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Functional Flexibility of the Honey Bee Hypopharyngeal Gland in a Dequeened Colony

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ABSTRACT—The role of the worker honey bee *Apis mellifera* L. changes depending on age after eclosion (age polyethism): young workers (nurse bees) take care of their brood by synthesizing and secreting brood food (royal jelly), while older workers (foragers) forage for nectar and process it into honey. Previously, we showed that the major proteins synthesized in the hypopharyngeal gland of the worker change from brood food proteins to α-glucosidase at the single secretory cell level in parallel with this age polyethism [Kubo *et al*., J. Biochem. 119, 291–295 (1996); Ohashi *et al*., Eur. J. Biochem. 249, 797-802 (1997)]. Here, we examined whether the function of the hypopharyngeal gland has flexibility depending on the colony conditions, by creating a dequeened colony in which the older workers were compelled to feed the drone larvae. It was found that most of the older workers in the dequeened colony synthesized brood food proteins as did nurse bees. Furthermore, the percentage of workers that synthesized brood food proteins was maintained at 80–90% of the total workers for at least two months, as in a normal colony. These results indicate that the function of the hypopharyngeal gland cells of the worker has flexibility and can, if necessary, be maintained as that of the nurse bee, depending on the condition of the colony.

INTRODUCTION

A change in age-dependent roles (age polyethism) is one of the most characteristic features of the honey bee *Apis mellifera* L. society. The average life span of workers is usually 30–40 days during spring to autumn of a year (Dayer, 1991). During this period, young workers (nurse bees, usually younger than 13 days after eclosion) take care of their brood by synthesizing and secreting brood food (royal jelly), while older workers (foragers, usually older than 18 days) forage for nectar and process it into honey by converting sucrose to glucose and fructose (Lindauer, 1952; Sakagami, 1953; Winston, 1987). In parallel with this age-dependent shift in the roles, physiological changes occur in certain organs of the worker. For instance, the hypopharyngeal gland, which synthesizes brood food (Rösch, 1930; Halberstadt, 1980; Knecht and Kaatz, 1990), is well developed in the nurse bee, whereas it shrinks in the forager and develops the enzymatic activity to hydrolyze sucrose (Simpson *et al*., 1968; Sasagawa *et al*., 1989).

Previously, we identified three major proteins from the hypopharyngeal gland of nurse bees, that are synthesized as brood food proteins (50-, 56- and 64-kDa proteins), and one major protein, α-glucosidase, from the forager-bee gland as an α-glucosidase (Kubo *et al*., 1996; Ohashi *et al*., 1996; Ohashi *et al*., 1997). In addition, we showed that mRNAs for two other carbohydrate-metabolizing enzymes (amylase and glucose oxidase) were expressed specifically in the forager-bee gland (Ohashi *et al*., 1999). Amylase is thought to convert starch of plant origin, which is found in nectar, into glucose, and glucose oxidase is needed to convert glucose to gluconic acid and hydrogen peroxide (Winston, 1987; White *et al*., 1963). We showed by *in situ* hybridization analysis that the change in the expression of mRNAs for the 64-kDa brood food protein and α-glucosidase occurred in a single secretory cell in the acini of the gland (Ohashi *et al*., 1997). Thus, the hypopharyngeal gland cell seems to exist in two distinct differentiation states, which can be characterized by protein expression, and the function of the gland switches along with the age-dependent change in worker's role.

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The role change of the worker honeybee is flexible to some extent and depends on various conditions such as colony demography (Rösch, 1930; Huang and Robinson, 1996), colony size (Winston and Fergusson, 1985), additional storage comb for the colony (Fergusson and Winston, 1988), and the season (Fukuda, 1983). However, it is still unclear whether the physiology of the worker, such as the function of the hypopharyngeal gland, is programmed so that workers are capable of performing certain tasks at certain ages, or whether colony conditions dictate the activity of the gland. To gain more insight into the functional flexibility of the hypopharyngeal gland of the worker, we examined whether individual older bees can synthesize brood food proteins when they are compelled to feed their brood, in an artificially dequeened colony.

