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Research Article

Histochemical Comparison of the Hypopharyngeal Gland in *Apis cerana* Fabricius, 1793 Workers and *Apis mellifera* Linnaeus, 1758 Workers

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Hypopharyngeal glands of honeybee are age-dependent structures that change with the size of acini and are correlated with various social behaviors. The histochemical structure of *Apis cerana* and *A. mellifera* worker hypopharyngeal glands in four different developmental stages wes stained with ninhydrin Schiff's and periodic acid Schiff's reagents (PAS) for localization of proteins and carbohydrates, respectively, and examined with light microscopy. Nurse bees of both honeybee species had significantly larger glands as compared to guards and forgers, but there were no statistically significant differences between these two species after accounting for caste. Gland protein concentration increased progressively in nurse bees, and this was correlated with the appearance of enriched protein granules in the cytoplasm. In addition, the hypopharyngeal gland protein concentration of *A. mellifera* was higher than that of *A. cerana* even though gland size was not significantly different between species. However, gland size was shown to have decreased progressively in foragers and guards.

1. Introduction

The development of hypopharyngeal glands (HPGs) in dwarf honeybee workers primarily depends on age. These glands begin to differentiate at pupal stage and are largely undeveloped at emergence [1]. When workers become nurse bees, they perform brood rearing that is associated with HPGs development. The size of HPGs is correlated with glandular production and generally increases with age from 6 to 18 days in nurse bees [2, 3]. HPGs synthesize and secrete proteinaceous substances and royal jelly that are fed to the queen and brood [3]. The highest rate of protein synthesis occurs during nursing ages from 8 to 16 days [4, 5]. In bees older than 18 days (guards and foragers), the HPGs decrease considerably in size and secrete enzymes such as α-glucosidases, leucine arylamidase and invertase [6–9]. Forager gland size is reduced and correlated with the gland activity [3, 4]. We previously demonstrated the location of proteins and carbohydrates within HPGs of dwarf honeybee workers among different ages [1]. The glands were composed of several secretory apparati. Each opened into a secretory duct and then passed through the mouthparts. In pupae, the secretory cells were irregular in shape with low concentrations of proteins and carbohydrates while the glands of nurse bees and foragers were fully developed with numerous secretory vesicles [1]. For this study, we measured the size of glandular acini, examined protein concentration in hypopharyngeal glands, and identified the location of proteins and carbohydrates in the hypopharyngeal gland workers of *Apis cerana* and *A. mellifera*.

2. Materials and Methods

2.1. Honeybees. *Apis cerana* and *A. mellifera* workers of different ages were collected from Samut Songkarm province, Thailand. Pupae (dark brown-eyed stage) were grasped from the cells. Nurse bees were taken from their colony while they were feeding brood. Guards were collected from in front of

Table 1: Hypopharyngeal gland sizes (mean ± S.E.) of *A. cerana* and *A. mellifera* workers in different stages of life: nurse, forager, and guard.

Honeybees	Workers	Hypopharyngeal gland sizes (mean ± s.e.)	
		width (μm)	length (μm)
A. cerana	Nurse	101.57 ± 4.68^{b}	128.55 ± 4.41^{a}
	Guard	83.59 ± 3.80^{cd}	108.28 ± 6.53^{bc}
	Forager	91.56 ± 3.33^{bc}	113.91 ± 6.28^{ab}
A. mellifera	Nurse	116.41 ± 4.25^{a}	122.51 ± 4.31^{ab}
	Guard	68.12 ± 2.54^{e}	94.06 ± 4.77^{c}
	Forager	74.69 ± 2.03^{ed}	97.66 ± 2.84^{c}

Note: Means ± S.E. followed with different letters in the same column denote significant differences (ANOVA-Duncan's multiple range test; $F = 24.98$, $df = 5$, $P < .0001$; $F = 7.32$, $df = 5$, $P < .0001$).

the hive entrance. Foragers (bees with pollen loads) were captured inside the colonies.

