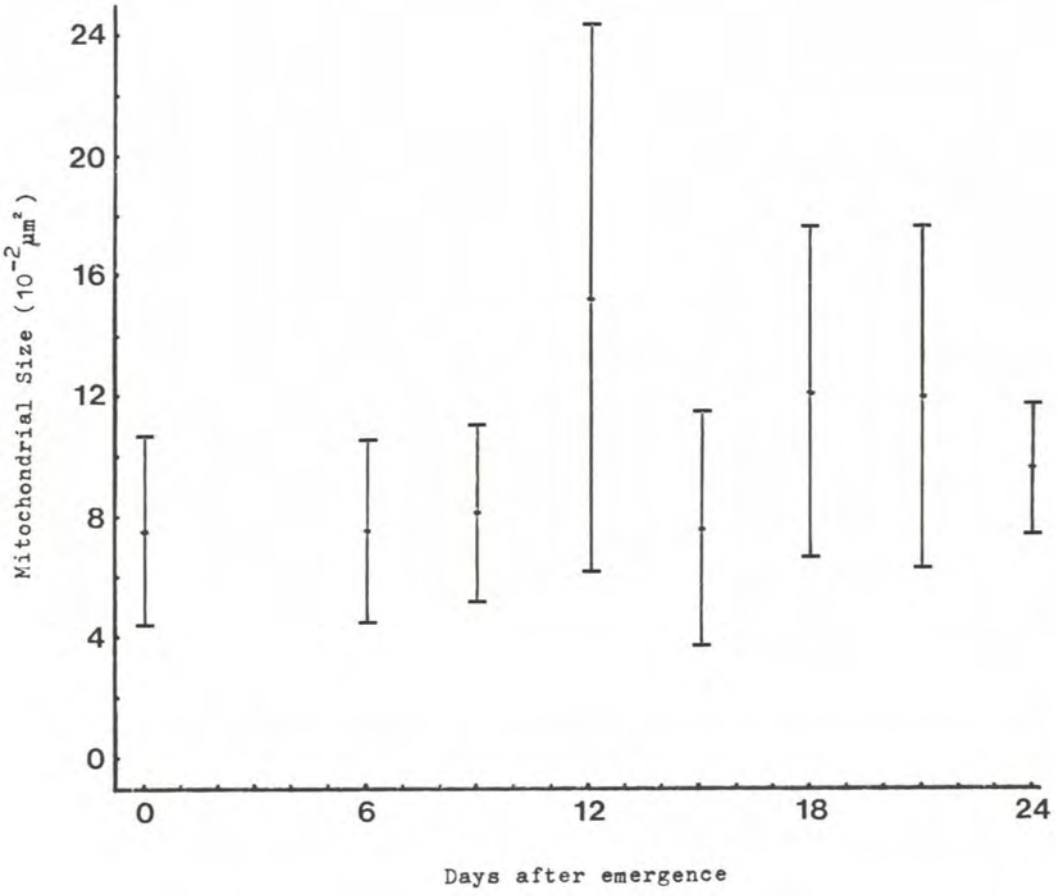
Although mitochondrial size might prove reliable as a quantifiable factor that can be related to corpus allatum activity, certain

precautions are necessary in size assessment. Microcinematography has revealed that a mitochondrion may change its shape as many as nine times in twenty minutes (Alberts et al., 1983). The same mitochondrion may become long and thin, round and fat, lumpy, and curved. The literature abounds with statements that imply that a certain mitochondrial shape is particular to a certain stage in the life cycle of a species. These "typical" shapes may have been extremely temporary in nature. Change of shape also affects measurements of size. A long thin mitochondrion will appear "small" in cross-section. A round and fat mitochondrion of the same volume might be recorded as large. The average of many measurements should overcome the problem of limited view, as a representative number of mitochondria will be cut in longitudinal as well as in cross section (unless all are similarly orientated.) However, at present, the literature on corpus allatum ultrastructure does not contain statistical support of statements concerning mitochondrial size.

In the present study, mitochondrial size is derived as an average of many measurements. The Jandel Digitizer and graphics tablet gives a two-dimensional measure (area) of each mitochondrion that accounts for irregularity of shape, which cannot be taken into account by one-dimensional measures (length and diameter.)

In fig. 5 are provided means ($\pm$SD) of mitochondrial size in the corpora allata of worker bees from adult ecdysis to Day 24 of adult life (on Day 30 mitochondria could not be detected on standard micrographs for digitizing.) The raw data failed Cochran's C test for homogeneity of variances and was therefore subjected to Kruskal-Wallis analysis (a non-parametric alternative to one-way ANOVA).

Fig. 5 Changes in mitochondrial size (mean ± SD) with age after adult emergence (Size = sectional area).
Sample sizes (n): 26 (Day 0); 25 (Day 6); 69 (Day 9); 78 (Day 12); 116 (Day 15); 48 (Day 18); 56 (Day 21); 13 (Day 24).



A test statistic of 88.58 ($P < 0.0001$) was obtained. Therefore, mean mitochindrial size can be said to change significantly with time. Scheffé tests (95%) indicate a significant difference between mean mitochondrial size on Day 12 and those on Days 0, 6, 9 and 15. Mean mitochondrial sizes on Days 18 and 21 were also significantly different from those on Days 9 and 15. The two peaks in mean mitochondrial size on Day 12 and Days 18/21 are therefore significant, suggesting two cycles of rise and fall in size during the sampling period. (See figs. 6 and 7 for examples of mitochondrial sizes.)

Cycles in mitochondrial size suggest cyclical metabolic activity. Cycles in JH production that occur during moulting cycles of larval insects are reflected in cyclical change in corpus allatum ultrastructure. Although there are no further moulting cycles in the life of the adult worker bee, one cannot assume that the nature of corpus allatum activity alters radically on reaching adulthood.

There is evidence in the literature for a cyclical nature of corpus allatum function in the adult insect. In female insects, cycles of ultrastructural change in corpora allata have been observed during cycles of oocyte development (see Tobe and Stay, 1985.) Furthermore, Fain-Maurel and Cassier (1969) reported cyclical changes in mitochondrial morphology in corpora allata of adult female Locusta migratoria which are associated with oocyte development.

