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IRON-CONTAINING CELLS IN THE HONEY-BEE (*APIS MELLIFERA*)

II. ACCUMULATION DURING DEVELOPMENT

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SUMMARY

The development of iron granules in honey-bee tissues was investigated using both anatomical and analytical methods. Iron granules are present only in the trophocytes of post-eclosion adults and have the same elemental composition as those in foraging adults. The granules increase in both size and number during ageing. Iron levels in developing worker honey-bees were measured by proton-induced X-ray emission spectroscopy. The rate of iron accumulation was directly related to iron levels in the diet, and the iron can be obtained from pollen and honey, both major food sources of the bee. In adults, the iron content of the fat body reached a maximum level ($2{\cdot}4 \pm 0{\cdot}15\ \mu g\ mg^{-1}$ tissue), regardless of the amount of iron available for ingestion. Maximal iron levels are reached at the time when honey-bee workers commence foraging behaviour, suggesting that iron granules may play a role in orientation. Alternatively, accumulation of iron in granules may be a method of maintaining iron homeostasis.

INTRODUCTION

The trophocytes of adult worker honey-bees (*Apis mellifera*) contain numerous iron-rich granules distributed throughout the cytoplasm of the cells (Kuterbach, Walcott, Reeder & Frankel, 1982). Approximately 7 % of the trophocyte cell volume is occupied by these granules in foraging adults, and the granules account for 0·1 % of the total body weight. We raise here questions about the origin of this iron, granule formation, and the time of granule formation in the developing honey-bee. Early investigations on iron metabolism suggest that insects obtain iron from their diet (Koschevnikov, 1900; Harnisch, 1925). Those studies, however, were performed on iron-loaded experimental animals, and the results cannot be compared to naturally occurring processes.

We have studied iron accumulation in developing honey-bees using both anatomical and analytical techniques. The Prussian Blue reaction (Hutchinson, 1953) and electron microscopy were used to detect iron granules within the trophocytes of

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developing bees. Proton-induced X-ray emission (PIXE) spectroscopy was used to measure iron levels both in whole bees and in isolated tissues and to quantify changes in iron concentration during development. The iron contents of the fat body of adult honey-bees raised on diets containing different iron levels were also compared to determine some aspects of iron accumulation.

MATERIALS AND METHODS

Specimen collection

Larvae and pupae were obtained from the brood combs of hives located on the North Shore of Long Island, and staged according to Thompson (1978). Specimens for microscopy were used immediately, while those for PIXE analysis were weighed, quick-frozen in liquid freon 22 (Balzers) at liquid nitrogen temperature, and stored at −80°C.

Newly eclosed adults were removed from the brood comb at the time of eclosion and maintained in the laboratory in plastic containers covered with cheesecloth within incubators at 28°C. Specimens were removed at 3-day intervals following eclosion and prepared for light and electron microscopy and PIXE analysis. Foraging adults were collected as previously described (Kuterbach & Walcott, 1986).

Microscopy

Larvae and pupae

Larval and pupal specimens were pierced with stainless-steel minuten pins before immersion in 2·5% glutaraldehyde in 0·1 mol l^{-1} sodium cacodylate buffer, pH 7·2 for 30 min. The specimens were then removed, slit lengthwise, and reimmersed in the fixative for another hour. Specimens were washed in buffer and dehydrated in a graded ethanol series before embedding in JB-4 glycol methacrylate medium. Thick sections (3 μm) were cut using glass knives, and every third section was collected.

Sections were tested for the presence of iron by the Prussian Blue technique following the method of Hutchinson (1953). All solutions were heated to 60°C prior to use. The sections were dehydrated in 95% and then absolute ethanol, cleared in xylene, and coverslipped using Permount mounting medium.

For electron microscopy, specimens were fixed as described above and post-fixed for 30 min with 1% OsO_4. Specimens were embedded and sectioned for electron microscopy as previously described (Kuterbach & Walcott, 1986).

Adults

Newly eclosed adults were maintained in the laboratory on a diet of comb honey, supplemented with 50% sucrose in distilled water, and comb pollen. Specimens were removed at 3-day intervals and dissected as previously described (Kuterbach & Walcott, 1986). Fat body tissue was fixed *in situ* with 2·5% glutaraldehyde in 0·1 mol l^{-1} sodium cacodylate buffer, pH 7·2. Fat body tissue was then excised and embedded in glycol methacrylate as above. Sections (3 μm thick) were treated for iron with the Prussian Blue reaction as above.

