**Mendel University in Brno**
**Faculty of AgriSciences**


# MendelNet 2020

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# PREFACE

Each year, the editors of the volume you are about to read are tasked with the responsibility of putting a coherent form to the proceedings from MendelNet, the international PhD Students Conference of the Faculty of AgriSciences of Mendel University in Brno.

The event which reached, this year, on November 11, 2020, its 27th edition, is traditionally aimed at both under and postgraduate students from the Czech Republic, Europe and beyond, and proudly welcomes the participants of various professional and cultural backgrounds. And while this time the people could not gather on-site due to globally-imposed COVID-19 restrictions, the conference swiftly transformed itself into a virtual and fascinating beehive of results, opinions and brand new research paths and ideas.

Here in Brno, under the spell of great genetician J. G. Mendel and the guidance of skilled senior researchers and supervisors, students can introduce, defend and discuss their scientific results while those who do not feel confident enough to present and pen their paper in English are invited to join as spectators and follow-up discussion participants.

The best submissions are, after rigorous peer-review process, collected here and range from plant and animal production to fisheries and hydrobiology to wildlife research while agroecology and rural development, food technology, plant and animal biology, techniques and technology and applied chemistry and biochemistry also belong to the core areas being investigated.

The collection as varied and huge as this can succeed only as a team effort, both on authors' and editors' side, so we would like to express our thanks and gratitude to all committees and reviewers both for their outstanding work and invaluable comments and advice. The final volume is, as always, sent to Clarivate Analytics to be considered for an inclusion in Conference Proceedings Citation Index.

*The Editors*

# Total haemolymph protein and hypopharyngeal glands in the honeybee

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*Abstract:* The development of the hypopharyngeal glands (HG) of honeybees depends on nutrition, which influences also the total concentration haemolymph proteins (THP). The objective of this study was to compare the size of hypopharyngeal glands and the total haemolymph protein in the honeybee workers. Unaffected natural ontogenesis of HG development and THP content was measured in four colonies of different strength. Correlation between HG and THP without age or colony sorting was weak. The moderately positive correlation between HG and THP was found in freshly emerged workers. Strong dependence was confirmed between the mean values of HG and THP of all colonies together sorted according to age of workers.

*Key Words: Apis mellifera*, colony, haemolymph protein, hypopharyngeal gland, superorganism

## INTRODUCTION

The honeybee is a social insect forming superorganism (a honeybee colony) with well-defined anatomical, physiological and behavioural cohort differentiation (Moritz and Southwick 2012). Worker honeybee cohorts are involved in age-dependent division of labour and ontogenesis of their physiological markers (Crailsheim 1986). Physiologically young workers, the hive bees, posse bigger hypopharyngeal glands and whole body weight, higher protein levels in the haemolymph, higher antioxidant ability and enzymatic activity (Halberstadt 1967, Fluri et al. 1982, González-Santoyo and Córdoba-Aguilar 2012) than physiologically old workers, the foragers rapidly approaching their death, which have high titre of juvenile hormone (Amdam and Omholt 2002). In addition, distinction of duties and lifespan between the summer and winter workers are typical for temperate climate regions (Fluri et al. 1982). The winter honeybee workers prepare to overwinter under temperate climates by entering a distinct physiological and behavioural state (Doeke et al. 2015). Workers developed within spring/summer show significantly faster physiological ageing/shorter lifespan than workers developed in autumn (Fluri et al. 1982).

The fitness of bee workers is not outwardly apparent due to external skeleton (Snodgrass 1925) as it is considerably easily evaluable in vertebrates with internal skeleton (Ullman-Culleré and Foltz 1999). As a worker bee condition decreases with the physiological ageing it may be determinable by biochemical parameters (Kunert and Crailsheim 1988). The total protein in worker haemolymph and the hypopharyngeal gland size are easily assayed age dependent parameters for evaluating the worker physiological status (Fluri et al. 1982, Kunc et al. 2019).

The regular development of the hypopharyngeal glands depends on nutrition and on the existence of a social system (Crailsheim and Stolberg 1989). The nutrition influences the total haemolymph protein content (De Jong et al. 2009). Therefore, the aim of this study was to compare the size of hypopharyngeal glands and the total haemolymph protein in the honeybee workers and to find whether there is dependence between these two physiological parameters that would be useful for determination of honeybee worker condition. These hypotheses were established: Does the total

haemolymph protein content in the worker correlate with its hypopharyngeal glands size? Do the honeybees of equal age have similar values of the total protein and hypopharyngeal gland size?