The steady age-dependent increase in JH production reported for adult worker bees (eg. Rutz et al., 1976) is determined by pooling corpora allata or haemolymph from several bees until there is sufficient JH for chemical analysis. Such pooling prevents detection

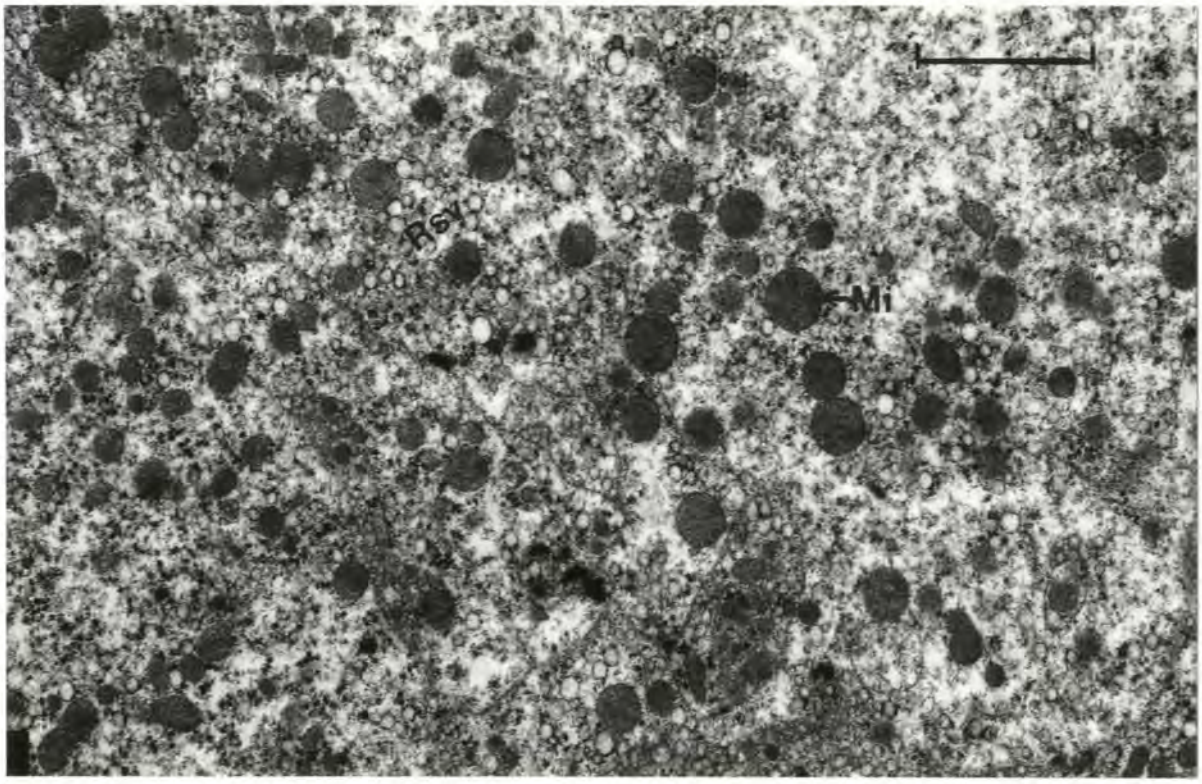


Fig. 6. Large electron-dense mitochondria (Mi) of sampling Day 12.
Ave. mitochondrial size = 0.153 μm^2
(Rsv) ribosome-studded vesicles. x11520
scale bar = 2 μm

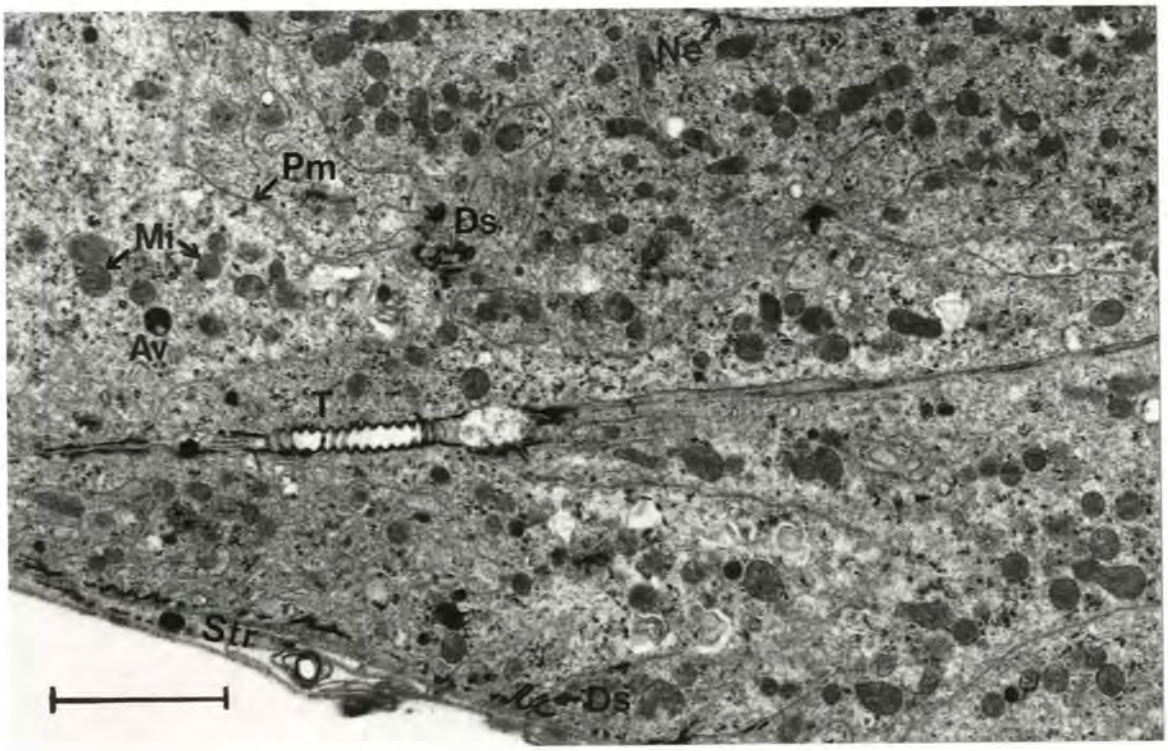


Fig. 7. Smaller mitochondria (Mi) of sampling Day 15.

