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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 in honeybee workers as a marker for the evaluation of colony condition?

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Abstract: The winter loses of honeybee colonies have been increasing in recent decades. Successful overwintering depends on abundance of the long-living bees in the colony. This study verified whether the total haemolymph protein content in the pooled sample from workers (TP) could be a useful marker to determine the overall colony condition and predict winter survivability. Here we show that the values of TP in autumnal workers did not correlate with the winter survival of colonies. The average TP did not differ between the collapsed and survived colonies. In the early spring, the average TP among apiaries did not differ in spite of very different health conditions of individual apiaries. These results do not support the hypothesis that TP assayed in the pooled sample prepared from unknown-aged workers is a reliable marker for prediction of the overwintering ability or the overall condition of colonies.

Key Words: Apis mellifera, condition, colony, haemolymph, protein

INTRODUCTION

Winter colony loses have been considered to be the most serious issue of recent beekeeping (Stokstad 2007, van Engelsdorp et al. 2009, Williams et al. 2010). This loses are caused multifactorially: diseases (Genersch et al. 2010), sublethal intoxication (Di Prisco et al. 2013) or malnutrition (Zhang et al. 2020). Devastating consequences arise mainly due to weakening and damage of winter bees (van Dooremalen et al. 2012). Overwintering is closely dependent on the number of long-living bees and their condition within wintering colony (Döke et al. 2015). There is a demanding issue to find predictive markers for the honey bee colony collapse (Dainat et al. 2012, Aurori et al. 2014, Steinmann et al. 2015). There were studied following physiological parameters in honey bee workers; a) in body: weight, storage proteins, size of hypopharyngeal glands, total antioxidant status, glutathione, activity of enzymes (superoxide dismutase, peroxidase, catalase, glutathione S-transferase), b) in haemolymph: total protein, vitellogenin, titre of juvenile hormone, lipids, carbohydrates, antibacterial activity, activity of phenoloxidase and number of haemocytes and c) a gene expression e.g. methyl farnesoate epoxidase (Fluri et al. 1981, Kunert and Crailsheim 1988, Otis et al. 2004, Farjan et al. 2012, Steinmann et al. 2015, Kunc et al. 2019).

Kunc et al. (2019) emphasised that the distinction of the summer and winter bees is important for prediction of the success of overwintering. The total haemolymph protein is basic physiological parameter correlating with several other physiological parameters (Kunc et al. 2019) in which their determination is more difficult (e.g. vitellogenin). Kunc et al. (2019) mentioned that their study was performed on only one colony, therefore, future experiments would involve efforts to measure

the parameters on a large number of colonies to correlate the results of analyses to the success of overwintering rate.

Therefore, following experiments on a large number of colonies were aimed on the total haemolymph protein in honeybee workers as a predictive marker of overwintering ability. The hypotheses were as follow: Is total haemolymph protein in autumn a predictive marker for overwintering of colony and its further growth in spring? Does the total haemolymph protein in early spring correlate with the strength of the colony?

MATERIAL AND METHODS

The experiments were carried out: a) in autumn 2019 at three apiaries in Lysicko region totally with 19 colonies and b) in early spring 2020 at three apiaries in Brno city, Brno – countryside region and Opava region totally with 59 colonies; totally 78 colonies of *Apis mellifera*.

Evaluation of colonies

The colonies included in the autumnal experiment were evaluated with respect to their ability to survive winter (survived/collapsed) in the early spring (March 2020) and then in spring (May 2020) with respect to their overall condition reflecting the ability to grow (strength of the colony). The colony strength was assessed according to number of occupied frames, supers and surface of brood area [dm^2]. Thus the colony population was divided into two groups: 1) colonies with good condition having standard spring growth and abilities to build combs and 2) weak colonies whose hive space had to be reduced in early spring and with substandard growth and limited abilities to build new combs.

The colonies included in the early spring experiment were evaluated in March 2020 with respect to their overall condition after winter period. The colony condition were scored with grades 1–10 (the grade 10 described a colony with excellent condition – occupied maximum frames and supers, had the largest areas of brood).

Haemolymph collection

The haemolymph for the autumnal experiment was collected on 23th October 2019 and for the early spring experiment on 18th March 2020. Twenty workers from each experimental colony (*Apis mellifera*) were sampled to plastic vials. The bees were taken from the broodless comb that was located straight next to the first brood comb of the brood nest (Kunc et al. 2019). The plastic vials with bees were cooled up to 4 °C and transported to laboratory for haemolymph collection. Haemolymph was collected from each sampled worker by micro-capillary pipette by incision between the 3rd and 4th abdominal tergites in volume 1 µl to create pooled haemolymph sample of total volume 20 µl representing a colony. For quantification of protein, 1 µl of haemolymph representatively taken from the pooled sample was mixed with phenylthiourea and phosphate buffer (pH = 7) to prevent haemolymph melanisation and to improve stability of the haemolymph proteins.