Fat body tissue for electron microscopy and analytical electron microscopy was excised and embedded as previously described (Kuterbach & Walcott, 1986). Measurements of cell and granule sizes were made on photographic montages of thin sections passing through the nuclear region of cells. Cell dimensions were measured at the widest points, and the area of the cell profile was determined by assuming an oval cell shape. Granule number was counted and expressed as the number of granules per area of cell profile. Nuclear area was not subtracted from the total cell area. Granule diameter was measured across the largest dimension.

Proton-induced X-ray emission

Proton-induced X-ray emission (PIXE) spectroscopy is a sensitive, non-destructive method to measure accurately elemental composition in samples (Johannson & Johannson, 1976) and has been used successfully in biological tissues (Van Rinsvelt, Duerkes, Levy & Cromroy, 1973). Therefore, PIXE was used to measure changes in tissue iron content during development.

Sample holders were constructed of 1 mm thick hard plastic (Lexan) squares (5 cm sides) with a centre aperture of 3·75 cm diameter. Formvar films were stretched across the aperture, and tissue samples were placed into the centre of the film. A second layer of Formvar was used to seal the sample, creating a sandwich. The samples were allowed to air dry for several days before analysis, resulting in a total specimen thickness of $\leq 1{\cdot}0$ mm.

Samples were taped over numbered openings in an aluminium rotating wheel, and placed in a scattering chamber which was then pumped to a vacuum of $\leq 10^{-6}$ Torr. The samples were excited with a 7 mm, 15–20 nA proton beam generated by a 2·5 MeV Van de Graff accelerator located in the Department of Applied Physics at Brookhaven National Laboratories (Upton, NY). The induced X-rays from the specimen were collected by a Si(Li) energy dispersive detector, and the spectra fed into a Nuclear Data 6600 Data Acquisition System for analysis. Precautions were taken to capture back-scattered electrons and transmitted protons by placing electrified apertures in front of and behind the samples. The size and placement of the beam were checked by visualizing the beam on a zinc sulphide film.

Standards were made by dissolving 2·54 mg $FeCl_2$ in 20 ml of honey-bee saline containing 2% agarose (designed to simulate the intracellular environment). Serial dilutions were made from the stock solution so that the final concentrations of iron were 5·4, 1·08, 0·54, 0·108, 0·054 and 0·0054 $\mu g\, 2\, \mu l^{-1}$. 2-μl samples were routinely used, and were measured with a Pipetman automatic pipette. Blanks, consisting of empty Formvar films, honey-bee saline and 2% agarose in saline, were assayed for possible contamination before each run.

All samples were run at 15–20 nA beam current, and X-ray counts taken for 10 min, resulting in a total sample excitation energy of 1500 μC. Each sample was assayed three times, after which it was removed from the Formvar film and weighed on a Cahn electrobalance to obtain the total dry weight.

The iron content was determined for each run using the following formula:

$$C = \frac{P-B}{mk},$$

where C is the concentration of iron in the sample, P is the total area under the iron kα peak (6·48 keV), B is the total area of the background counts under the iron kα peak, m is the slope of the line generated by plotting the total counts obtained for the series of standards on a logarithmic scale, and k is the total area equal to 1·0 μg iron (obtained from the standards).

The iron content was the average of the three measurements divided by the total dry weight of the sample. Student's *t*-test was applied to differences in iron content between tissues to determine whether these differences were significant.

Larval specimens were either placed whole on the Formvar films (length $\leq$ 1·0 mm) or mounted on brass stubs with distilled water and cryosectioned (50–80 μm) using a glass Ralph knife. Pupal and adult tissues were dissected and rinsed in fresh saline before placement on the Formvar films.

Iron accumulation in bees raised on diets varying in iron content

Adult workers were obtained at the time of eclosion and maintained in the laboratory as previously described. The bees were divided into two groups, one receiving 50% sucrose in distilled water (iron-deficient), and one receiving 10 μmol l^{-1} $FeCl_2$ in 50% sucrose solution (iron-enriched) as the liquid portion of their diet. Both groups had free access to commercially gathered pollen (Long Island Beekeepers' Association).

Two to six adults from each group were removed at 3, 6, 9, 12, 15 and 20 days post-eclosion and the iron content of the fat bodies was examined by PIXE as above.

Food sources such as honey and pollen were examined for iron content by PIXE. 2-μl samples of comb honey, and individual pollen pellets of commercially gathered pollen were analysed. The pollen pellets are removed from forager pollen baskets as the foragers crawl through a mesh to enter the hive. Since each forager is faithful to the species of plant it visits (Singh, 1950), each pollen pellet represents pollen gathered from a single species of plant. Pollen was identified merely by colour.