Ave. mitochondrial size = 0.076 μm^2

(Av) autophagic vacuole; (Ds) desmosomes; (Ne) nuclear envelope;

(Pm) plasma membrane of interdigitating cellular processes;

(Str) stromal sheath; (T) trachea. x11520

scale bar = 2 µm

of a cyclical nature of JH production by individual corpora allata. When a method has been devised for the continuous monitoring of JH production in the individual bee, relationships between corpus allatum activity and ultrastructural changes will be clarified.

Occasionally, mitochondria were found that were swollen in appearance (fig. 13). It has been suggested that the swelling of mitochondria is an artefact of tissue preparation for EM. However, Scharrer (1978) found swollen mitochondria hand in hand with normal mitochondria in the corpora allata of L. maderae. Swollen mitochondria are not common in worker bee corpora allata. They were omitted from the assessment of mitochondrial size discussed above.

Ribosomes

Free ribosomes and small clusters of ribosomes (polyribosomes) are abundant in the cytoplasm of worker bee corpus allatum cells at all sampling ages in the present study. Most polyribosomes are in the form of small clusters (fig. 3). Fig. 4 shows a coiled polyribosome in which it can be seen clearly how the ribosomes are linked in a chain, which is known to be held together by a piece of m-rna. This arrangement indicates that protein synthesis is in process. Free ribosomes also produce protein (Reid and Leech, 1980.)

Rees (1977) states that low weight carrier proteins have been found in corpus allatum tissue. The ribosomes in honey bee corpora allata are probably producing JH-carrier proteins. Carrier proteins function to increase solubility of JH in the haemolymph, and to facilitate cellular recognition of JH at the target tissue. Ordinarily, another major function of carrier protein would be to protect JH from esterases (degenerative enzymes in the haemolymph that regulate JH titre.) However, de Kort et al. (1977) suggest that carrier proteins are unlikely to be involved in JH protection in honey bee larvae. Their reasoning stems from the finding that the half-life of JH in honey bee larvae is exceptionally high: it is 5 to 6 hours, compared to the average of 30 minutes in other insects. It appears that JH titre in honey bee haemolymph is not regulated by esterase activity. Bühler et al. (1983) have provided confirmation of this in adult bees with their finding that injected [^{3}H]-JH-III is not degraded in the haemolymph of adult workers.

Vacuoles

Two types of vacuoles were found in the corpora allata of adult worker honey bees: "extracted vacuoles" and autophagic vacuoles.

The first type I have called "extracted vacuoles" as in most cases they appear as empty spaces in the cytoplasm (figs. 10, 11, 12a & b, 13). Christensen (1975) reported lipid vacuoles in human Leydig cells which were usually similarly extracted during tissue preparation. Lipid vacuoles are storage sites for the exogenous substrates of testosterone biosynthesis, including fatty acids and cholesterol. Fatty acids are substrates of JH biosynthesis (Tobe and Stay, 1985). Extracted vacuoles are therefore likely to be remnants of lipid vacuoles in adult worker bee corpora allata. They are large and very numerous in the emerging worker bee (fig. 8 - in this example the vacuole membranes have not been extracted.) They are numerous, but smaller, on Days 3 and 6, and present in moderate numbers on Days 15, 21 and 24.

Christensen states that lipid vacuoles are largest and most abundant when the endocrine cells are least active, becoming smaller and fewer in number as their contents are consumed in more active cells. The large and numerous vacuoles of Day 0 suggest low levels of JH biosynthesis in the emerging worker. The intermittent reappearance of extracted vacuoles on Days 15 and 21 suggest periods of decline in JH biosynthesis. The overall picture is one of cycles in lipid storage, which suggests cycles in JH biosynthesis.