MATERIALS AND METHODS

Preparation of a dequeened honey bee colony and sampling of workers

An *Apis mellifera* L. Italian race colony consisting of about 10,000 workers, which was located in the Experiment Station for Medical Plant Studies (Chiba, Japan) of the University of Tokyo, was used. The queen and resident queen cells were removed from the colony on March 23, 1997. Some cells containing young larvae were reformed into so-called "emergency queen cells" by the workers within a few days for regeneration of new queens. Since the total developmental period from egg hatching to adult emergence for a queen is 14–17 days (Winston, 1987), these "emergency queen cells" were also removed 10 days later (April 2). In this way, we created a completely dequeened colony, where no young larvae that could differentiate into queens were present. Ten days later, all the resident pupae emerged as workers. The day when the last worker emerged (April 12) was designated as "day 0" (Fig. 1). After this time, the resident workers were older than indicated by the "day". For example, at day 28 (May 10), the youngest workers were 28 days old, while most of the workers were older than 28 days. Thirty to forty workers were randomly sampled from the inner side of the hive in the evening of days 28, 35 (May 17), 47 (May 29) and 61 (June 12). The workers collected on March 11 (day –32) beforehand were used as control samples.

Three different colonies, termed "A", "B" and "C", were used as control colonies in our experiment. Nurse and forager bees were collected on May 19, 1993, from a normal colony "A", located at Tamagawa University, according to their behavior (Kubo *et al.*, 1996). Ten workers whose behavior had not been identified were collected from colony "A" on May 5, 1993, which was 29 days after eclosion (Kubo *et al.*, 1996). Twenty eight workers were collected on July 5, 1997, from a normal colony "B", located in the Experiment Station for Medical Plant Studies of the University of Tokyo, as an additional control (Table 1).

For preparation of 6- and 40-day-old workers (Table 2), newly emerged workers in a normal colony "C", located at Tamagawa University, were marked with paint on the dorsal thorax on May 7, 1998, and 30 workers each were collected 6 or 40 days thereafter, respectively.

Preparation of hypopharyngeal gland homogenates

Hypopharyngeal glands collected from workers were homogenized in 0.1 ml of buffered insect saline (10 mM Tris-HCl buffer pH 7.4, containing 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$) in a glass homogenizer on ice as described previously (Kubo *et al.*, 1996). The homogenate was centrifuged at 700 x *g* for 10 min to remove debris, and the resulting supernatant was stored at –80°C.

Electrophoresis, immunoblotting and dot-blot analyses

Electrophoresis in SDS-polyacrylamide slab gels was carried out by the method of Laemmli (1970). Samples were denatured for 20 min at 75°C in 1% (w/v) SDS and 2% (v/v) 2-mercaptoethanol. After electrophoresis, the gel was stained by the method of Fairbanks *et al.* (1971).

For immunoblotting analysis, proteins separated by electrophoresis were transferred electrophoretically to a polyvinylidene difluoride filter (Immobilon, Millipore). For dot-blot analysis, proteins were blotted directly on a nitrocellulose filter (Schleicher and Schuell). Then, the filters were washed for 15 min at room temperature with TBS-T (10mM Tris-HCl buffer, pH 7.4, containing 130 mM NaCl and 0.1% Triton X-100) to remove SDS. The filters were immersed in skim milk solution (TBS-T containing 5% skim milk) for at least 1 hr, transferred to 5 ml of rinse solution containing affinity-purified antibody against either 50-kDa brood food protein or α-glucosidase (diluted 2,000-fold) (Kubo *et al.*, 1996) and shaken gently for 1 hr at room temperature.