2.2. Glandular Size Measurement. Measurements of glandular size were made from nurse bees, guards, and foragers. Under a stereomicroscope, HPGs of each life stage were removed from the head using modified blades and then transferred into insect saline solution (NaCl 8.766 g., $CaCl_2$ 0.188 g., KCl 0.746 g., $MgCl_2$ 0.407 g., $NaHCO_3$ 0.336 g., sucrose 30.807 g., and trehalose 1.892 g., pH 7.6). Using a micrometer, gland diameters were measured (width and length) under light microscopy.

2.3. Preparation of Protein Sample. Ten worker bees were collected to represent each species and anesthetized on dry ice. Protein extraction from HPGs was modified from the work of Li et al. [9]. Glands were transferred to 50 μL 0.1 M phosphate buffer (PB) pH 7.8 in a 1.5 mL microcentrifuge tube, homogenized for 10 min on ice, sonicated for 2 min, then centrifuged at 1500 rpm for 10 min at 4°C. The supernatant was transferred to another tube. The pellet was resuspended in 10 μL PB and then centrifuged at 1500 rpm for 10 min at 4°C. The supernatant from this resuspension was removed and added to the previous supernatant and stored at 4°C.

2.4. Protein Assay. Glands were homogenized and then centrifuged at 1000 rpm for 2 min. The supernatant was analyzed using the Bradford protein assay [10]. Standard curves were prepared using bovine serum albumin (BSA) and absorbance measured at 595 nm against a blank reagent using a Shimadzu UV-visible spectrophotometer (UV-1610). Concentrations of protein (BSA) were plotted against the corresponding absorbance value to generate a linear regression standard curve.

2.5. Histochemical Study. Bee heads of each developmental stage were dissected in insect saline (NaCl 7.5 g/L, and Na_2HPO_4 2.38 g/L, and KH_2PO_4 2.72 g/L) and then fixed in Bouin's solution for 24 h. Samples were dehydrated through an ethyl alcohol series: 70%, 90%, 95%, and 100% for 10 min each. Samples were soaked in xylene for an hour and then embedded in paraffin wax. The tissues were sectioned into 6 μm thickness using a rotary microtome (Leica, Germany), stained with hematoxylin and eosin, and submitted to periodic acid Schiff's reagent (PAS) and ninhydrin Schiff's reagent for localization of carbohydrates and proteins, respectively.

2.6. Data Analysis. Statistical differences between hypopharyngeal mean gland size and protein concentration were compared using ANOVA and Duncan's multiple-range test (DMRT).

3. Results

3.1. Glandular Size. Acini of the glands started to develop at the pupal stage and increased in size at the adult stage. The size (width and length) of the acini of both species was the largest in nurse bees and then gradually decreased from nurses to guards (Table 1). However, the acini slightly increased in size when they changed their tasks to become foragers. There was no significant difference in the width of acini between *A. cerana* and *A. mellifera* ($F_{1,3} = 2.63$, $P < .1120$). However, there were significant width differences among different life stages ($F_{1,2} = 31.24$, $P < .0001$, and $n = 48$). In contrast, the length of the acinus of HPGs showed statistically significant differences between these two species and developmental stages ($F_{1,2} = 8.48, 12.92$; $P < .0048, .0001$; $n = 48$).

3.2. Protein Content. The mean total protein content of the hypopharyngeal glands taken from all three developmental stages of *A. mellifera* was significantly higher than those of *A. cerana* ($F_{1,2} = 38.88$, $P < .0001$) (Figure 2). The highest protein concentration was found in nurse bees of *A. mellifera* which were 1389.6 ± 158.9 μg/μL (69.5 ± 8.0 mg/bee). Proteins (revealed with ninhydrin Schiff's reagent) decreased in concentration in later developmental stages. In contrast, the lowest protein concentrations were found in guards of *A. cerana* which were 413.8 ± 5.1 μg/μL (20.7 ± 0.3 mg/bee, Figure 2). There was a significant difference in protein concentration among the developmental stages of *A. mellifera* and *A. cerana* ($F_{2.5} = 217.82$, $P < .001$, and $n = 49$, Figure 1).