Wirtz (1973) reported similar fluctuations in the numbers of large

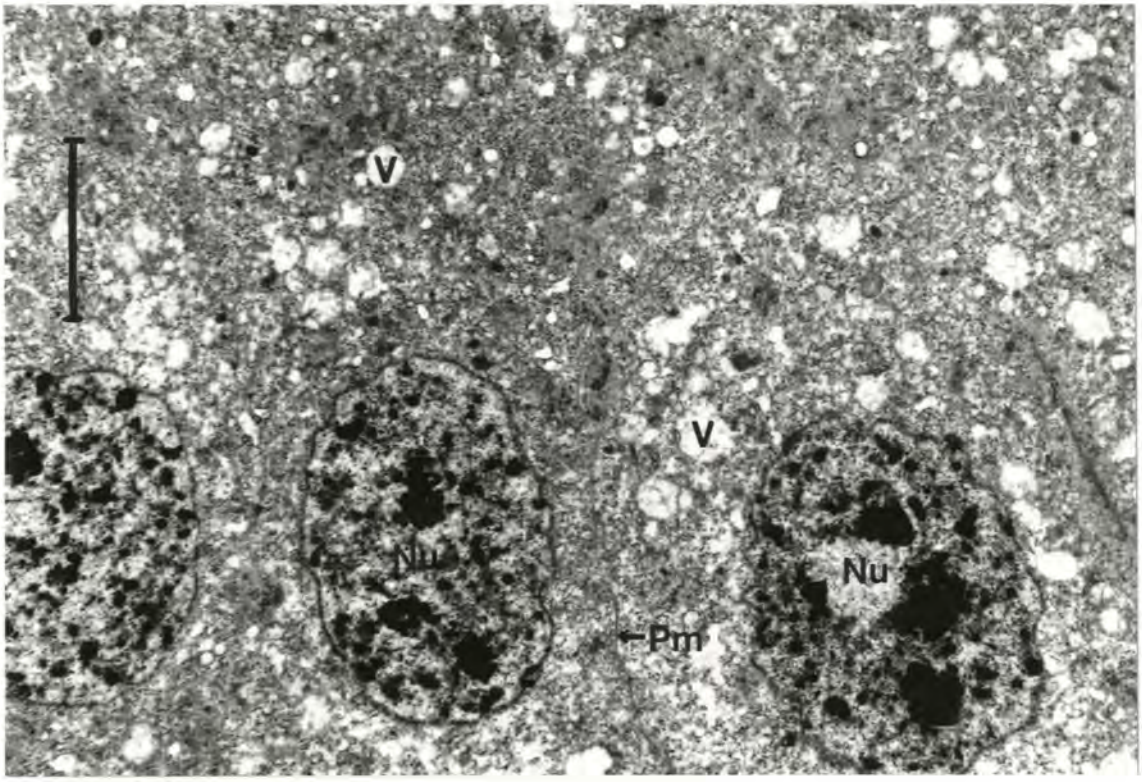


Fig. 8. Numerous large and small extracted vacuoles (V).
(Nu) nucleus; (Pm) plasma membranes of two adjacent cells.
x 5760
scale bar = 4 µm

vacuoles in the corpus allatum of the worker bee larva. At that time it was thought that such extracted vacuoles were the remnants of lipid vacuoles, but also that they were the storage sites of JH.

Autophagic vacuoles

Autophagic vacuoles make occasional appearance (fig. 7). They are known to be involved in the breakdown of membrane organelles (ER) and mitochondria. In the present study they were too infrequent to make any generalizations about their role in corpus allatum activity.

Nuclei

It has been reported that in the cell nuclei of the corpora allata of larval honey bees, the euchromatin undergoes cycles of condensation and decondensation (Dogra et al., 1977). Decondensation was seen under the light microscope as the dispersal of fragments of light staining euchromatin through the darker staining heterochromatin. It was reported to occur immediately before an inter-larval moult. It was taken as an indicator of corpus allatum activity.

Cruz-Höfling and Cruz-Landim (1977) also regarded chromatin decondensation as a sign of corpus allatum activity when they reported that the corpora allata of worker honey bees seemed more active after 20 days of adult life. However, a recent study of corpus allatum cell nuclei in the honey bee larva reported "a homogeneous distribution of nuclear chromatin during all larval instars" (Yaginuma et al., 1990). No trends of decondensation of nuclear euchromatin were observed in the present study. Changes in nuclear outline, ranging from smooth to undulating nuclear membranes, were seen, but did not avail of functional interpretation.

The study of cell nuclei in the corpus allatum of the larval honey bee has reached new heights of sophistication. Ulrich and Rembold (1983) demonstrated the uptake of radio-labelled nucleotides by cell nuclei in the larva during interstadial moulting. Photometric measurement of the DNA content in such nuclei showed increases occurring in the ratio 1 : 2 : 4, indicating that endomitosis was taking place. Yaginuma et al. (1990) demonstrated that the cells of the larval honey bee corpus allatum are in classes of ploidy, ranging from 2C (diploid) to 32C. The number of nuclei in higher classes

of ploidy increases as the honey bee larva passes through its progressive moults. Endomitosis is more rapid at an earlier age in queen larvae than in worker larvae, and since the corpora allata of queen larvae are known to produce higher levels of JH at an earlier age than those of worker larvae, it appears that DNA content is related to levels of production of JH. Measurement of nuclear DNA would be worthwhile in further research into the corpus allatum of the adult worker honey bee.

"Intercellular spaces" / light and dark cells

In a light microscope study of sequential changes in the retrocerebral glands of the female honey bee larva, Canetti et al. (1964) reported that in the fifth larval stage "intercellular spaces" appeared in the corpora allata. They suggested that these spaces indicated corpus allatum inactivity. Rembold (1987) has shown that a marked drop in haemolymph JH titre occurs during the fifth larval stage of worker and queen honey bees, confirming the timing predicted by Canetti et al. of a possible decline in corpus allatum activity.

In another light microscope study of the larval honey bee, Dogra et al. (1977) state that in the inactive corpus allatum "the cell shape can be distinctly identified," and that "the cell borders disappear" during heightened corpus allatum activity directly before a moult. Their photographs show that the appearance and disappearance of cell borders at the light microscope level is brought about by the appearance and disappearance of the "intercellular spaces" originally noticed by Canetti et al. These spaces provide contrast which makes cell borders visible under the light microscope. Dogra et al. have therefore provided confirmation of the association between "intercellular spaces" and corpus allatum inactivity.

At the level of the electron microscope, van Laere and Lagasse (1973) presented micrographs of real intercellular spaces in worker bee corpora allata that are found between the plasma membranes of adjacent cells. These spaces were found in the present study, occasionally containing stromal matrix that is continuous with the stromal sheath (fig. 9). Genuine intercellular spaces are far too

