

CHARACTERISTICS OF HYPOPHARYNGEAL GLANDS IN HONEYBEES (*Apis mellifera carnica*) FROM A NURSE COLONY

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Summary: The development and size of hypopharyngeal glands (HPGs) in workers sampled from the nurse colony was compared to workers from the control queen-right honeybee colony. The diameter of the acini in workers (age 1 to 30 days) from the nurse colony ranged from 109.2 to 180.9 μm , and in workers of the same age from the control colony was between 100.8 and 158.2 μm . We found that nurses from the nurse (cell builders) colonies aged 15 to 27 days had significantly larger acinar diameter ($p < 0.0001$) than the workers in the control colonies of the same age. We described the morphological and histological characteristics of the HPGs in nurse workers aged 1 to 27 days and found that HPGs secretion in brood feeding nurses was extended in comparison to workers from the control colony. Moreover, we described the HPGs in a worker pupa before the emergence and in winter bees from the control colony. Results show that HPGs in worker pupae consist of clusters of irregularly shaped secretory acini. Cell cytoplasm is not structured and is homogeneous, nuclei are dense and oval in shape. Winter bees had hypertrophied HPGs and cells containing numerous vesicles accumulating secretion. We found larger acini with a dense structure and milky-white colour. Physiological function of the glands and age related tasks of nurse worker bees is also discussed.

Key words: acinar diameter; age; morphology; nurse bee; winter bee; worker pupa

Introduction

Hypopharyngeal glands (HPG) produce and secrete the components of royal jelly which is the most important food for brood and queen. In young workers, the glands are well developed with large actively secreting acini. Development of HPGs, which produce royal jelly, i.e. food for larvae, is of great importance in the quality queen rearing. HPGs are located in a head of a honeybee worker and a queen but only develop in workers (1, 2).

Active secretion reaches its peak in nurse-workers at the age of 6 to 13 days and HPG were mostly studied in workers. Active secretion was observed in nurse workers, newly-emerged bees and older workers (3). Reduced glandular activity was detected using a histochemical method of localising acid phosphatase activity in cells of adult workers and queens, despite the fact that the glands were anatomically in the regression phase (4). Furthermore, comparing the gland structure and morphology of *Polistes versicolor* (Hymenoptera, Vespidae) and other groups of Hymenoptera, HPGs in different age groups of *P. versicolor* have

similar morphological characteristics with the exception in the size of secretory cells that differ between the individuals and does not depend on age (5). The reason for such age independence is a hierarchy which occurs in a linear way in *Polistes* without morphological differences between castes.

The HPGs glands in the honeybee are a paired organ, located in the head of the worker bee, in front of the brain between the compound eyes. The ducts open into the sub-oral part of the hypopharynx. Each of the glands is composed of numerous small oval bodies or acini, short parts attached to axial or terminal secretory duct. Each acinus is determined by 10 to 15 individual cellular bodies and each cell is connected to the axial duct via a thin duct (6). The gland size increases and decreases with age of a worker. In newly-emerged workers the glands are inactive and the secretory vesiculi develop by the age of 3 days (7). Six-day old bees produce secretion in the glands and at the age of 9 days the glands secrete the enzymes and cooperate in royal jelly production (8). However, at the age of 15 days secretion activity increases and the cells already start to form lysosomes which are involved in the degeneration processes (7). In winter, the bees' glands are hypertrophied and it is assumed that the secretion is stored till spring. Glands activate in the presence of a young brood and are less active in protein synthesis in comparison to middle sized glands of young workers (9). Younger summer workers with larger acini actively produce proteins more than older foragers, which have smaller gland size (10), with clear signs of structural degeneration and regression indicating cell death (11). Glandular development and royal jelly production depend on protein vitellogenin that slows down aging in workers and queens (12), but has an effect on HPGs only in nurse workers (13). In previous research, the ultrastructural changes during HPGs development were described at different age groups of bees (2, 7, 9, 14, 15, 16, 17). Rough endoplasmic reticulum in the cells, characteristic for protein synthesis, starts to expand as the worker emerges and the maximum size is reached in nurse workers, and decreases in foragers. Intracellular ducts, which function to store and transport secretion material, expand from the space between the endocuticle and gland cell membrane into the secretion vesicles (2). Within septa of the secretory cell there are filamentous actin (F-actin)-decorated tubular structures (actin