3.3. Histochemical Structure for Localization of Protein. The histochemical structure of proteins from *A. cerana* and *A.*

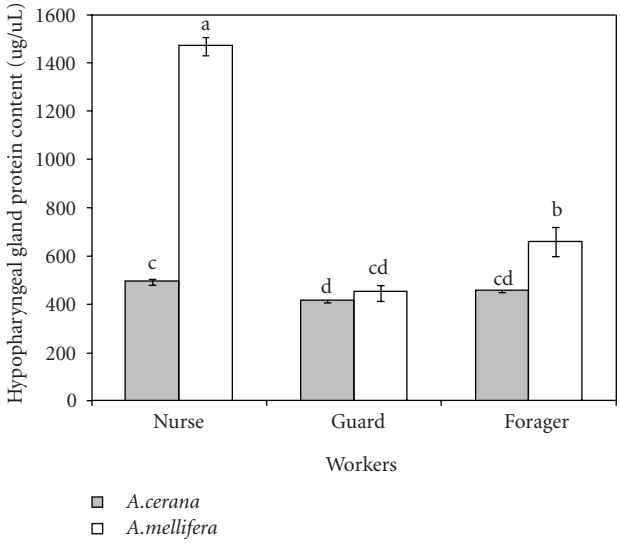


Figure 1: Mean ± S.E. protein concentration of *A. cerana* and *A. mellifera* hypopharyngeal glands in different developmental stages: nurse, guard, and forager. Vertical bars with different letters represent significant differences (ANOVA-Duncan's Multiple Range Test; $F = 217.82$, $df = 5$, $P < .0001$, $n = 49$).

mellifera was relatively similar among life stages. In both species, several clusters of acini were connected to a long slender secretory duct. Each acinus was composed of 8–10 aggregated secretory cells and the glands consisted of an incomplete irregularly shaped secretory unit with unequal secretory cells. However, there were differences among stages of either *A. cerana* or *A. mellifera*. The histochemical staining shows a glandular cell cytoplasm rich in secretory vesicles and presenting glycoprotein secretions.

3.3.1. Pupae. The hypopharyngeal glands developed and formed an acinus. The glands consisted of an incomplete structure of secretory units which were irregular in shape and composed of 8–10 secretory cells. Each cell had an oval nucleus which stained green. The slightly pink cytoplasm can be seen in this stage, but secretory vesicles do not appear (Figure 2(b)).

3.3.2. Nurse Bees. Each acinus consisted of numerous vesicles that were not stained with Schiff's reaction. These white and clear vesicles were found surrounding the nucleus of acinar cells. Strongly positive Schiff's staining was found in the periphery of secretory cell cytoplasm. Ninhydrin Schiff's staining clearly distinguished the unstained vesicles from the peripheral area of the cytoplasm that included the nucleus that was strongly positive with pink staining. Nevertheless, areas of some vesicles stained magenta (Figures 2(c) and 2(d)).

3.3.3. Guards. In the guards, some HPGs demonstrated the beginning of retrogression through the formation of irregular secretory cells. Guard cytoplasm stained weakly with Ninhydrin Schiff's reagent as compared to the stronger staining of nurse bees and foragers. Even the secretory vesicles of guards were smaller in size than nurse bees or foragers, the vesicles were elongated and cylindrical in shape. The wider extracellular space between two adjacent secretory cells was clearly seen in this stage (Figure 2(e)).

3.3.4. Foragers. The glands were composed of several acini smaller than those of nurse bees but larger than those of guards. Each acinus consisted of 8–10 pyramidal secretory cells, with oval nuclei staining positive using Ninhydrin Schiff's reagent in the area of euchromatin. The cytoplasm of secretory cells, except vesicles, was strongly magenta positive when stained with Ninhydrin Schiff's reagent (Figure 2(f)).

3.4. Histochemical Structure for Localization of Carbohydrate. Carbohydrate levels in the HPGs of the four stages of *A. cerana* and *A. mellifera* workers were similar to each other. The glands stained strongly positive to PAS, indicating the existence of carbohydrates (Figures 3(a) and 3(b)). However, there were clear differences among developmental stages in both species.