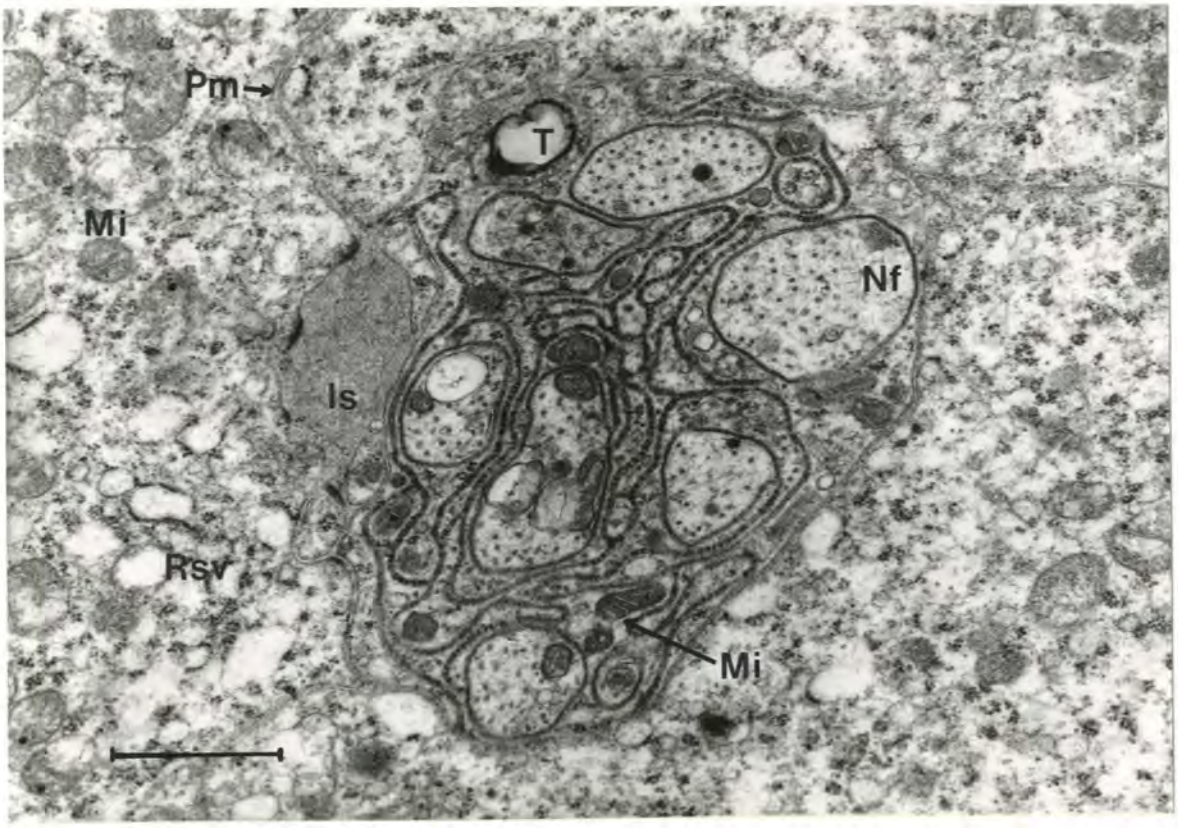


Fig. 9. Intercellular spaces (Is) accompanying a bundle of nerve fibres. (Mi) mitochondrion; (Nf) nerve fibre; (Pm) plasma membrane; (Rsv) ribosome-studded vesicles; (T) trachea.

x22400

scale bar = 1 µm

small to be seen under the light microscope. They are not the phenomenon reported by Canetti et al.

Under the electron microscope in the present study there appeared an ultrastructural feature that provides an explanation for the "intercellular spaces" reported by Canetti et al. in corpora allata of the larval honey bee.

Each corpus allatum cell has processes that extend and interdigitate with the processes of neighbouring corpus allatum cells. Such cytoplasmic extensions are seen to arrive in numbers at the stromal sheath (fig. 10). Fig. 11 shows the meeting of interdigitating processes from several cells in the central area of the gland. At certain sampling ages (Days 3, 9, 15 and 24) interdigitations within the body of the corpus allatum exhibit cytoplasm with an electron density far lower than that of the surrounding cells (figs. 12a and 12b). Such "light" interdigitations give the impression of intercellular spaces under the light microscope. Corpora allata with light interdigitations resemble those with "intercellular spaces" in the photographs of Canetti et al. and Dogra et al. For convenience and accuracy these "intercellular spaces" will be referred to as "light interdigitations" from now on in this thesis.

Furthermore, on Days 15 and 24 whole cells with cytoplasm of lighter electron density than that of surrounding cells were found (fig.13). Such "light and dark cells" have been found in the corpora allata of other insect species (Sedlak, 1985). Van Laere and Lagasse (1973) state: "in exceptional cases a cell is found with less dense hyaloplasma" in adult worker bee corpora allata. They did not sample bees of various ages, which might account for their limited observation

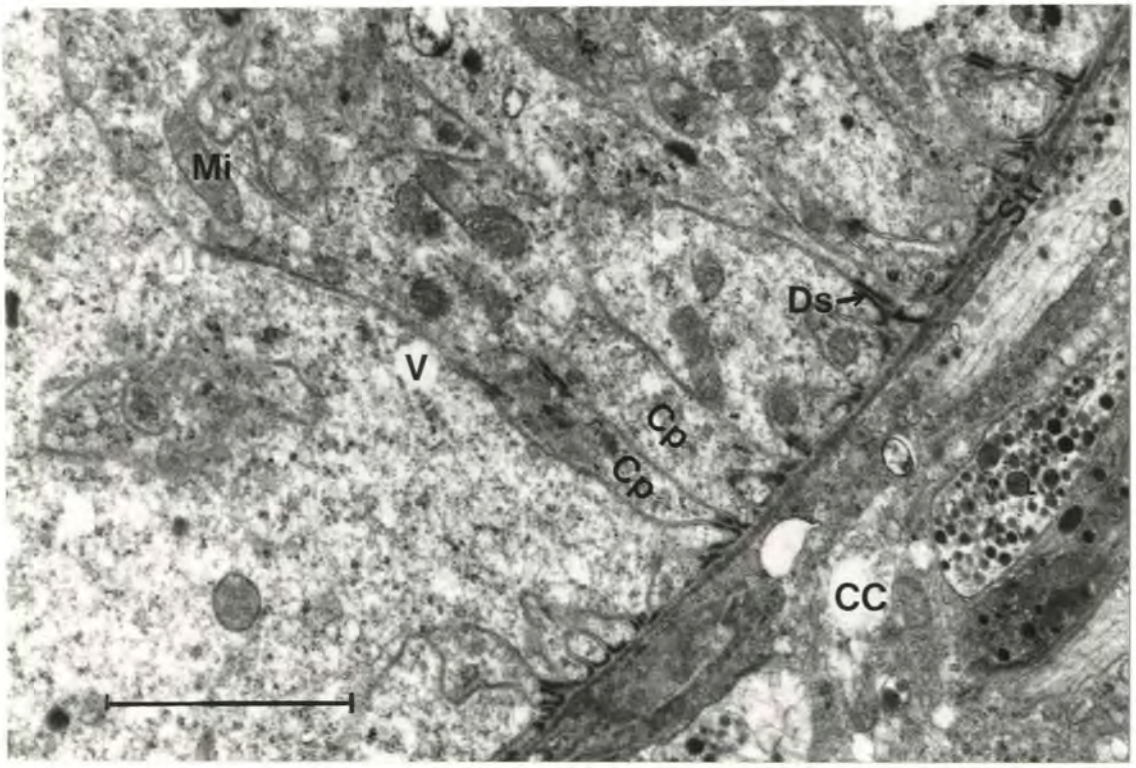


Fig. 10. Cellular processes (Cp) arriving at the stromal sheath. (CC) corpus cardiacum tissue adjacent to the corpus allatum; (Ds) desmosome; (Mi) mitochondrion; (Str) stromal sheath; (V) extracted vacuole. x16000