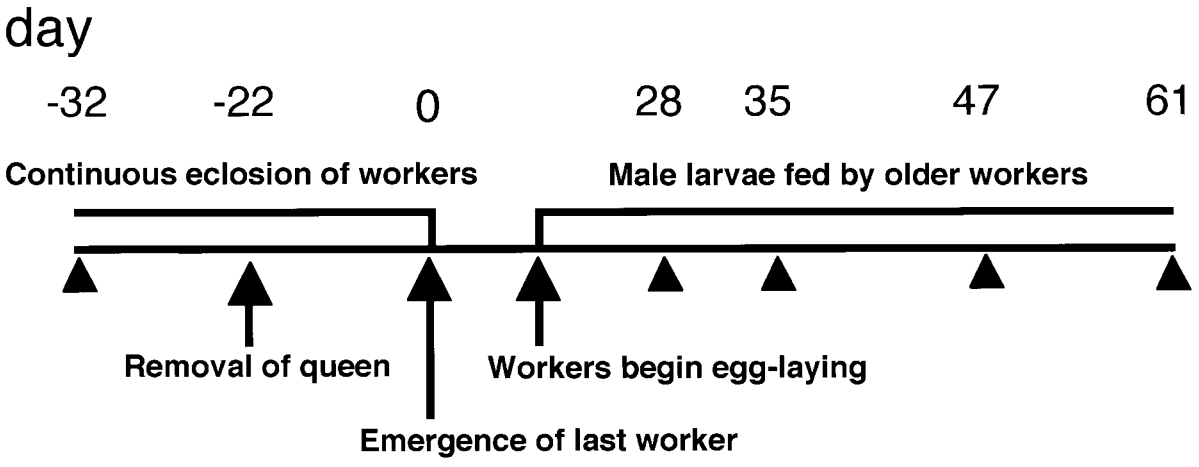

Fig. 1. Schematic representation of the schedule for preparation of a dequeened colony and sampling of the workers. The horizontal line indicates the time course, where "day 0" indicates the day when the last workers emerged. The queen and queen cells were removed from the colony on March 23, 1997 (day -22), and 30–40 workers were randomly collected from the colony on day -32, 28, 35, 47 and 61 as indicated by arrowheads. Some workers were assumed to start laying eggs around day 10. The upper horizontal line to the left of day 0 indicates the period for which eclosion of workers continued, whereas the upper horizontal line to the right indicates the period during which drone larvae were fed by old workers.

Table 1. Ratio of workers whose hypopharyngeal glands exhibited the nurse-type protein profile to the total workers in the dequeened colony[a]

		Protein profile			
Day	n	N[b]	F[b]	Neutral[c]	Ratio of N (%)
–32	40	33	7	0	83
28	30	27	2	1	90
35	30	27	0	3	90
47	30	27	2	1	90
61	30	24	4	2	80
c[d]	28	26	1	1	93

[a] Hypopharyngeal glands dissected from 28 to 40 workers from the dequeened colony on each of days -32, 28, 35, 47 and 61. [b] Nurse-type (N) and forager-type (F) protein profiles were judged according to the expressions of 50-kDa brood food protein and α-glucosidase. [c] Samples with both of these proteins were classified as neutral types. [d] "c" indicates a sample of workers collected from a different normal colony "B" as an control.

Table 2. Ratio of workers whose hypopharyngeal glands exhibited the nurse-type protein profile relative to the total workers in a normal colony [a]

		Protein profile			
Age	n	N	F	Neutral	Ratio of N (%)
6	30	30	0	0	100
40	30	0	29	1	0

[a] Hypopharyngeal glands dissected from each group of 30 workers 6 or 40 days after eclosion. Details are the same as for Table 1.

They were then washed well with TBS-T, transferred to 10 ml of skim milk solution containing a second peroxidase-conjugated antibody against rabbit immunoglobulin fragment (diluted 4,000-fold) (Amersham) and incubated for 1 hr at room temperature. After washing with TBS-T, signals were visualized by ECL (Amersham) according to the manufacturer's instructions.