## MATERIAL AND METHODS

The field experiment was realized on 4 bee colonies (1 apiary) in the spring 2020 (May, June). Bees of known age were collected and sampled to measure their total haemolymph protein content and hypopharyngeal gland size.

### Evaluation of colonies

Strength of all colonies involved in the experiment [n = 20] was assessed according to their overall condition on April 15$^{th}$ 2020. The colonies were divided into 4 groups with respect to number of bee-occupied frames (combs), supers and brood area [dm$^2$]. The colonies were scored with grades 1–10: a) very week colonies with no growth potential and with less than 3 occupied inter-frames gaps (grades 1–3, the colonies were excluded), b) weak colonies with substandard development (grades 4–5), c) good condition colonies with standard growth (grades 6–7), d) strong colonies with above standard development and growth abilities (grades 8–10). Four colonies were selected for the experiment: colony No 1 (grade 4, weak condition), colonies No 2 (grade 6) and No 3 (grade 7) both of good condition and colony No 4 (grade 8, strong condition).

### Marking and sampling of the bee workers

In each of the experimental colonies a comb with the oldest stadium of the sealed brood was isolated on April 24$^{th}$ 2020 into plastic net bag and returned back to the colony for 24 hours. Emerged workers were marked by colour pen (brand POSCA) and both the comb and marked workers were returned back to the maternal colony. The first sampling was made at the marking of workers and the other ones at weekly intervals until the marked workers vanished from the colony; totally 5 sampling dates with workers up to 4week-old. A sample with 10 workers from each of the experimental colonies was collected into a plastic vial and cooled to 4 °C directly after sampling.

### Haemolymph collection

Haemolymph was collected as soon as the cooled workers became motionless. The haemolymph was collected individually from every worker using micro-capillary pipette by incision between 3$^{rd}$ and 4$^{th}$ abdominal tergites in volume 1 µl. The haemolymph sample was immediately pippeted into 1.5 ml Eppendorf tube containing 49 µl of phosphate buffer (pH = 7) and homogenized.

### Hypopharyngeal glands

Each of the workers was decapitated and the head was put into 0.5 ml Eppendorf tube containing 30 µl of 75% ethyl alcohol. The head samples were stored at room temperature until dissection. The hypopharyngeal gland dissection was performed by cutting off the forehead part of the cuticle by micro-scalpel. The cuticle and the tracheal sacs were removed by tweezers. The size of hypopharyngeal gland was scored on a scale:1 = almost invisible shrunken gland, 2 = oval shaped pseudoacini spread within a one layer, 3 = oval shaped pseudoacini spread within more than one layer, 4 = polygonally shaped pseudoacini filling fully space between brain and frontal cuticle with regular convex formation of the gland on its surface. Transitional forms were scored with a semi-degree, e.g. gland between the 2$^{nd}$ and the 3$^{rd}$ size was scored with degree 2.5.

### Total protein quantification

The protein concentrations were estimated by Bradford assay in micro-titration plates. Briefly: 5 µl of protein sample (50× diluted haemolymph) was mixed with 250 µl of Bradford reagent (Sigma-Aldrich, Merck). After 5 min of incubation at room temperature, the absorbance at 595 nm was measured using FUOstar Omega plate reader (BMG Labtech). Concentrations were calculated using calibration curve. The calibration curve for each measured plate was done using bovine serum albumin (Sigma Aldrich) as standard. Each measurement was performed in triplicate.