scale bar = 2 µm

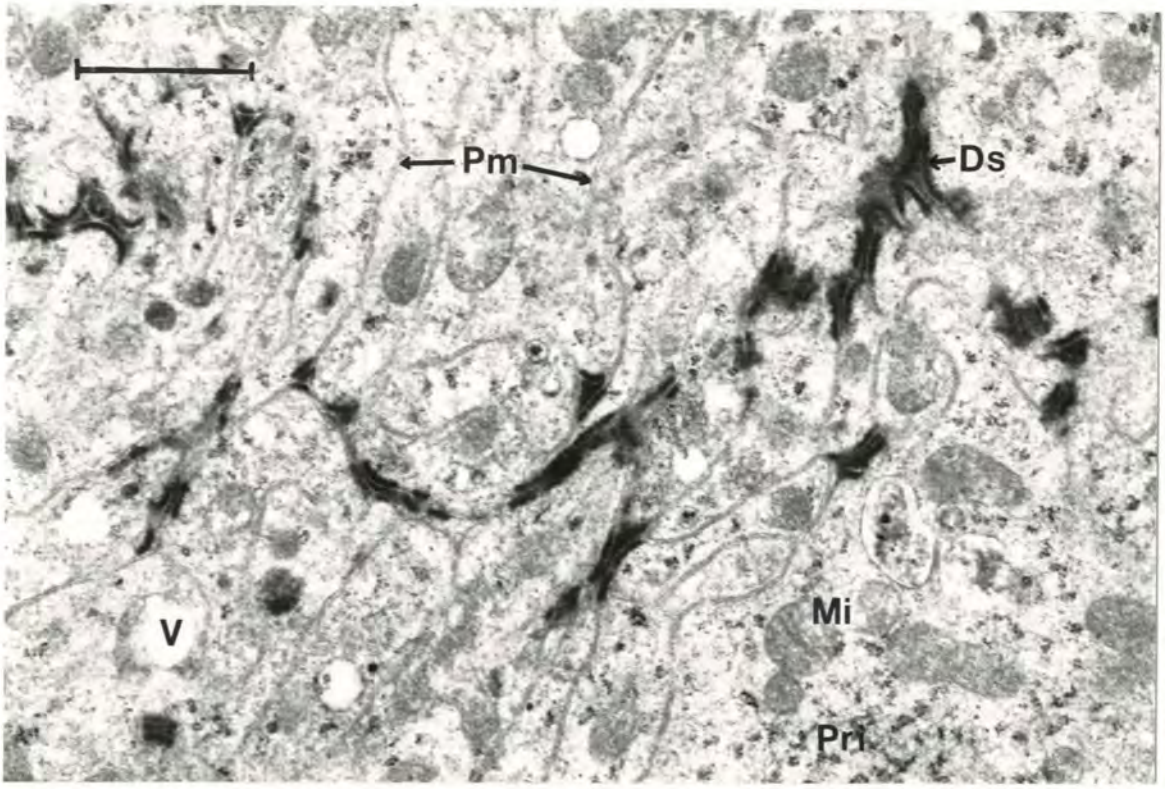
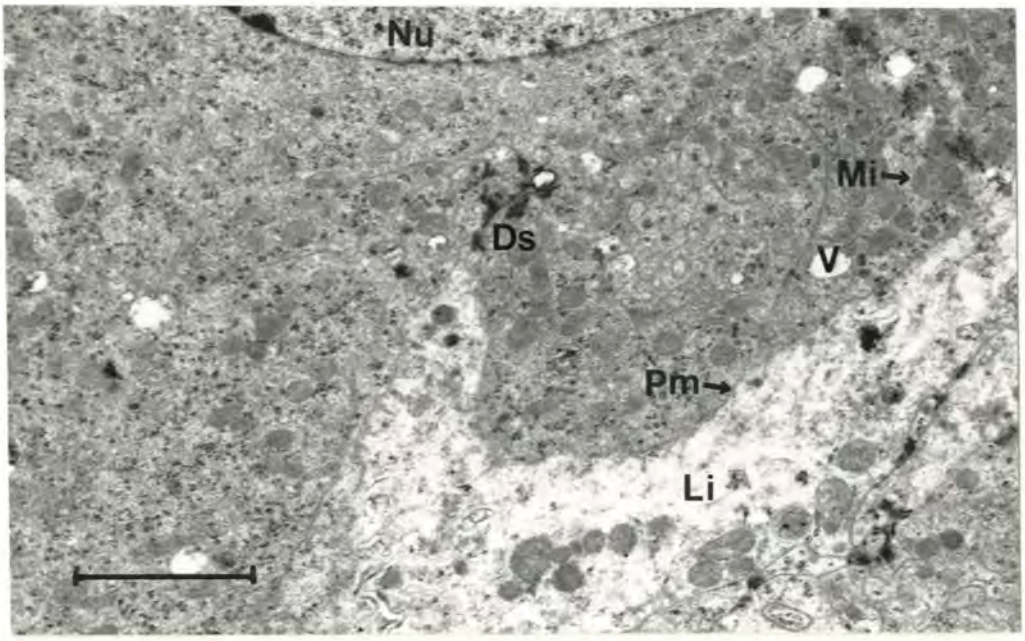