RESULTS

Around day 10 (April 22, 1997), some workers, known as "laying workers", seemed to have started laying eggs in our dequeened colony. As the "laying workers" had not mated with drones, they laid unfertilized eggs that developed into drones. That some worker layers started oviposition was clear from the following observations: [1] several eggs were often laid in a single cell, which is typical for worker laying (Winston, 1987); [2] all the pupae sampled in the "dequeened" colony were male; and [3] many small drones actually emerged about 20 days after oviposion. In a normal colony, eggs for drones are usually laid in large cells, whereas in the dequeened colony they are laid randomly in normal-size cells prepared to produce workers, and consequently develop into small drones (Winston, 1987). Thus, around day 20 (May 2) the colony consisted of older workers, which would have become foragers if they had been in a normal colony, and larvae for drones.

No obvious decline in colony activity was observed in the dequeened colony during the experimental period. Even on day 61, many workers that had foraged for nectar and pollen returned to the hive with pollen loads, and many drone larvae

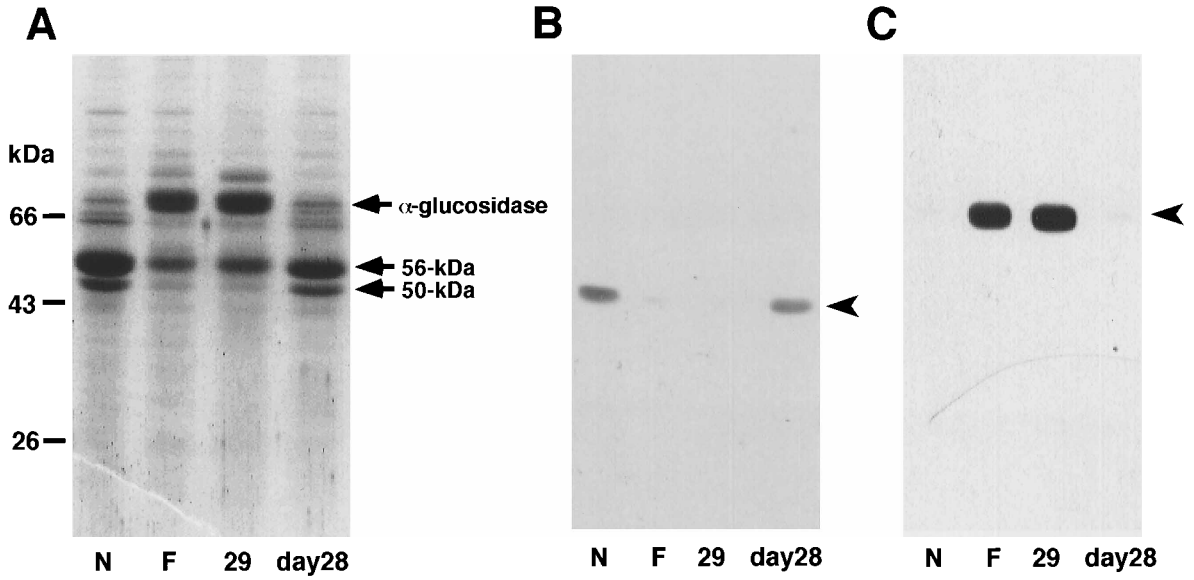

Fig. 2. Profiles of proteins synthesized by the hypopharyngeal glands of workers collected on day 28 from the dequeened colony. (A) Hypopharyngeal glands were dissected from ten workers collected randomly on day 28, homogenized and subjected to SDS-polyacrylamide gel electrophoresis (lane "day 28"). "N" and "F" indicate the homogenates of hypopharyngeal glands from ten nurse and forager bees from normal colony "A", respectively (Kubo *et al.*, 1996). "29" indicates a sample of ten workers of unidentified behavior that were collected from normal colony "A", 29 days after eclosion (Kubo *et al.*, 1996). Molecular mass makers used were bovine serum albumin (66 kDa), ovalbumin (46 kDa) and α-chymotrypsinogen (25 kDa). Ten micrograms of protein was applied to each lane. (B) and (C) The same samples as those used in (A) were subjected to immunoblotting analysis using affinity-purified antibodies against 50-kDa blood food protein (B) and α-glucosidase (C). The bands for 50-kDa brood food protein and α-glucosidase are indicated by arrowheads on the right of panels (B) and (C), respectively.

and pupae grew up in the colony. However, on day 66 (June 17), the colony activity declined drastically, and the dequeened colony came to be composed of a small number of workers and many drones, without drone larvae or pupae.