RESULTS

Neither light microscopic examination of 3-μm plastic sections treated with the Prussian Blue technique nor electron microscope sections showed any blue granular reaction product in any tissues of larvae or pupae. The fat bodies of newly eclosed adults were also non-reactive. Fat bodies of ageing post-eclosion adults, however, show the Prussian Blue reaction product at about 6 days post-eclosion (Fig. 1 A–D). The reaction product gradually increases between 6 and 12 days post-eclosion, but beyond 12 days the cells containing reaction product have the same distribution and intensity as in foraging adults (Kuterbach *et al.* 1982). No other adult tissues showed this accumulation.

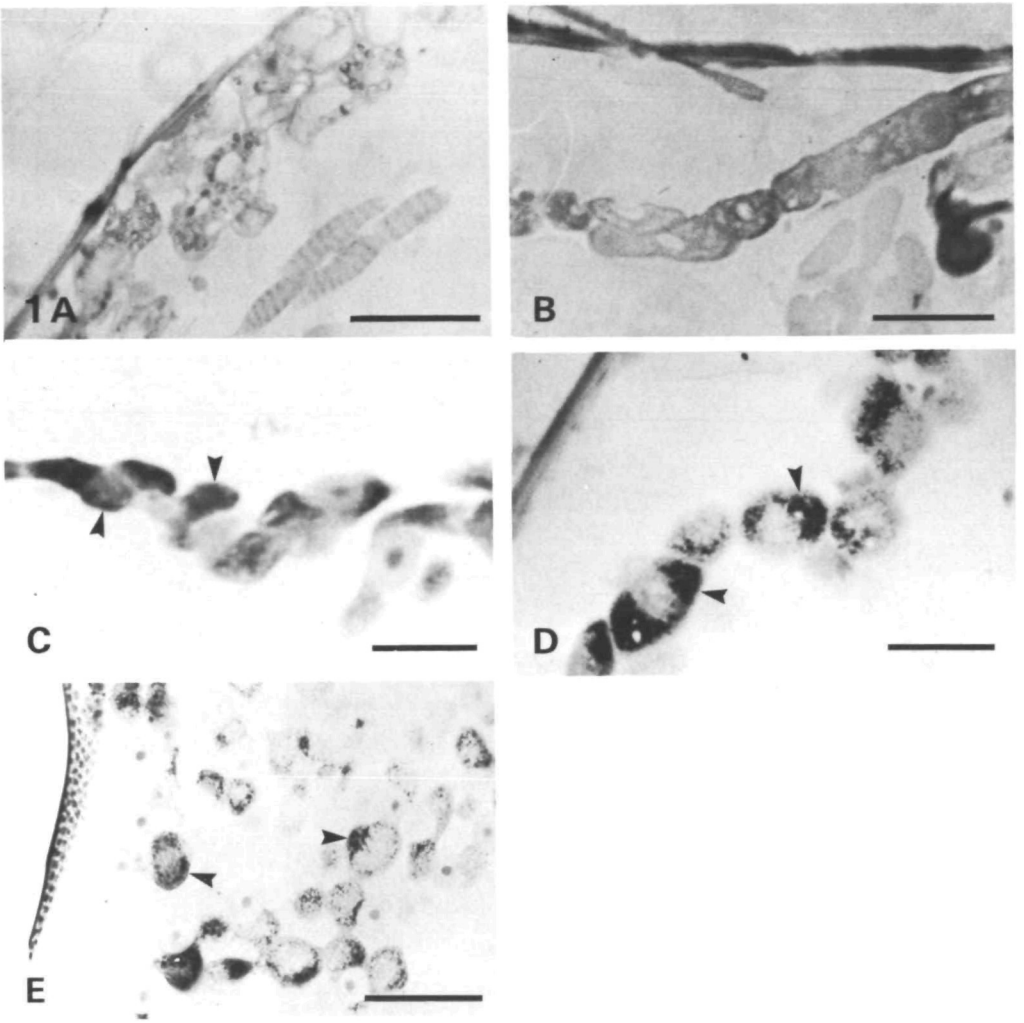

Fig. 1. Prussian Blue reaction of post-eclosion worker trophocytes. There is a gradual increase in observable Prussian Blue reactivity (arrowheads) within the trophocytes of ageing bees from 6 to 12 days post-eclosion which resembles that of the foraging adult in distribution and intensity. (A) 0 days; (B) 3 days; (C) 6 days; (D) 12 days; (E) foraging adult. Scale bars: A,B,E, 100 μm; C,D, 50 μm.

Electron microscopy

The trophocytes of larvae and pupae undergo gross cytological changes during development as has been described by Bishop (1922, 1958). The cytoplasm of the larval and pupal trophocytes was never seen to contain electron-dense iron granules, even when examined at high magnification (Fig. 2A–D). Although some cells contain dark granular structures, these did not shatter when sectioned and they were not visible in unstained sections of unosmicated tissue, indicating they are probably the albuminoid granules described by Bishop (1958).