Fig. 11. Interdigitating cellular processes. (Ds) desmosome; (Mi) mitochondrion; (Pm) plasma membrane; (Pri) polyribosomes; (V) extracted vacuole. x92800

scale bar = 0.25 μm

a)



b)

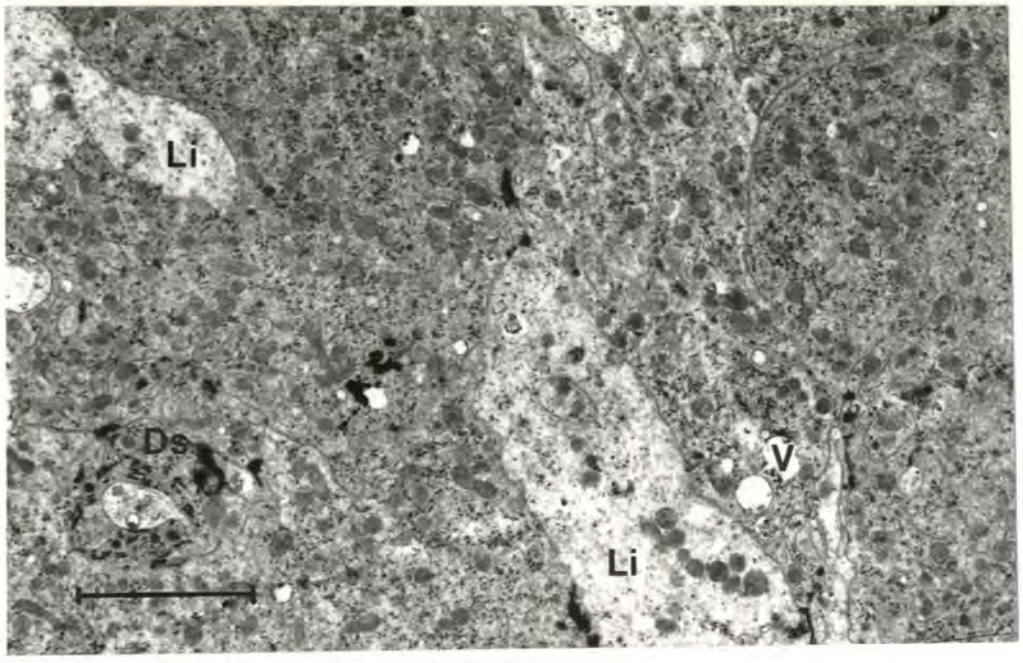


Fig. 12a) and b). "Light interdigitations" (Li).

(Ds) desmosomes; (Mi) mitochondrion; (Nu) nucleus; (Pm) plasma membrane; (V) extracted vacuole.

12a) x11520; 12b) x7680

12a) scale bar = 2 µm; 12b) scale bar = 3 µm

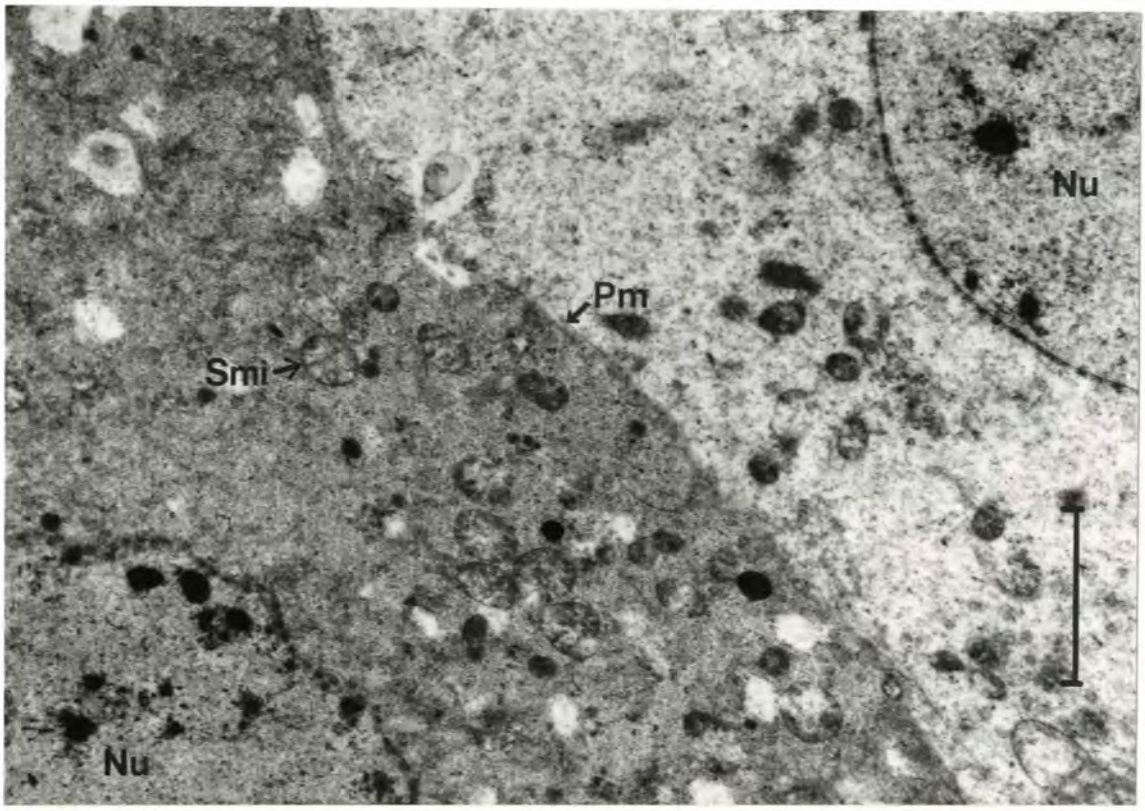


Fig. 13. Light and dark cells. (Nu) nucleus; (Pm) plasma membrane; (Smi) swollen mitochondrion. x11520

scale bar = 2 μm

of light cells. Deleurance and Charpin (1978) suggested that light and dark cells indicate differential degrees of cellular activity in the corpora allata of C. angustata. Dorn (1973) noticed variation in relative number of light and dark cells in the corpora allata of Oncopeltus fasciatus and suggested that light are the active cells because dark cells were prevalent in the last larval instar of this species. However, Leichty and Sedlak (1978) found that in adult O. fasciatus dark cells were prevalent in corpora allata deemed to be active by their maturity and by a correspondingly advanced state of yolk deposition in the insects' oocytes. When such mature O. fasciatus with active corpora allata were treated with precocene, an anti-JH drug known to retard corpus allatum development, the corpora allata were found to contain light cells.