rings) connected to an extracellular ductus (18). The authors speculate that the function of the actin rings is important for the secretion process. Foragers have smaller amounts of proteins in the HPGs in comparison to older workers, which have the ability to reactivate gland secretion. This only happens as a response to needs inside and/or outside the brood nest (2).

Histological structure in the HPGs was only presented in a normal queen-right colony, therefore we wanted to analyse morphological and histological changes in the HPGs in nurse bees of a different age in a nursing colony and compare the size of the acini with the HPGs in a normal, control colony. Moreover we described the HPGs in a worker pupa and winter bees.

Material and methods

For the experiment, two colonies were established, a normally developed honeybee colony as a control and a nurse colony, both without any visible disease symptoms. The nurse colony was obtained with plenty of young nurse workers. The experiment was conducted in the experimental apiary of the Agricultural Institute of Slovenia in Senično situated in the Gorenjska region. Worker honeybees (*A. m. carnica*) were obtained as a mixture from three colonies. Bees emerged from their brood comb in an incubator at 34.5 °C (± 1.0) and 60 % relative humidity. Around 100 newly emerged workers (age 0–24 hrs) were marked on the thorax with a marker of the same colour to define their age and inserted into the control colony. The procedure with introducing the newly emerged bees, marked with the same colour on the same day into the honeybee colony, was repeated every three days. At the beginning of the experiment, the nurse colony of two hive boxes was established, with a queen excluder in the middle. The queen was kept in the lower box. Into the upper box we added a honey comb, a comb with bee bread and a comb with a sealed brood arranged from both outer sides. Finally, approx. 2 kg of young nurse workers were added into the upper box and among these bees approximately 100 marked young workers were added into the box. The presence of workers and sealed brood was checked and the combs with honey or pollen were replaced. When the first marked workers were inserted into the colonies, 10 larvae (aged

12–24 h) were grafted and inserted into the middle of the upper nest box of the nurse colonies. The next and consecutive insertion of grafted larvae was performed on the third, eighth and tenth days after the first insertion. During the same periods, approx. 100 newly emerged and marked bees were put into each of the control and nurse colonies.

Bee sampling

Nurse bees at different ages, from 1 to 30 days, including nurse bees feeding queen larvae in the nurse colony, were sampled regularly at the time of introduction into the colonies and every five days thereafter. Three workers were sampled from each age and experimental group. Worker pupae with dark coloured compound eyes, aged around 17 days, were sampled from the brood comb cells. In November of the same season, winter bees of unknown age from the control colony were also sampled.

Dissection of the glands

Bees were immobilized with CO₂ and the head in Hyes' solution (NaCl 9.0 g, KCl 0.2 g, CaCl 0.2 g, NaHCO₃ 0.1 g, 1 l distilled water, pH 8.5) was fixed with two entomological needles on a rubber base (Xantopren® L blue and Activator universal, Heraeus Kulzer, Germany) in a Petri dish. Dissection was performed using the stereomicroscope (SterEO Discovery.V12, Zeiss). The external chitinous exoskeleton of the facial region of the head was removed between the compound eyes, and prepared for the further histological procedure.