However, electron-dense granules were seen in the trophocytes of newly eclosed adults. These granules were smaller, averaging 0·10 ± 0·03 μm in diameter, but were morphologically similar to those in foraging adults (Fig. 3A). When examined by

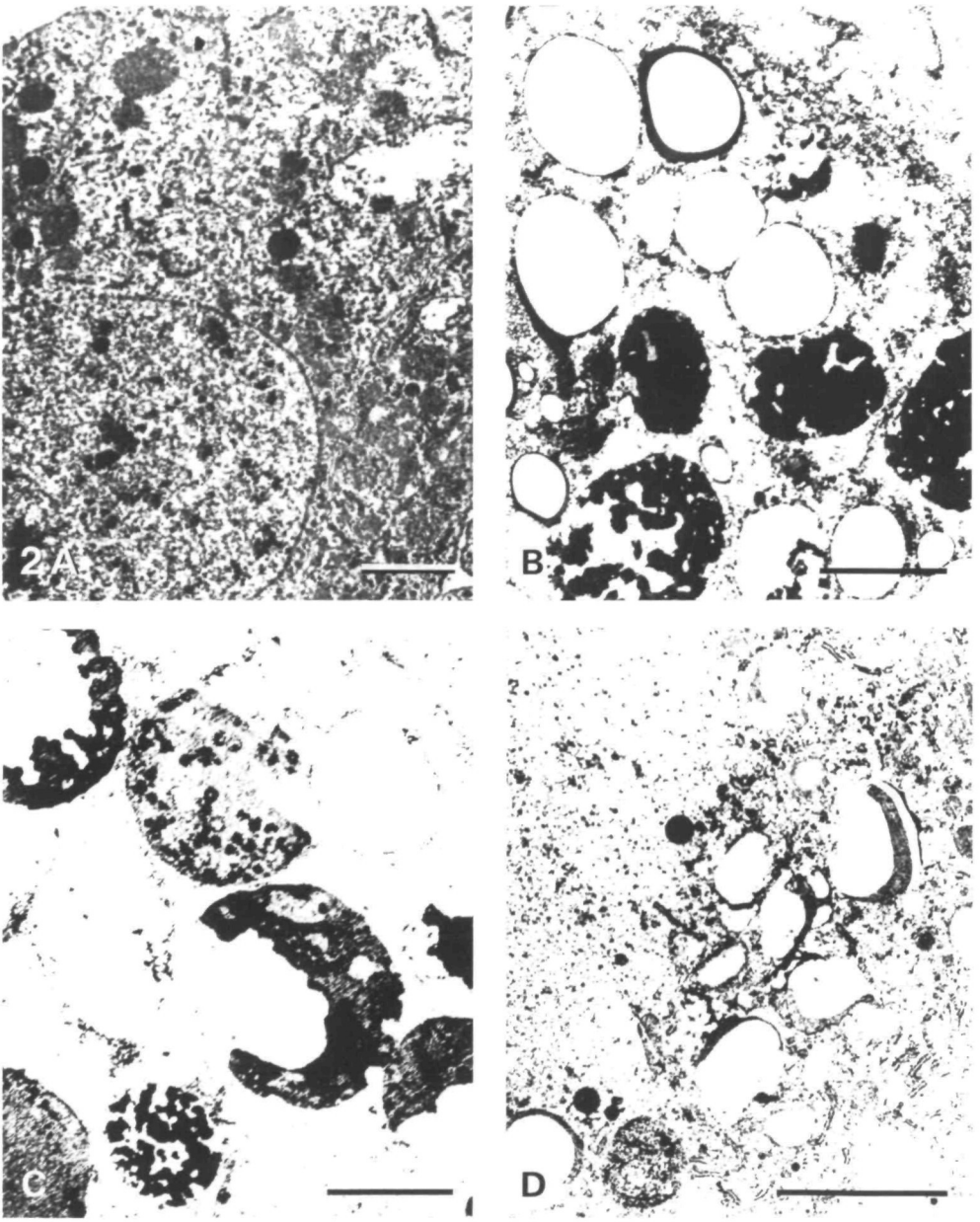

Fig. 2. Electron micrographs of the cytoplasm of larval and pupal trophocytes. Although some dark spherical bodies can be seen in these sections, no electron-dense granules can be seen in unstained sections of unosmicated tissue. (A) 2 mm larva; (B) prepupa; (C) brown-eyed pupa; (D) imago. Scale bars, 5 μm.