Further understanding of the meaning of light and dark cells in corpora allata might be attained if we remember that the difference between light cells and dark cells is that their cytoplasm has taken up different quantities of osmium tetroxide. Stoeckenius and Mahr (1965) studied the reactions of osmium tetroxide with lipids and fatty acids commonly found in biological tissues and drew up a chart which listed the quantities of osmium tetroxide that reacted with each compound. Farnesylacetone demonstrated an above-average affinity for osmium tetroxide - three moles of osmium tetroxide react with one mole of farnesylacetone. This is appreciably higher than the amount required for a detectable increase in contrast in the electron microscope image. High affinity for osmium tetroxide is a consequence of the degree of unsaturation of the lipid: one molecule of osmium tetroxide being taken up per double bond. Farnesol is a precursor of JH and is an unsaturated lipid with three double bonds per molecule.

Its location during the biosynthesis of JH is in the cytosol. The cytosol in active corpus allatum cells is rich in JH precursors, which are unsaturated lipids (see Tobe and Stay, 1985, p. 342). Hence, the cytoplasm of cells that are actively producing JH would be expected to appear dark in the electron microscope image.

Conversely, the cytoplasm of light cells must be lacking in unsaturated lipids. Light cells can therefore be considered inactive as far as JH production is concerned.

In conclusion, light interdigitations are cellular extensions of light cells. They give the impression of "intercellular spaces" under the light microscope. The presence of light cells and/or light interdigitations indicates a decline in JH production by the corpus allatum. The appearance of light cells/interdigitations on Days 3, 9, 15 and 24 indicate periodic decline in JH producing activity in the corpora allata of adult worker honey bees.

SUMMARY AND CONCLUSION

Endoplasmic reticulum is not a reliable indicator of cellular activity in the corpus allatum of the adult worker honey bee. SER is difficult to find. RER is identifiable but is also obscure in comparison with the well developed membrane organelles in the corpus allatum of the larval honey bee. Since adult worker bee corpora allata produce JH without well developed SER, RER and Golgi apparatus, it is possible that in the larva these membrane organelles are involved in a metabolic activity other than JH production. A secretory product of the honey bee corpus allatum that is not JH has in fact been discovered. It is a gonad repressor substance (Dixon and Moser, 1972; Liu and Dixon, 1973).

Mitochondrial size is a readily quantifiable indicator of metabolic status in corpus allatum cells. New methodology provides scientific assessment of fluctuations in size of this organelle. A cyclical pattern of increase and decrease in mean mitochondrial size has been found in the corpora allata of the adult worker honey bee. Since large mitochondria have been associated with increased levels of activity in the corpora allata of a number of insect species, the alternations of large and small mean mitochondrial size found in this study suggest alternations in high and low levels of corpus allatum activity.

The periodic appearance of extracted vacuoles suggests a periodic storage of exogenous fatty acids, raw materials of JH biosynthesis. Storage of raw materials suggests low levels of biosynthetic activity in hormone glands. With the exception of a few scattered vac-

uoles on sampling Day 21, the presence of extracted vacuoles coincides with smaller mitochondria (Table 1).

Furthermore, light cells, proven indicators of decreased JH production in corpora allata fixed with osmium tetroxide, are only found on the same sampling days as smaller mitochondria.

Since the two indicators of decreased JH production co-exist with smaller mitochondria, they confirm the opinion that larger mitochondria are associated with increased JH biosynthesis. Hence, fluctuations in mean mitochondrial size suggest cycles of JH production in individual corpora allata of the adult worker honey bee.

At the outset of this ultrastructural study, it was hoped that morphological indicators would be discovered that would explain the well known age-dependent increase in output of JH by corpora allata in adult European honey bee workers during summer. As already discussed, pooling of haemolymph for JH determination smothers detection of any possible cyclical fluctuations in JH titre of individual bees. However, even if JH production is cyclic, the higher JH titre in older bees remains to be explained in ultrastructural terms. Since the cytosol is heavily involved in the majority of chemical reactions that take place during JH biosynthesis, overall volume of cytosol in a corpus allatum determines how much JH is produced at the peak of a production cycle. Therefore, corpus allatum size remains as the most reliable indicator of the quantity of JH that can be produced at any given time in the life of the adult worker honey bee.

Table 1. Occurrence and nature of indicators of corpus allatum activity during the first thirty days of worker honey bee adult life.

SAMPLING DAY:	0	3	6	9	12	15	18	21	24	30
Mean mitochondrial size	S	S	S	S	L	S	L	L	S	S
L - large ($\geq 0.12\mu m^2$) S - small ($\leq 0.08\mu m^2$)										
"Light" cells/interdigitations	P	P		P		P			P	P
P - present										
Extracted vacuoles	P+	P+	P+			P		P	P	
P - present P+ - abundant										

Note: On each sampling day the sample number is one corpus allatum. This is because no two corpora allata were found to be in identical condition on the same sampling day. For example, on Day 30 one member of a pair of corpora allata was found to have light cells, whilst light cells were absent from the other member. Pooling of mitochondrial size data from separate corpora allata would have prevented detection of the statistically significant size classes. Furthermore, when the mitochondrial size data was displayed in fig. 5, the two "peaks" in mean mitochondrial size were sandwhiched quite fortuitously between the "troughs" of smaller size, thus conveying the impression of cycles. More than two cycles of rise and fall in mean mitochondrial size might be found during a thirty day period in a corpus allatum *in vivo*.

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