3.4.1. Pupae. The glands began forming a cluster of paired irregularly shaped secretory units that were composed of 8–10 cell aggregations. The cells gave weak positive staining to PAS with pink in cytoplasm.

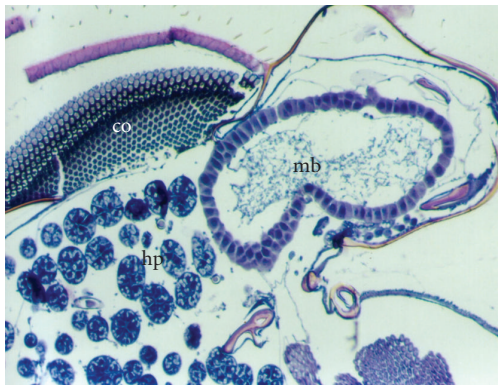
3.4.2. Nurse Bees. The HPGs were fully developed and composed of several secretory units made up of 8–10 aggregated pyramidal acinar cells. Within the acinar cells there were numerous red-pink secretory vesicles indicating a strong positive with PAS reaction and corresponding to glandular sizes (Figure 3(c)) (Table 1).

3.4.3. Guards. For guards, the glandular structure and histochemistry were slightly different to foragers. For example, each acinus was composed of 8–10 cells with small secretory vesicles and also lower number than those of foragers. However, they showed large extracellular space between adjacent cells, unlike the glands of nurse bees and foragers where there was minimal space (Figures 3(d) and 3(e)).

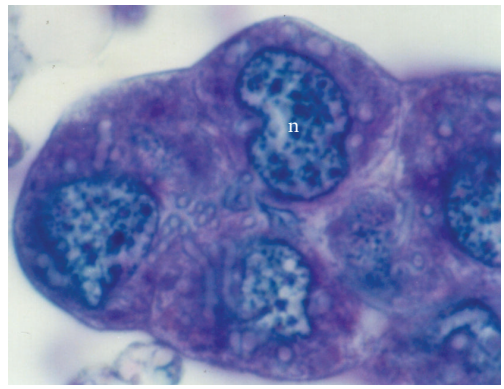
3.4.4. Foragers. The glands of foragers showed complete structure consisting of 8–10 aggregated acinar cells. The secretory vesicles of foragers were slightly larger than those of guards. The secretory vesicles of foraging bees gave strong positive staining with PAS which was similar to those of nurse bees (Figure 3(f)).

4. Discussion

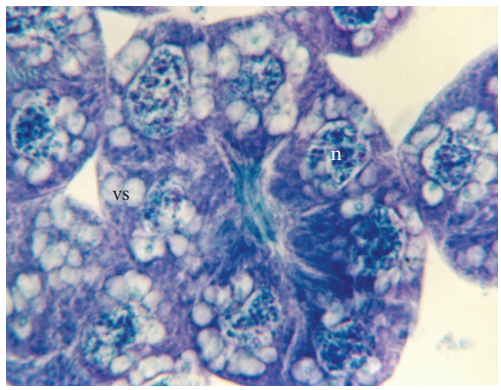
The structure of honeybee hypopharyngeal glands depends on the development and age of individuals, which corresponds with age-specific tasks and is known as age polyethism [3, 11]. The results of this study showed that the histochemical structure of carbohydrate and protein in *A. cerana* and *A. mellifera* was correlated with honeybee



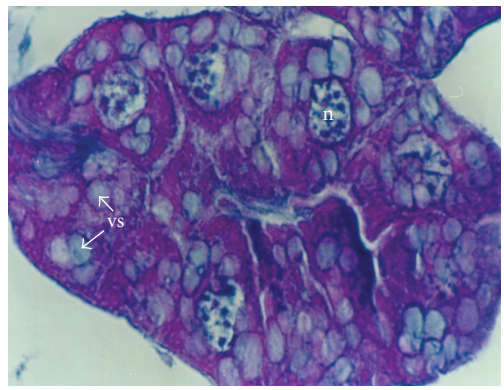
(a) A section of a nurse bee head in *Apis cerana* showing the location of the hypopharyngeal gland located beside the compound eye and close to the mandibular gland (40x)