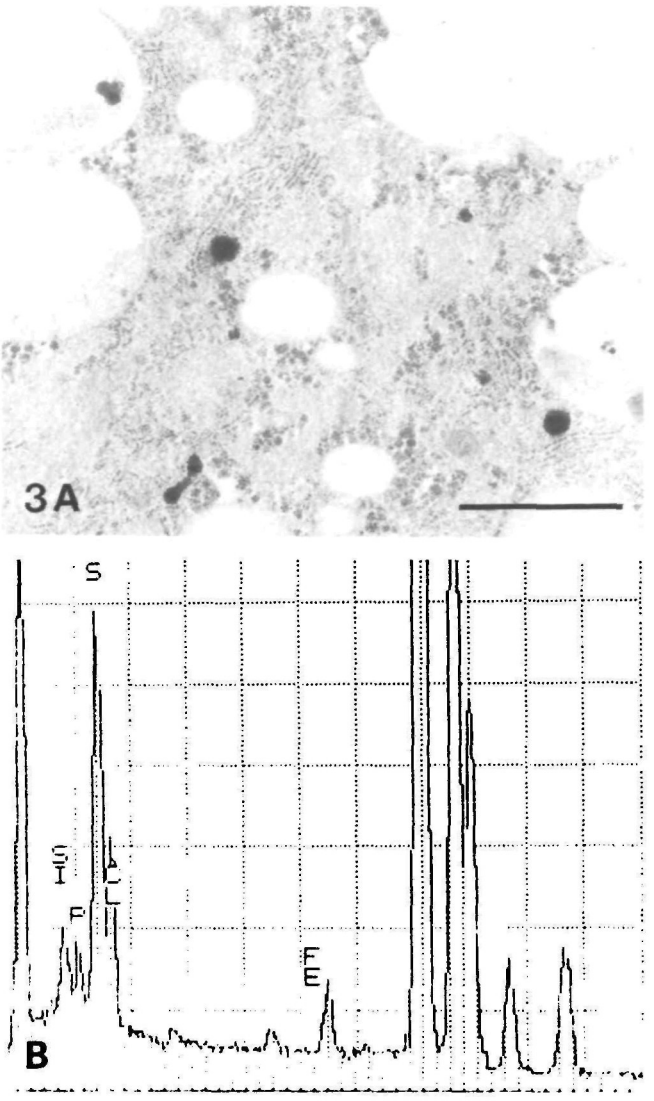

Fig. 3. Electron-dense granules in newly eclosed workers. (A) High magnification electron micrograph of the cytoplasm of a trophocyte from a newly eclosed worker. Small (0·1 μm), electron-dense granules can be seen. Scale bar, 0·5 μm. (B) Energy-dispersive X-ray spectrum of an electron-dense granule in an unstained, thick section of unosmicated fat body from a newly eclosed worker. Peaks at 2·0 and 6·4 eV show the presence of iron (FE) and phosphorus (P) in approximately the same ratios as was found in iron granules of adult foraging bees (Kuterbach & Walcott, 1986). Other peaks present are from the embedding media (EPON 812, DuPont) and the copper support grid; 80 kV, 200 s, 35° tilt.

energy-dispersive X-ray microanalysis, these small granules were found to contain iron and phosphorus in approximately the same proportions as in the adult granules. The calcium peak, seen in the adult granules, was, however, absent (Fig. 3B). The granules increased in size [average diameter $0{\cdot}10 \pm 0{\cdot}03\,\mu$m ($N = 100$) to $0{\cdot}32 \pm$

0·07 μm ($N = 900$), Fig. 4] and the number of granules per cell also increased (Fig. 5) with age. The increase in number followed a sigmoidal curve, with the most rapid change in numbers occurring between day 3 and day 6 post-eclosion. After day 9, the number of granules reached a plateau.

Proton-induced X-ray emission

Normal development

The anatomical methods revealed that iron granules were present in the trophocytes only after the adults had emerged from the comb. However, these techniques would not reveal the presence of iron in a soluble, protein-associated form. Therefore, proton-induced X-ray emission (PIXE) was used to quantify changes in tissue iron content during development.

The total iron content in honey-bee larvae increased with age from 0·039 ± 0·015 μg immediately after hatching from the egg to 1·764 ± 0·339 μg in the prepupae. Although the total iron content increased, the concentration of iron per milligram of tissue did not vary (Table 1). During pupation, there is no intake of dietary iron and, therefore, the total iron level in the imago (late pupa) would not be expected to differ from that of the prepupa (1·764 μg). Calculations of the total iron present in the imago (1·477 μg) showed that this is indeed the case.