Preparation of the tissue for histological analyses

The dissected tissue was fixated in 10% Formaldehyde solution (100 ml Formaldehyde solution, min 37 %; 8.5 g NaCl; tap water; 0.165 M, pH 7.1) for 24 hours (± 1), dehydrated in different concentrations of alcohol (70%, 90%, 100%, 100% alcohol; Ethanol 96 vol. %; demineralised water) and in xylene for 24 hours (± 1) each time. Samples were moved into a plastic histosettes, put into an incubator at 60 °C with three glass tubes containing xylene and wax (3:1, 1:1 and

1:3, xylene:wax), for 24 hours each time. Samples were transferred in the glass tube containing wax and incubated for 24 hours (± 1) and finally in the fresh wax for 6 to 12 hours. The samples were then embedded in wax using metal moulds and cooled on the cold plate. Sections of 5 μ m were then cut on a microtome, floated on distilled water (42 °C) and collected on cleaned slides. The tissue on the slides was first dewaxed with xylene 3 times for 5 minutes, rehydrated in 100% alcohol (Ethanol 96 vol %) 3 times for 3 minutes and then stained with hematoxylin and eosin. A drop of water-soluble fluid (Faramount Aqueous Mounting Medium, Dako) was put on a slide with a stained tissue and covered with cover glass. The prepared material was analysed using light-microscope.

Acini measurements

Morphological measurements of the gland lobes (acini) in nurse workers, pupae and winter bees were performed 'in vivo' using stereomicroscope (SterEO Discovery.V12, Zeiss) and camera (Zeiss). The images were stored and later measured using the program AxioVision Rel. 4.6. In three workers of the same age, diameters of 30 randomly selected acini were measured perpendicular to the longer axis of the oval acinus. The shape of an acinus is oval, so only the length of the shorter axis was measured and used for calculation.

Data analysis

Data were statistically evaluated with SPSS version 13.0 (SPSS Inc.; Chicago, IL, USA). Basic statistical parameters were calculated with Means and Descriptive. Significant differences in acini diameter were calculated using One-way ANOVA, with a filter as a cell bar introduction (1 to 3) and type of the colony (nurse colony, control colony). Data of differently aged workers were compared. Mean acini diameter of HPGs was compared between workers from the nurse colony and the control colony, using One-way ANOVA with age as a factor and further compared with cell bar introduction as a factor. For data testing Scheffe test was applied. Acini diameter in HPG of pupae and winter bees was measured. Basic statistical parameters were calculated with Means and Descriptive.