To analyze the profiles of proteins synthesized by the hypopharyngeal glands of the workers in the dequeened colony, we randomly sampled 30-40 workers from the inner side of the hive on days -32, 28, 35, 47 and 61. When a homogenate of the hypopharyngeal glands from 10 workers collected on day 28 was examined by SDS-polyacrylamide gel electrophoresis (lane "day 28"), its protein profile resembled that of nurse bees (lane "N"), and two major 50- and 56-kDa brood food protein (Kubo *et al.*, 1996) was detected (Fig. 2A). By contrast, the protein profile for the hypopharyngeal gland homogenate from 10 29-day-old workers from a normal colony as a control (lane "29") resembled that of foragers (lane "F"), and a major 70-kDa α-glucosidase (Kubo *et al.*, 1996) was detected in the homogenate. We confirmed these results by immunoblotting analysis using antibodies against 50-kDa brood food protein and α-glucosidase, since expression of the former protein had been shown to be restricted to the gland of the nurse bee and that of the latter protein to the gland of the forager (Kubo *et al.*, 1996).

As shown in Fig. 2B, bands for 50-kDa brood food pro-

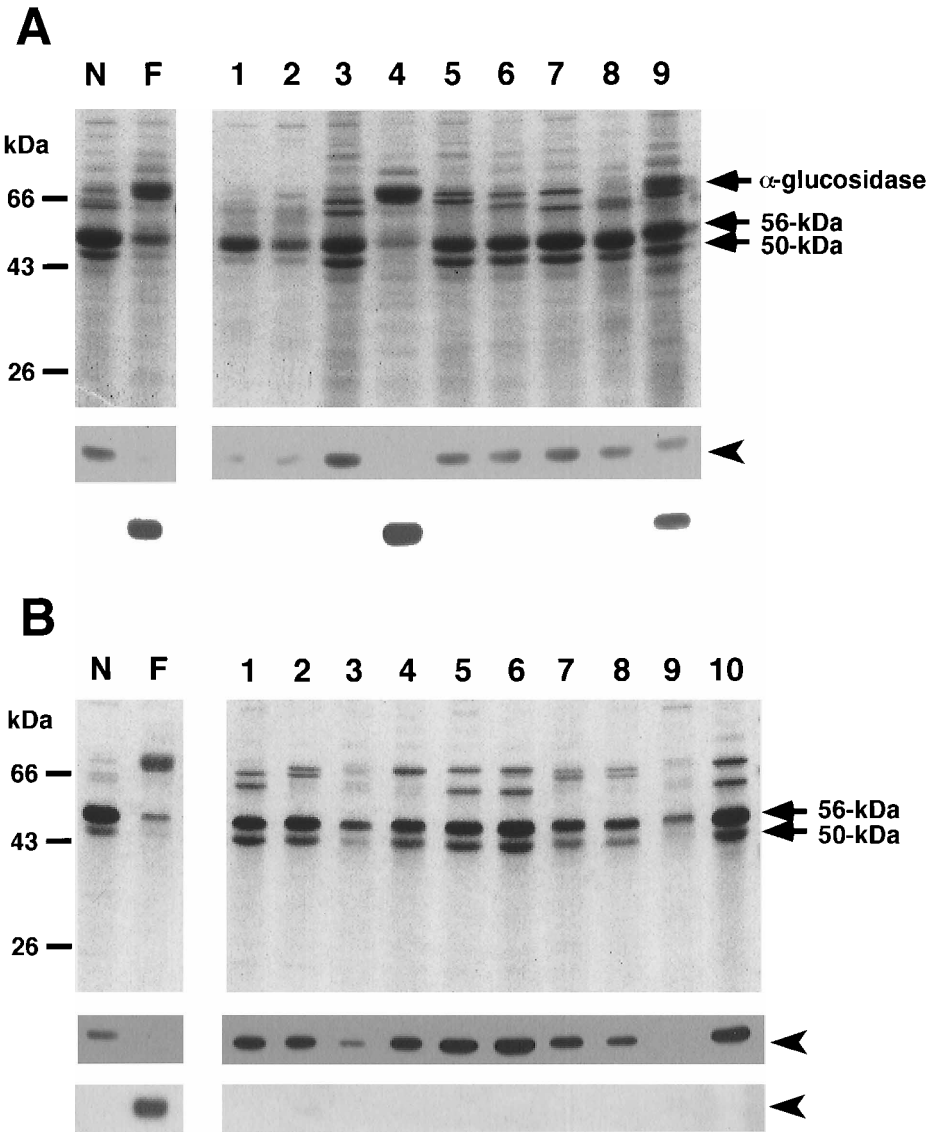

Fig. 3. Analysis of the protein profiles of individual workers collected from the dequeened colony. (A) Hypopharyngeal glands from nine workers collected on day 28 from the dequeened colony were homogenized separately and subjected to SDS-polyacrylamide gel electrophoresis (upper panel) followed by immunoblotting analysis using affinity-purified antibodies against 50-kDa blood food protein (middle panel) and α-glucosidase (lower panel). (B) Hypopharyngeal glands from ten worker bees exhibiting nursing behavior ("nurse bees"), that were collected on day 47 from the dequeened colony, were homogenized separately and subjected to SDS-polyacrylamide gel electrophoresis (upper panel) followed by immunoblotting analysis using affinity-purified antibodies against 50-kDa brood food protein (middle panel) and α-glucosidase (lower panel). Molecular mass makers used were the same as those in Fig.2. The bands for 50-kDa brood food protein (middle panels) and α-glucosidase (lower panels) are indicated by arrowheads on the right.