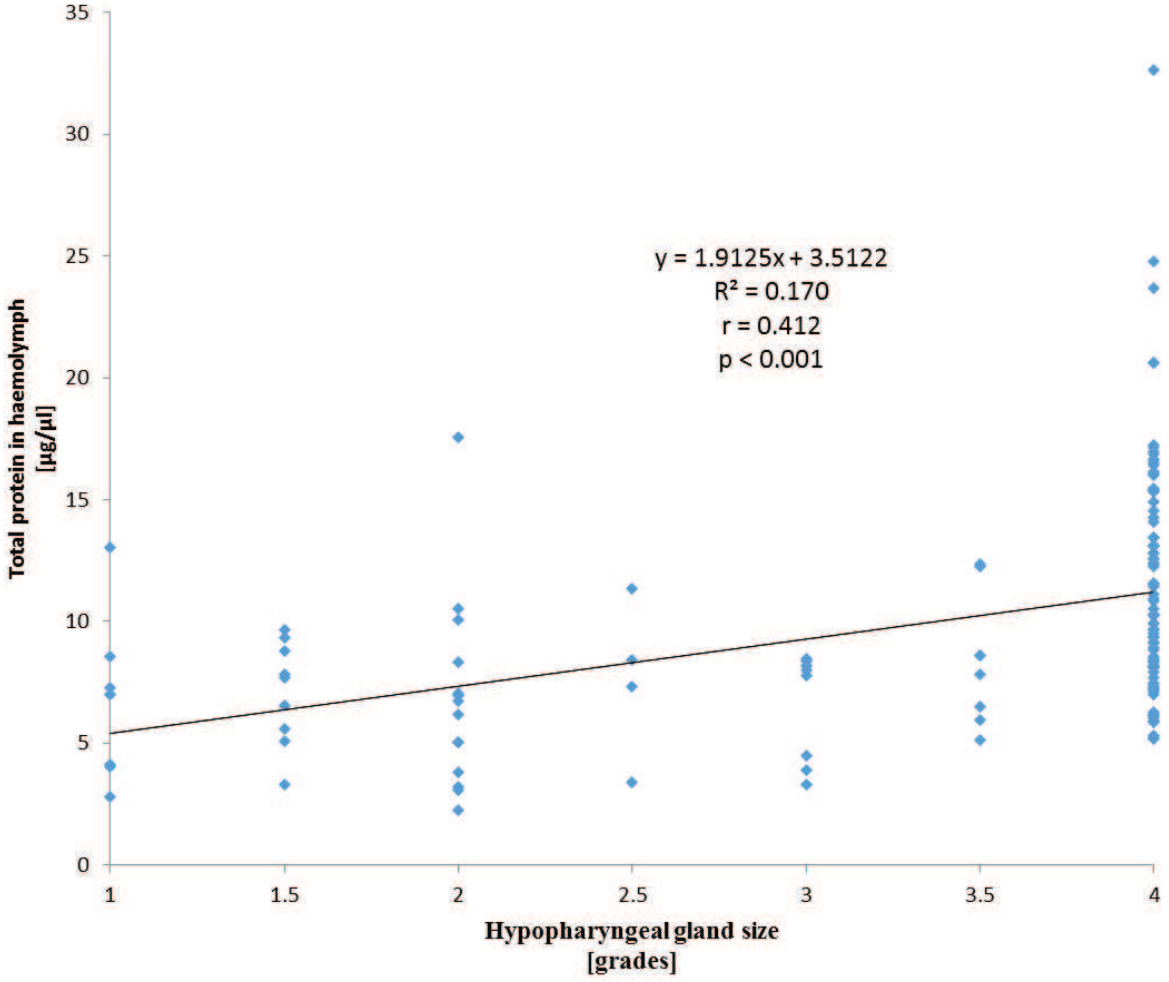
### Statistical evaluation

The abscissae around means in charts represent the standard deviation. The data were evaluated by regression analysis and Pearson's correlation.

## RESULTS AND DISCUSSION

Regression analysis between the total haemolymph protein (THP) and the hypopharyngeal gland (HG) size in all of the sampled workers (all individuals from all colonies) is depicted in Figure 1. The analysis showed only medium positive correlation (r = 0.412). The workers with the 4th degree of HG size showed THP values in range of 5 to 33 µg/µl. The workers with smaller HG showed this THP range in approx. 3–18 µg/µl.

*Figure 1 Regression analysis between the total protein contents and the hypopharyngeal glands sizes of all sampled worker honeybees*




The correlation coefficients in the emerged workers and workers older than two weeks are summarized in Table 1. The dependence of middle importance was detected only in the freshly emerged workers. The older analysable samples of workers showed only weak dependence. The correlation in one- or two-week-old workers was not possible to calculate due to the fact that workers had HGs developed to 4th degree.