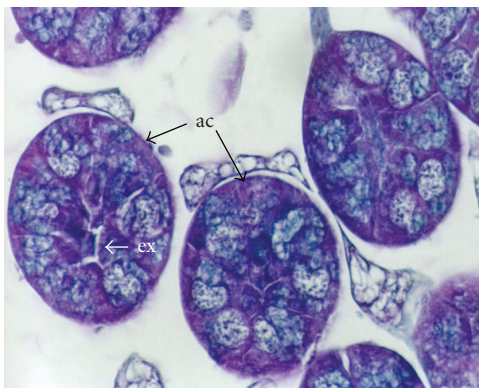
(b) A section of the hypopharyngeal gland of pupa of *Apis cerana* worker showing the incomplete irregular shaped secretory units (1000x)



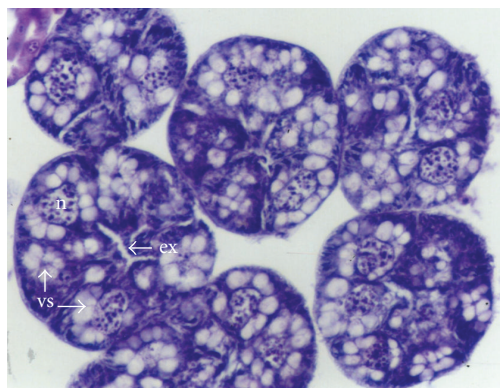
(c) The micrograph illustrates the secretory units of the complete hypopharyngeal gland of *A. cerana* nurse bee, the cytoplasm containing a large amount of proteins is characterized by staining purple-pink color with NHS (200x)



(d) The micrograph has been stained by a histochemical method NHS to demonstrate the presence of proteins which are stained magenta to purple pink in the secretory cells of the glands of *A. mellifera* nurse bee (200x)

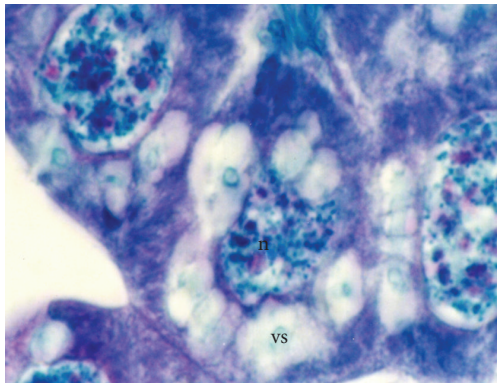


(e) A micrograph showing the histochemical appearance of the secretory units of the complete developed gland of an *A. cerana* guard; the cytoplasm is stained pink with NHS technique showing the narrow extracellular space between adjacent acinar cells seen by white color separating them from each other (100x)

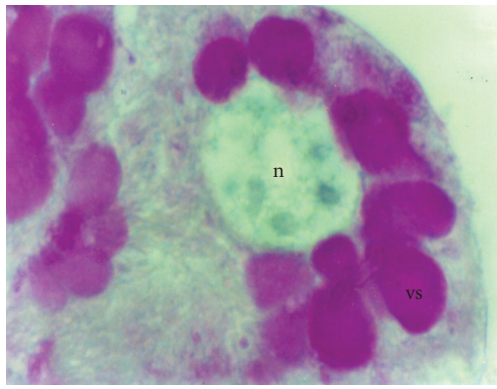


(f) A histochemical micrograph of an *A. mellifera* forager showing the cytoplasm of the secretory cell seen to contain a variable numbers of secretory vesicles that are almost unstained with NHS (100x)

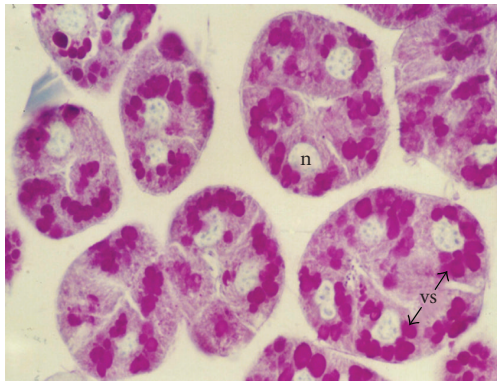
Figure 2: Light microscope micrographs of hypopharyngeal gland acini stained with ninhydrin Schiff's reagent (NHS). *Abbreviations:* ac: acinus; co: compound eye; ex: extracellular space; hp: hypopharyngeal gland; mb: mandibular gland; n: nucleus; vs: vesicle.