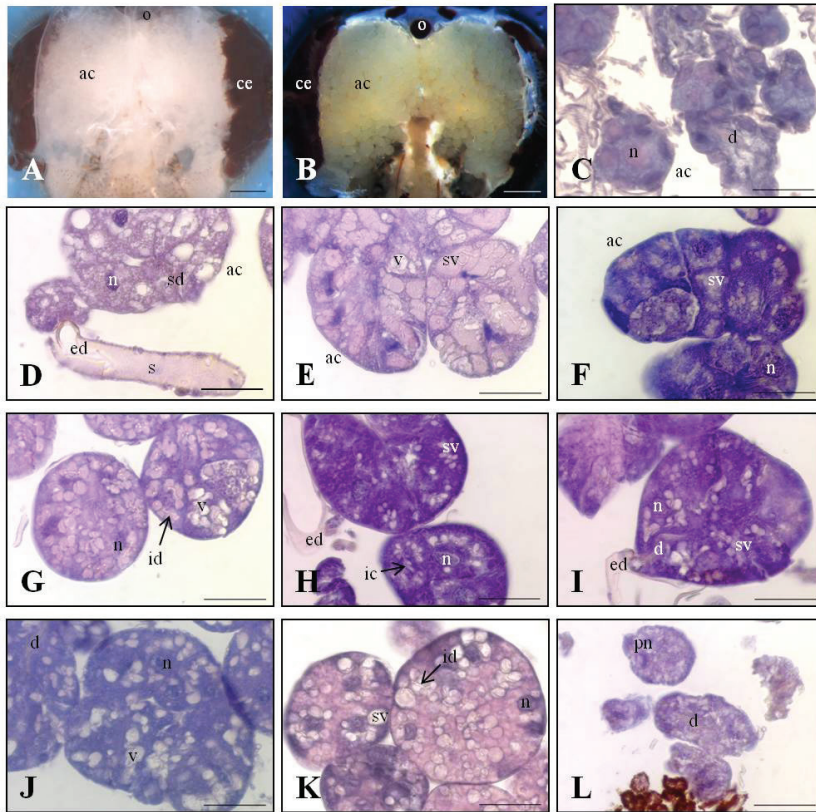


Figure 1: A - HPGs in honeybee pupae in vivo are transparent and lobular structure is not clearly seen. B - In winter bees HPGs with a milky-white colour, lobular structure with acini (ac), compound eye (ce) and ocellus (o) can be seen. Scale bar 0.5 mm. Histological section of the HPG gland acini, stained with Hematoxylin and eosin. Acini in worker pupa (C) and winter bee (D and E). Acini of (F) 1-day, (G) 3-day old workers from nurse colony and (H) 6-day, (I) 9-day and (J) 12-day-old workers from control colony. Acini of 18 day-old worker from (K) nurse colony and (L) control colony. Ac - acinus, n - nuclei of the gland cell, v - vesicle, sv - secretory vesicle, sd - secretory duct, s - secret, id - intracellular duct, ed - excretory duct, d - ducts, pn - picnotic nuclei. Scale bar 50 µm

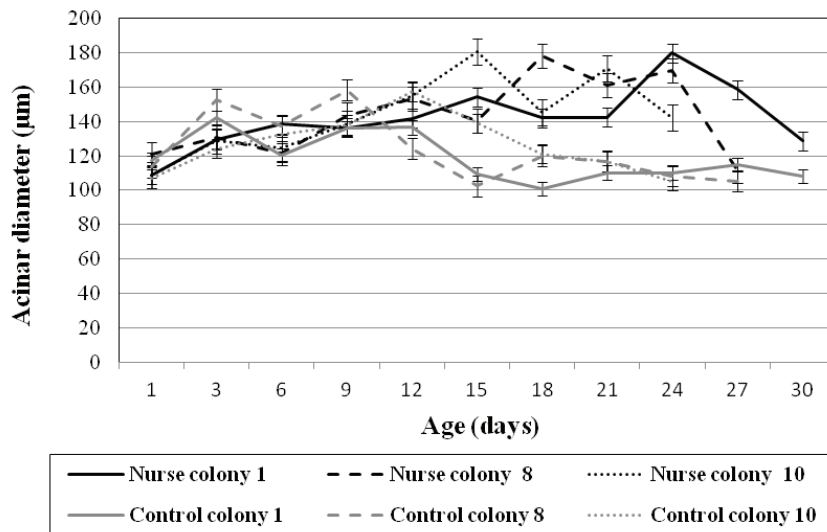


Figure 2: Average acini diameter ($\pm$ SD) of the HPGs in 1 to 30 days old workers from the nurse colony and normally developed (control) honeybee colony which was not included in queen brood feeding. Newly emerged marked workers and grafted larvae were inserted into the nurse colony on day 1 (Nurse colony 1), 8 (Nurse colony 8) and 10 (Nurse colony 10). Newly emerged workers were marked and inserted into the control colony as well

Results

Morphological characteristics of hypopharyngeal glands *in vivo*

HPGs in worker pupa consist from transparent acini (Fig. 1A). In late summer and in early fall, "long-lived" workers accumulated body fat and

the morphological structure of the HPGs starts to change. In these workers we found large acini with a dense structure and milky-white colour (Fig. 1B).

The acini diameter in workers sampled in the nurse colony aged from 1 to 30 days, ranged from 109.2 to 180.9 µm, and acini in workers from the control colony ranged between 100.8 and 158.2 µm (Fig. 2). Acini diameter of 15 and 27 day-old (1st

cell bar introduction) nurses and acini diameter of 15, 18, 21 and 24 day-old nurses (2nd and 3rd cell bar introduction) originated from the nurse colony, was significantly larger in comparison to the acini diameter in workers originating from the control colony ($p < 0.0001$).