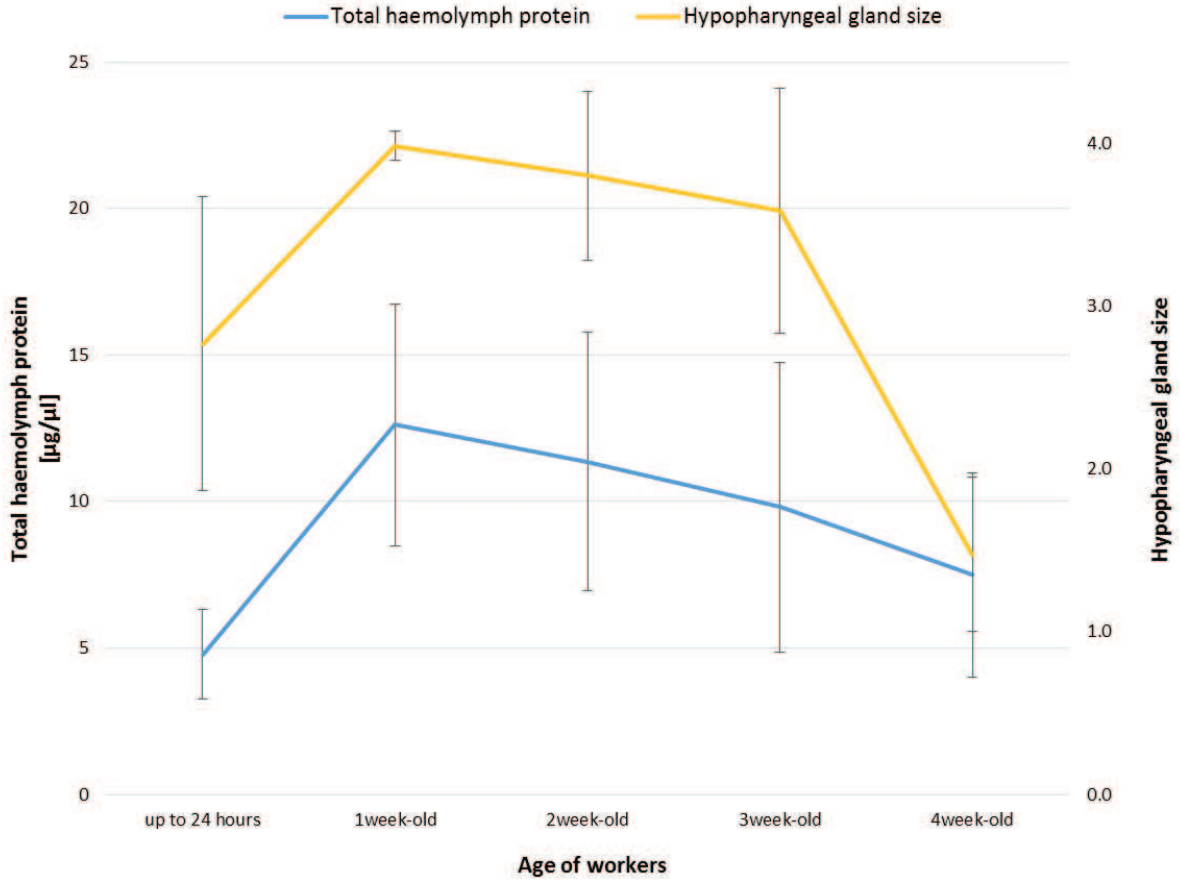
*Table 1 Correlation between THP and HG size according to the worker age*

| Age of workers | Up to 24 hours old (n = 40) | 3week-old (n = 40) | 4week-old (n = 40) |
|---|---|---|---|
| r<br>total haemolymph protein<br>-<br>hypopharyngeal gland size | 0.58<br>p < 0.001 | 0.26<br>p < 0.105 | 0.17<br>p < 0.294 |

The analysis of ontogenetic changes according to the worker age expressed as a mean value for the experimental colonies together is depicted in Figure 2. Dependence between these mean values

($n_{yz}$ = 5) was very high and positive (r = 0.703, p = 0.185). The premise that workers with well-developed HG have concurrently high protein concentration in haemolymph is based on the fact that the injection of high doses of juvenile hormone into adult honeybee worker causes size reduction of HG and lowers the THP concentration (Rutz et al. 1976). However, the premise was confirmed by the results only up to the colony level instead of the individual level. This finding is in accordance with the superorganism postulates (Moritz and Southwick 2012) and points to the existing integrity in the honeybee colony (Crailsheim and Stolberg 1989). Since, there were lower levels of THP in workers with smaller HG we suppose that the workers with the low level of THP and fully sized HG could show only low secretion activity in their HGs as it was found by Brouwers (1982). However, three- or four-week-old workers did not show this dependence in our experiment. There are probably other factors involved in the investigated relationship because the size of HG is concurrently closely related physiological parameter to labour-division and age of the summer workers (Pridal et al. 1997). One of them may be a fact a shrunken HG produces proteins too, mainly enzymes (Sasagawa et al. 1989).

*Figure 2 Ontogenetic course of changes in THP and HG*




## CONCLUSION

The hypopharyngeal gland size and the total haemolymph protein are physiological parameters related probably only at the honeybee colony level. The size of HG and THP were increasing from emerging of worker to age of the hive worker and then decrease to age of the forage only on average. This premised relationship at the level of the individual workers was not detected in spite of the size of HG is concurrently closely related to labour-division and age in the summer workers. Thus, the physiological context between development of HG and THP is not still fully understood. The given findings are in accordance with the superorganism postulates of the honeybee colony.

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