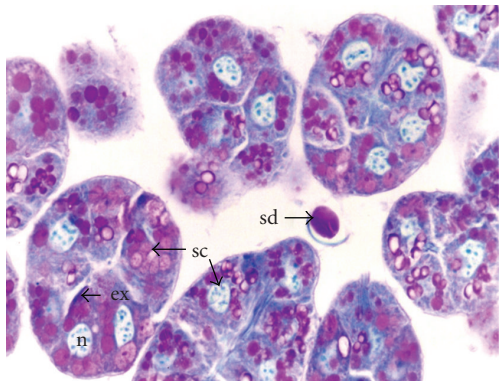
(a) High-resolution micrograph of the *A. cerana* nurse bee hypopharyngeal gland stained pink-purple surrounding the secretory vesicles (1000x)



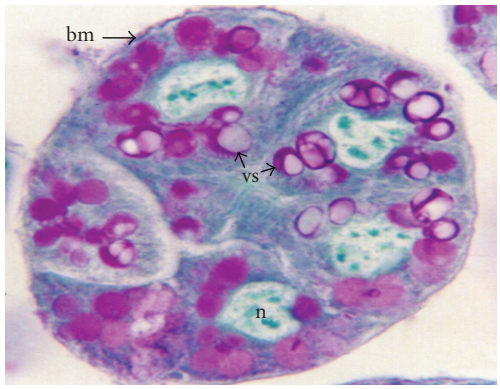
(b) A section of the hypopharyngeal gland of an *A. mellifera* worker nurse bee showing the cytoplasm of the secretory cell containing a variable number of secretory vesicles which is stained red-pink with PAS. The oval nucleus stains pale greenish with light green (1000x)



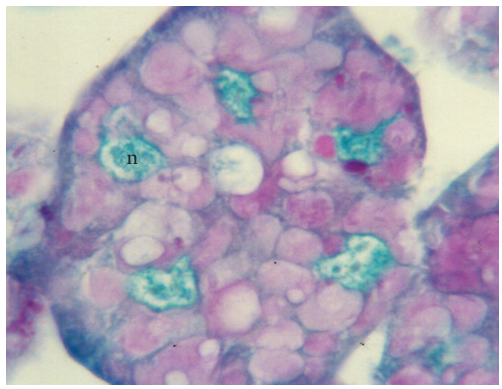
(c) A medial section of the completely developed acinar gland *of A. cerana* nurse bee showing a cluster of several secretory units. The secretory cell cytoplasm is stained pink with PAS and the cell has a large nucleus. (100x)



(d) The secretory units of the hypopharyngeal gland of *A. mellifera* forager showing the smaller size of secretory vesicles containing less carbohydrate. These are characterized by staining slightly red-pink with PAS and showing the wide extracellular space between adjacent acinar cells which is seen by white color separating it from the other secretory cells (100x)



(e) With a higher magnification of *A. mellifera* guard, the secretory cell contains various sizes of secretory vesicles which are less stained to PAS than that of nurse bee (200x)



(f) A medial section of the hypopharyngeal gland of an *A. cerana* guard showing the shrinkage and damaged plasma membrane (400x)

Figure 3: Light microscope micrographs of hypopharyngeal gland acini stained with PAS. *Abbreveations:* bm: basement membrane; ex: extracellular space; n: nucleus; sc: secretory unit; sd: secretory duct; vs: vesicle.

age-specific tasks of the colony. Young worker nurse bees care for and feed their brood with royal jelly that is synthesized and secreted from the hypopharyngeal glands. These hypopharyngeal glands were strongly positive to PAS and Ninhydrin Schiff's reagent reactions in this study. However older workers, when they became guards, had less positive staining to PAS as compared to nurse bees. This may be related to the development of the hypopharyngeal glands which are fully developed when young workers take care of their brood by synthesizing and secreting royal jelly. Older workers no longer feed the brood, and thus gland atrophy is expected [3, 4, 8]. However, when bees become foragers, the hypopharyngeal gland contains significant amounts of carbohydrate and protein. This finding is consistent with the finding that the hypopharyngeal glands are the site of conversion of nectar to simple sugars by enzymes [6–9].