tein were detected specifically in the lanes for both nurse bees (lane "N") and workers collected on day 28 (lane "day 28"). Bands for α-glucosidase were detected specifically in the lanes for both foragers (lane "F") and 29-day-old workers (lane "29") (Fig. 2C). These results indicated that the change in the function of the worker bee hypopharyngeal gland, which usually occurs with age in a normal colony, had not occurred in our dequeened colony. No significant difference was detected between the protein profiles of the hypopharyngeal glands from nurse bees (lane "N") and those of workers collected on day 28 (lane "day 28"), suggesting that the protein components of the brood foods for workers and drones were almost the same.

Next, we examined the protein profiles of nine individual workers of unidentified behaviour collected randomly from the "day 28" colony by SDS-polyacrylamide gel electrophoresis and immunoblotting analysis. As shown in Fig. 3A, seven of the nine workers examined expressed 50-kDa brood food protein but not α-glucosidase (lanes 1–3 and 5–8) like the nurse bee, whereas one worker (lane 4) expressed α-glucosidase but not 50-kDa brood food protein like the forager. The remaining one worker (lane 9) was found to express both 50-kDa brood food protein and α-glucosidase. These results indicated that most of the workers in our dequeened colony synthesized brood food proteins like nurse bees.

Many workers that exhibited nursing behavior were observed in this colony throughout the experimental period. They often thrust their heads into cells to feed larvae brood food secreted from their hypopharyngeal glands. Therefore, on day 47 we collected these "nurse bees" to examine whether they actually synthesized brood food proteins. As expected, nine of ten "nurse bees" were found to synthesize 50-kDa brood food protein (Fig. 3B). No workers examined expressed α-glucosidase.

Using samples collected on days -32, 28, 35, 47 and 61, we performed dot-blot analysis with antibodies against 50-kDa brood food protein and α-glucosidase to examine the ratio of workers whose hypopharyngeal glands exhibited nurse-type to total workers. The percentage of the workers whose hypopharyngeal glands exhibited the nurse-type profile remained within the range of 80–90% of the total workers throughout the experimental period [day -32 to 61], which is similar to that in a normal colony (93%) (Table 1). The percentage was 80% even on day 61 when the age of the residual workers had reached almost double the life span of a normal worker.