The reorganization of tissues during pupation allows individual tissues to be assayed for iron content and compared to foraging adult tissues (Table 1). Tissues such as intestinal tract and flight muscle contained the same amount of iron as in the

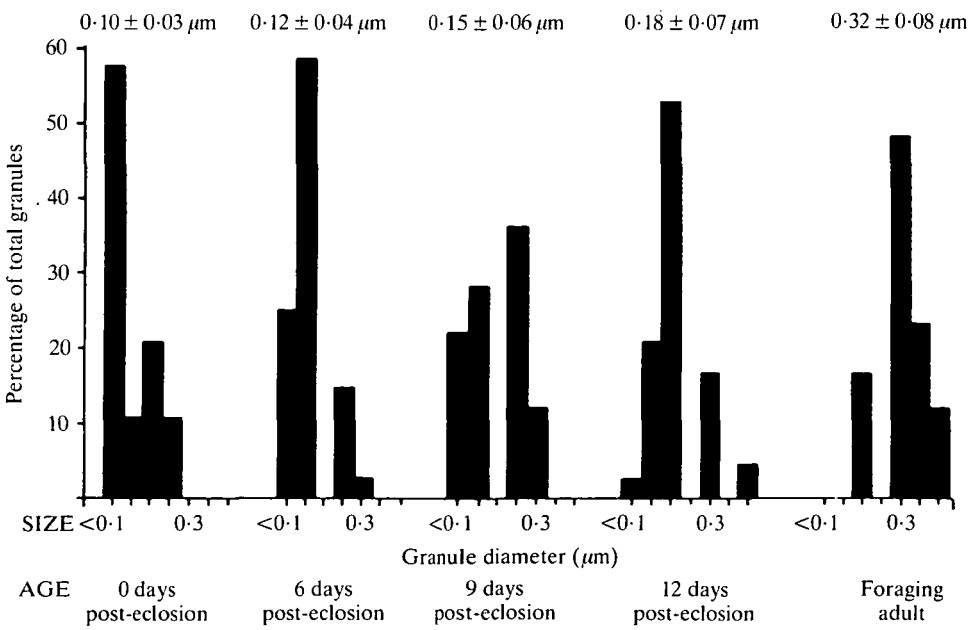

Fig. 4. Honey-bee granules increase in size with age. Histogram showing the distribution of granule sizes at 3-day intervals post-eclosion. A gradual increase in granule size is seen. Average granule diameters ± S.E. are given above the bars.

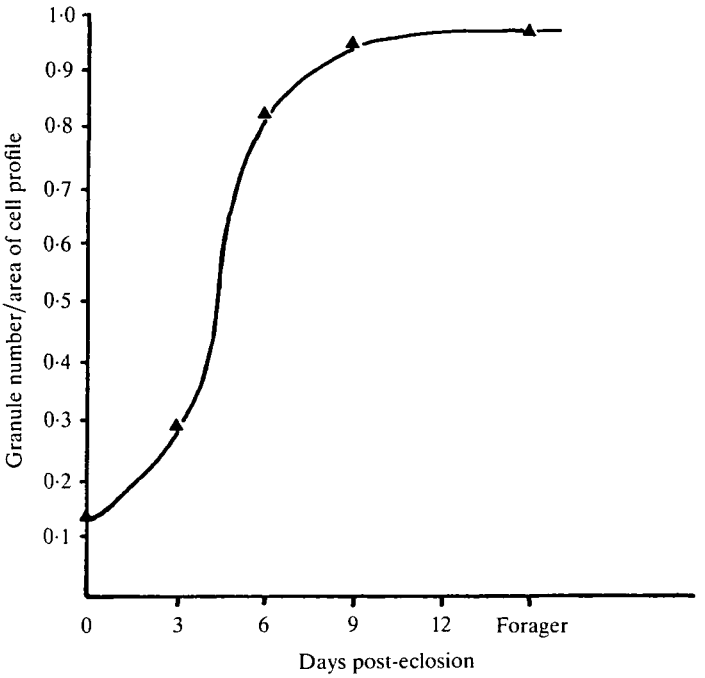

Fig. 5. Granule number per trophocyte increases with age. The most rapid increase in granule number occurs between 3 and 6 days post-eclosion. After day 9, the total granule number reaches a plateau.

adult. By contrast, the fat body of the imago contained only one-tenth the amount seen in foraging adults. Thus, iron was specifically accumulated by the fat body of post-eclosion worker honey-bees.

Honey-bee food sources were also assayed for iron content (Table 1). Four different pollen samples from commercially gathered pollen and three samples of comb honey were measured. Pollen contained the highest levels of iron, with amounts ranging from $0{\cdot}011 \pm 0{\cdot}009$ to $1{\cdot}642 \pm 0{\cdot}037\ \mu g\ mg^{-1}$ depending on the plant species. Honey contained $0{\cdot}002 \pm 0{\cdot}001\ \mu g\ mg^{-1}$ (approximately 0·35 $\mu mol\ l^{-1}$). Thus, pollen is the most likely source of iron in honey-bees.