The hypopharyngeal gland has been studied in several ways, including its ultrastructure, protein complement, and histochemical structure in *Apis andreniformis* and *A. florea*. [1, 3, 5, 8, 9, 12]. In this study, we found similarities in glandular structure between species, and the glands were fully developed in nurses, guards, and foragers. Moreover, the secretory units of the HPGs were filled with numerous vesicles that gave a strong positive staining with PAS and Ninhydrin Schiff's reagent [1]. This indicates that the glands of nurses, guards, and foragers in four species of honeybees (i.e., *A. andreniformis*, *A. florea* [1], *A. cerana,* and *A. mellifera,*) play an important role not only in secretion of carbohydrate rich substance but also in the secretion of enzymes for converting nectar to honey [1, 13, 14]. However, there were also differences found in structure of the glands between the hive cavity nest honeybees, *A. cerana* and *A. mellifera*, and the single open nest honeybees, *A. andreniformis* and *A. florea*. The structure of the extracellular space between adjacent cells of *A. andreniformis* and *A. florea* was wider than that of *A. cerana* and *A. mellifera*. In addition, the secretory units of hypopharyngeal glands of *A. mellifera* from this study were different from results in the study of Deseyn and Billen [3] who showed that the volume of acini decreased in foragers or displayed degenerative structure while that was not found in this study.

In the present study, the development and histochemical aspects of the hypopharyngeal glands were evaluated in *A. cerana* and *A. mellifer*a. The findings are in agreement with our previous study [1]. Additionally, glandular sizes were the greatest in nurse bees. Hypopharyngeal glandular size is known to be positively correlated with gland activity and is influenced by larval feeding [2, 4, 15, 16]. These glands gradually decrease in size when honeybees become guards, cease feeding, and begin defending the colony [3]. However, the hypopharyngeal gland size of foragers was significantly larger than that of guards. The results in this study indicate that glandular development corresponded well with total protein synthesis in the hypopharyngeal glands at different adult life stages. A number of reports indicate that the HPGs produce enzymes that are used to hydrolyze nectar into honey, including amylase, α-glucosidases, glucosidase oxidase, galactosidase, esterase, leucine arylamidase, and invertase [4, 6–9]. It can be inferred that the HPGs perform two functions: first producing protein rich royal jelly for the nursing brood (by nurse bees) and then enzyme production (by foragers). However, the function of hypopharyngeal glands has flexibility depending on the colony condition and the need for feeding brood [4, 16]. The protein concentration of the hypopharyngeal glands peaked in nurse bees and declined in older workers. This corresponds to the degree of protein synthesis and is correlated with the abundance of rough endoplasmic reticulum (RER) found in adult workers. RER of acinar cells of worker glands developed and increased significantly a few days after emergence Most of the cytosol space was filled with RER stacks a few days after emergence, while in foragers the RER decreased [5]. However, the total mean protein content of the hypopharyngeal glands of *A. mellifera* workers was significantly higher than that of *A. cerana* workers ($F_{1,2} = 38.88$, $P < .0001$). This is related to glandular sizes. In *A. mellifera*, the hypopharyngeal glands and average body sizes are larger than those of *A. cerana*. In summary, the hypopharyngeal glands of *A. cerana* and *A. mellifera* workers are fairly similar in terms of the histochemical structure. Each gland begins development as pupae and had a fully forming acinus in nurse bees. Gland regression occurred when the bees developed into foragers and guards. Protein concentration peaked in nurse bees and declined in guards. In the hypopharyngeal glands, the histochemical staining of carbohydrates and protein corresponded well with the glandular size and protein content.

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