Next, we analyzed the profiles of proteins synthesized by young (6 days old) and old (40 days old) workers in a normal colony, to compare the ratio of workers whose hypopharyngeal glands exhibited nurse-type and forager-type protein profiles with that in the dequeened colony. For this end, newly emerged workers in a normal colony were marked with paint on the dorsal thorax and collected 6 or 40 days after eclosion for dot-blot analysis. It was found that the hypopharyngeal glands of 6-day-old workers (n=30) all expressed 50-kDa brood food protein but not α-glucosidase. By contrast, in 40-day-old workers (n=30), the hypopharyngeal glands expressed α-glucosidase but not 50-kDa brood food protein, except for one that expressed both 50-kDa brood food protein and α-glucosidase (classified as a "neutral" worker). This result was quite different from that in the dequeened colony, where 80% of workers expressed 50-kDa brood food protein even on day 61.

DISCUSSION

A number of reports have so far indicated the effects of colony conditions on the development (size) of the hypopharyngeal gland (Rösch, 1930; Milojevic, 1940; Huang and Robinson, 1996). For example, younger foragers can work as nurse bees when all nurse bees are removed from a colony, and their shrunken hypopharyngeal glands redevelop, while young nurse bees can work as foragers when all the foragers are removed (Rösch, 1930). However, the actual cellular function of the hypopharyngeal gland was not examined in these studies, since no appropriate molecules were identified as indices of the function of hypopharyngeal gland cells. The present study is the first attempt at direct analysis of the function of the hypopharyngeal gland cells of individual workers, by comparing the expression of major secretory proteins that are characteristic of the age-dependent roles of the workers in a manipulated and a normal colony.

Our results clearly showed that the function of the worker hypopharyngeal gland cells has flexibility: the gland function of foragers can be maintained, if necessary, as that of nurse bees, depending on the colony conditions. However, it is still uncertain whether the function of the hypopharyngeal gland in the same workers was maintained throughout the experimental period, or whether gland function was reversed in some foragers. We and others have shown that the concentration of the juvenile hormone (JH) in the hemolymph is closely related to the age-dependent role change of worker honey bees (Sasagawa *et al*., 1989; Robinson *et al*., 1989). JH is also reported to be related to hypopharyngeal gland development *in vivo*: application of JH or its analogue induces premature shrinking of the gland (Rutz *et al*., 1976). However, it remains unknown whether JH is directly involved in the change in the function of the worker hypopharyngeal gland.

The life span of the European honey bee is about 30 days during spring to autumn in Japan (Sakagami, 1958; Sakagami and Fukuda, 1968). The survival curve of workers declines drastically about 20 days after eclosion and almost no workers survive later than 50 days after eclosion (Sakagami and Fukuda, 1968). Thus, a normal colony is composed of more nurse bees and fewer foragers, which is consistent to our observations (Table 1, day -32 and c).

Many workers in our dequeened colony survived even on day 61, suggesting that the life span of the residual workers had been significantly prolonged. Our results indicate that the nurse bee phase may have been abnormally prolonged in the dequeened colony to maintain the ratio of nurse bees to foragers at a level similar to that in a normal colony, and therefore the total life span of the worker was prolonged. Possibly, some pheromone-like signal from the nurse bees, foragers

(Huang *et al*., 1996) and/or larvae might function to hold the physiological age of the older workers close to that of nurse bees to maintain the ratio of the nurse bees in the dequeened colony. Analysis of the mechanisms for modulating the function of the hypopharygeal gland according to changes in colony conditions will be important for understanding the cellular basis of the flexibility of the worker physiology, which is characteristic to the age polyethism of the honey bee (Rösch, 1930; Milojevic, 1940; Huang and Robinson, 1996).

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