Iron accumulation during iron deprivation

The total fat body iron and total body iron content of bees raised on an iron-deprived diet was compared with the total iron content in the prepupae (Table 2). The total iron content of the fat body in these bees increased from $0{\cdot}037 \pm 0{\cdot}005$ to $0{\cdot}128 \pm 0{\cdot}013\ \mu g$ during the first 3 days post-eclosion. From 3 to 12 days post-eclosion, the iron content in the fat body fluctuated between $0{\cdot}128 \pm 0{\cdot}013$ and $0{\cdot}312 \pm 0{\cdot}003\ \mu g$; however, the total body iron content during this time remained below the total iron content of the prepupae. Beyond 15 days post-eclosion, both the total fat body iron and the total body iron increased, with the total body iron reaching a maximum of $2{\cdot}377\ \mu g$.

Iron accumulation during iron loading

The iron content of the fat bodies of bees raised on an iron-enriched diet was compared with the iron content of those raised on an iron-deprived diet (Fig. 6). In both groups, an increase in fat body iron content follows eclosion. The accumulation of iron was more rapid and the levels of iron initially accumulated were much greater in the group fed the iron-enriched diet. Both groups reached maximum iron levels, and by day 20 the iron levels in the fat bodies of the two groups were not significantly different ($P > 0{\cdot}05$). These maximum iron levels were also not significantly different from the iron levels measured in the fat bodies of wild foraging adults ($P > 0{\cdot}05$).

DISCUSSION

Honey-bees are holometabolous insects, and their development includes larval and pupal forms. Immediately following hatching from the egg, the larvae begin a period of growth during which they are fed by the nurse bees with a mixture of honey and pollen known as bee bread (Snodgrass, 1925). The larvae increase in both size and mass between the time of hatching until just prior to pupation. The prepupal larvae (prepupae) stop feeding and are sealed into the cell where they undergo pupation. At the time of eclosion (emergence from the comb), the honey-bees are considered to be adults, and thereafter show only behavioural changes (Rosch, 1925). The newly

Table 1. *Iron content in honey-bee tissues and food sources as revealed by PIXE analysis*

Sample	*N*	Iron content ($\mu g\,mg^{-1}$ tissue ± S.E.)
Larva (2 mm)	3	0·052 ± 0·026
Prepupa	3	0·044 ± 0·007
Imago		
Intestinal tract	3	0·045 ± 0·002
Flight muscle	5	0·184 ± 0·021
Fat body	12	0·247 ± 0·045
New adult		
Intestinal tract	3	0·040 ± 0·006
Fat body	6	0·147 ± 0·021
Foraging adult		
Intestinal tract	3	0·017 ± 0·009
Brain	9	0·124 ± 0·029
Flight muscle	7	0·208 ± 0·033
Fat body	12	1·845 ± 0·255
Pollen		
Orange	3	0·011 ± 0·009
White	3	0·038 ± 0·012
Yellow	3	0·067 ± 0·013
Grey	3	1·642 ± 0·037
Comb honey	3	0·002 ± 0·001

Table 2. *Iron content in post-eclosion adult workers raised on 50% sucrose and pollen (measured by PIXE analysis)*

Age (days)	N	Total iron content (μg ± S.E.) Fat body	Total body*
0	6	0·037 ± 0·005	1·456
3	6	0·128 ± 0·013	1·546
6	5	0·312 ± 0·003	1·730
12	5	0·156 ± 0·031	1·575
15	3	0·429 ± 0·059	1·848
17	2	0·958 ± 0·049	2·377
20	3	0·774 ± 0·171	2·193
Prepupa	2	—	1·764 ± 0·339

* Estimated by adding total fat body iron and total flight muscle iron (1·419 μg).