Morphological characteristics of acini

In worker pupae (from a control colony) clusters of irregularly shaped secretory acini were found. Cytoplasm is not structured and is homogeneous, nuclei are dense and oval (Fig. 1C). In winter bees, there are numerous large secretory vesicles present in the glandular cells. The size of the vesicles varied between 1.5 and 22.81 μm (winter bees from the control colony, $n=3$; 30 acini per bee were measured). Cell nuclei are regularly or irregularly shaped and dense, with a dense secretory material visible in the ducts (Fig. 1D).

One-day-old workers have small-sized glands, homogenous and well structured cell cytoplasm. Nuclei are large and spherical with an evident chromatin granulation and smaller vesicles around the nucleus (Fig. 1E). In three-day-old bees, vesicles are larger, especially in workers from the nurse colony, and there is secretion present in vesicles and ducts (Fig. 1F). Workers from the nurse and control colony have similarly larger acini at 6 to 12 days of age and there are numerous secretory vesicles in cell cytoplasm (Fig. 1G, H and I). In 12-day-old workers from the control colony, cell nuclei already have irregular shape, some are picnotic but in the majority of the nuclei the chromatin is still evidently granulated (Fig. 1J). In 18-day-old workers from the nurse colonies, acini are large and indicative for active secretion (Fig. 1K). Acini in workers of the same age from the control colony are small in the size, cell cytoplasm is unstructured, without secretion. There are picnotic nuclei of irregular shape (Fig. 1L), and some of the nuclei are dilated.

Discussion

HPGs start to develop in worker pupae about a week before emergence (19) and continue after bees emerge from the brood comb, changing structure until they die. In summer bees, glands

are flexible and can reactivate the HPG activity when needed. In our research, we demonstrated the ability of glands to prolong their activity and enlarge their size which does not depend on the age of the workers. When the honeybee colony has a need of rearing a new brood or new queens, as in our case, workers stay in the hive for a longer period and feed larvae. In the meantime, the acini size increases after the age of 12 days and, as shown in our study, decreases after the age of 24 days when workers usually forage. This contrasts with previous observations which found the peak of gland secretion is at the age of 6 days (20, 7). According to our findings it is evident that workers have the ability to increase glandular activity, and that older nurse workers from the nursing colony, compared with workers from the normal (control) colony, have well developed HPGs with larger acini. Gland cells have numerous secretory vesicles accumulating secretion, they actively secrete and cell nuclei remain spherical containing granulated chromatin. Glands in older forager bees, as comparable to our workers from a normal colony can be reactivated when needed and start to secrete (2). Acini size in workers from nurse and honeybee colonies is correlated with their activity, which is in accordance with the previous research (20, 7).

We found that young nurse workers have larger glands when there is an abundance of queen cells in the nursing colony compared to the nurses from the control colony. In addition to that, the nurses from the breeder aged over 15 days had a larger acini diameter than the same-aged nurses from the control colony. According to the morphological and histological findings, the glands have an active secretion at the age of 27 days and it indicates that the older workers keep their task as nurses and continue intensive brood feeding.

When the gland cells are not developed, they are less productive than the glands with the middle-sized acini (10). The structure of the HPGs changes with the age of individual worker bees and the tasks in the hive. The division of labour and related changes in the organism are called the age polyethism (21, 7). We found that nurse workers stayed in the hive for a longer time in comparison to workers in normal, control colonies and fed the brood. The queen was present in the nursing colony, but separated with the queen excluder. It is possible that the presence of the