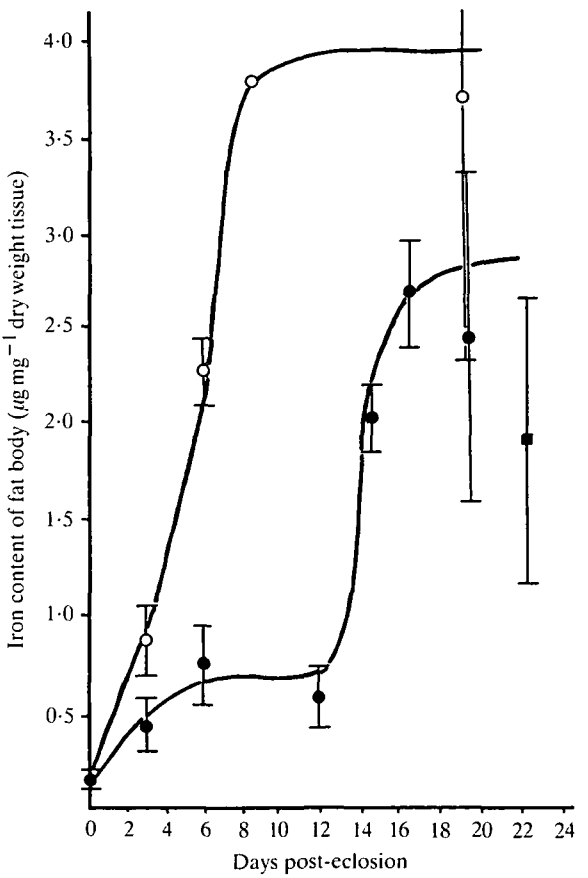

Fig. 6. The effects of dietary iron on iron accumulation. Bees fed an iron-enriched diet (open circles) accumulate iron more rapidly than those fed an iron-deficient diet (closed circles). Accumulation in both groups continues until similar ($P > 0{\cdot}05$) maximum levels are reached. These are not significantly different from the levels found in wild foraging honey-bees (closed square) ($P > 0{\cdot}05$). Bars show ±S.E.

eclosed bee is responsible for cleaning the hive and rearing the brood. At approximately 6 days post-eclosion, the workers begin to take short orientation flights which increase in length and duration as the bees age. By day 12, the workers are fully fledged foragers. The results presented in this paper were obtained using laboratory-reared bees. While this allows for control of the diet, it disrupts the normal behavioural activity of the worker. Therefore, these results may differ from colony-raised bees.

Iron granules are present in honey-bee trophocytes only after the adults emerge from the comb. Although PIXE analysis of prepupae shows that there is iron present, granule formation does not occur. This suggests that, during the larval and pupal periods, iron is found only in a protein-associated form and is perhaps used for metabolism. Once the pupal period has ended, these enzymes may no longer be needed in such large quantities, and the iron may be released to form granules.

The iron granules in newly eclosed adults are smaller than those in foraging adults, but X-ray microanalysis reveals a similar composition. The granules increase sigmoidally in number as the adults age, with the most rapid increase occurring between 3 and 6 days post-eclosion (see Fig. 5). After this, the number remains constant. Since the trophocytes are not increasing in size, this plateau in granule number suggests that there may be a finite number of sites within the cell available for granule formation. Granule size also increases with age (Fig. 4), indicating that material is added to existing granules during maturation of the bee.

The iron in the trophocytes is derived, in part, from internal stores. This is shown by comparing the total iron content of the fat bodies of adults raised on an iron-deprived diet with the total iron levels in the prepupae (Table 2). Total iron levels in the fat body of iron-deprived bees increased following eclosion from $0{\cdot}037 \pm 0{\cdot}005\,\mu g$ (0 days) to $0{\cdot}312 \pm 0{\cdot}003\,\mu g$ (6 days). Between day 6 and day 12, the iron levels remained constant. The total body iron (estimated by summing the total fat body iron and total flight muscle iron) of adults between 0 and 12 days post-eclosion never exceeded the total body iron of the prepupae. Since the pupae do not feed, the iron accumulated by the fat body during that time must have been derived from internal stores.

Although some iron comes from internal stores released after pupation, a large fraction of it, located in the fat body, appears to be obtained from pollen in the diet. Bees which were raised on an iron-rich diet accumulated iron more rapidly than those raised on an iron-deficient diet. However, the final plateau levels of iron that were accumulated in both groups were not significantly different from each other. These final levels were also not significantly different from iron levels in wild, foraging adults. Thus, the total accumulation of iron appears to be a directed action, and not merely a response to high levels of iron in the diet. Iron levels in bees fed the iron-deficient diet increased sharply after 12 days post-eclosion. Since the liquid portion of their diet contained no iron, this may be caused by ingestion of pollen.

The role of iron granules in the trophocytes of honey-bees is unknown. As discussed in the companion paper (Kuterbach & Walcott, 1986), they may be involved in the detection of magnetic fields. In this light, it is interesting to note that

the number of iron granules reached a maximum at approximately the time when the honey-bees begin foraging behaviour (9–12 days post-eclosion).

Alternatively, the iron granules may play a role in maintaining iron homeostasis. Many of the enzymes required for energy production and respiration are iron-containing enzymes (e.g. cytochrome oxidase, NADPH oxidase). These enzymes are present in large quantities in the flight muscle, where they are used for energy production during flight (Crabtree & Newsome, 1972). Thus, flight activity creates a potentially high requirement for iron. The composition of the iron granules in the trophocytes shows that these granules are similar to some forms of iron storage granules (Kuterbach & Walcott, 1986); however, the availability of this iron has not been investigated.

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