queen secreting mandibular pheromone and young larvae in queen comb cells had an effect on the workers to remain in the nest and feed the queen brood. It was also important to have a sufficient number of young nurse workers in the nursing colony. HPGs have flexible secretion activity which depends on the needs of the brood rearing (22). Nurse workers have also larger acini in the active phase of secretion. Glandular acini remain enlarged in winter bees (9). Around the cell nuclei there are numerous secretory vesicles and secretion containing carbon hydrates (23). In young worker bees, secretory vesicles appear in the gland cells. At the age of 6 days, the peak amount of accumulated secretion is found in the secretory vesicles (7). However, later with increasing bee ages the size of the acini decreases and cells still contain some vesicles. It was found that at the age of 18 days the ultrastructure of the glands shows electronic dense secretion (7). After a worker emerges from the comb cell, the endoplasmatic reticulum (ER) starts to expand in the gland cells and increases when the worker feeds the brood. Later on, when workers age, it vigorously decreases. In fact, the proteins are intensively synthesized in younger workers but their productivity is decreasing with age. Knecht and Kaatz (2) suggest that the reduced activity of granular ER also decreases the size of the acini and secretory vesicles.

In regression processes of glands, the juvenile hormone (JH) increases and workers consequently begin to forage. Young workers start to forage after they were topically treated with JH (24) as was confirmed with the high concentration of JH in the haemolymph of foragers (25, 21), additionally the effect of diet on the HPG development is also confirmed (26). Le Conte et al. (27) suggested that the brood pheromone is a primary regulator of the behavioural development in workers and thus we can explain why workers remain for a prolonged period of time in the area with the queen brood. When a colony is not able to provide sufficient amount of nurse workers, the older workers change their tasks and start taking care of the brood (21).

To produce high quality royal jelly and to breed vital honeybee queens, one must consider the technology of breeding, pasture, technology of feeding and diet supplements, and also the presence of pathogens, i.e. honeybee viruses (28) and *Nosema* spp. (29). HPGs are a flexible organ

in young worker bees and are able to actively respond to the needs of the colony, which is very important for brood rearing in swarms or queen supersedure, in periods of inclement weather conditions, as well as for massive queen breeding at the breeding stations.

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ZNAČILNOST HIPOFARINGEALNIH ŽLEZ PRI KRANJSKI MEDONOSNI ČEBELI (*Apis mellifera carnica*) IZ VZREJNE ČEBELJE DRUŽINE

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Povzetek: V raziskavi smo primerjali velikost hipofaringealnih ali krmilnih žlez (HPG) pri delavkah, ki smo jih vzorčili iz vzrejne čebelje družine in kontrolne družine s čebeljo matico. Premer acinusov je pri delavkah v starosti 1 do 30 dni iz vzrejne družine znašal 109,2 do 180,9 μm , pri delavkah iste starosti iz kontrolne družine pa od 100,8 do 158,2 μm . Ugotovili smo, da so imele čebele krmilke iz vzrejne družine (graditeljice celic), stare od 15 do 27 dni, statistično značilno večji premer acinusov ($p < 0.0001$), kot smo ga izmerili pri delavkah iste starosti iz kontrolne skupine. Opisali smo morfološke in histološke značilnosti krmilnih žlez pri krmilkah v starosti od 1 do 27 dni in ugotovili, da je bilo izločanje pri delavkah, ki so krmile zalego, podaljšano v primerjavi z delavkami iz kontrolne skupine. Nadalje smo opisali krmilne žleze pri bubah delavk pred izleganjem in pri zimskih čebelah iz kontrolne družine. Rezultati so pokazali, da so krmilne žleze pri bubi sestavljene iz skupkov neenako oblikovanih acinusov. Celična citoplazma ni strukturirana in je homogena, jedra so gosta in ovalne oblike. Pri zimskih čebelah smo našli hipertrofirane krmilne žleze, ki so vsebovale številne vezikle, v katerih se je nalagal izloček. Acinusi zimskih čebel so večji, goste strukture in so mlečno bele barve. V prispevku smo opisali tudi fiziološko funkcijo krmilnih žlez in s starostjo povezana opravila delavk krmilk.

Ključne besede: premer acinusov; starost; morfologija; krmilka; zimska čebela; buba delavke