Total protein quantification

Bovine serum albumin (BSA) was used as a standard and to validate the method. Validation of the method using the BSA standard always took place before each determination (the standard was always prepared fresh). Ready-to-use protein reagent Dry Reagent Concentration (BIO-RAD, California, USA) was purchased. This reagent was diluted 1:4 with MilliQ water before analysis (the reagent was always prepared fresh). Determination procedure: 10 µl of the sample was pipetted into a 96 well microtiter plate Nunc Immuno (Fisher Scientific, Pardubice, Czech Republic). Subsequently, 200 µl of diluted protein reagent was added to 10 µl of the sample. Followed by incubation for 5 min at room temperature and absorbance measurement at 595 nm using instrument Infinite M200Pro (Tecan, Männedorf, Switzerland). Each sample was analysed three-times (triplet).

Statistical evaluation

The abscissae around means in charts represent the standard deviation. Relations between the total protein and overwintering/early spring condition were evaluated by Spearman's/Pearson's correlation or regression analysis. The statistical significance of differences of the mean values was analysed by t-test (two groups) or ANOVA (more than two groups) with post-hoc analysis by Tukey's test. A difference with p-value above 0.05 ($p > 0.05$) was considered statistically insignificant.

RESULTS

Autumn

Relationship analysis between the total haemolymph protein in autumnal workers and the winter survival resulted only in low and positive coefficient of correlation ($r = 0.348$, $p = 0.144$). There was a tendency of colonies with a higher content of the protein in haemolymph to overwinter successfully, however, this dependence was weak. This tendency according to individual colonies is depicted in Figure 1. Both extreme values of the total protein belonged to the collapsed colonies. The collapsed colonies are crowded namely in the central and the right parts of the chart, i.e. with middle and low protein content. However, the survived colonies are evenly distributed over entire width of the chart. The survived colonies with weak early spring condition had, paradoxically a high protein content in autumn (yellow columns, the left part in Figure 1).

Figure 1 Total autumnal protein content in individual colonies in descending order with colour marking their overwintering ability and early spring development

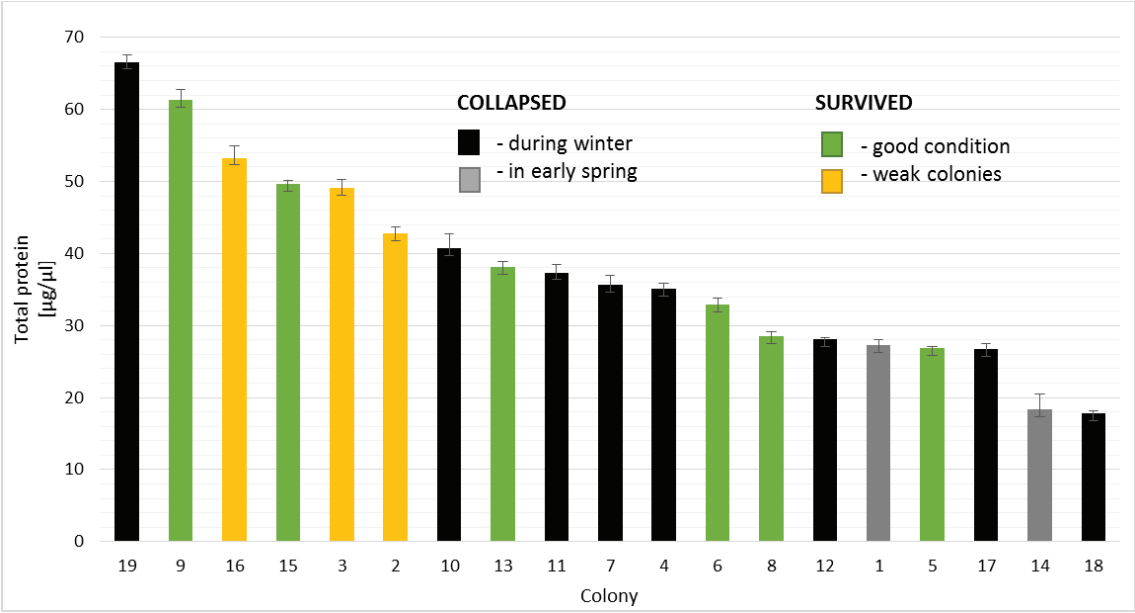
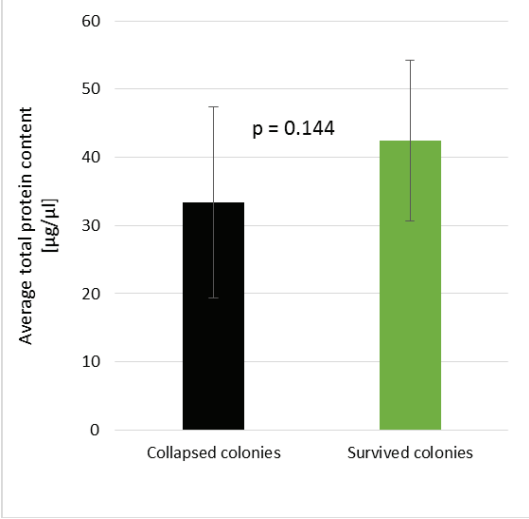


Figure 2 Average total autumnal protein content in collapsed and survived colonies



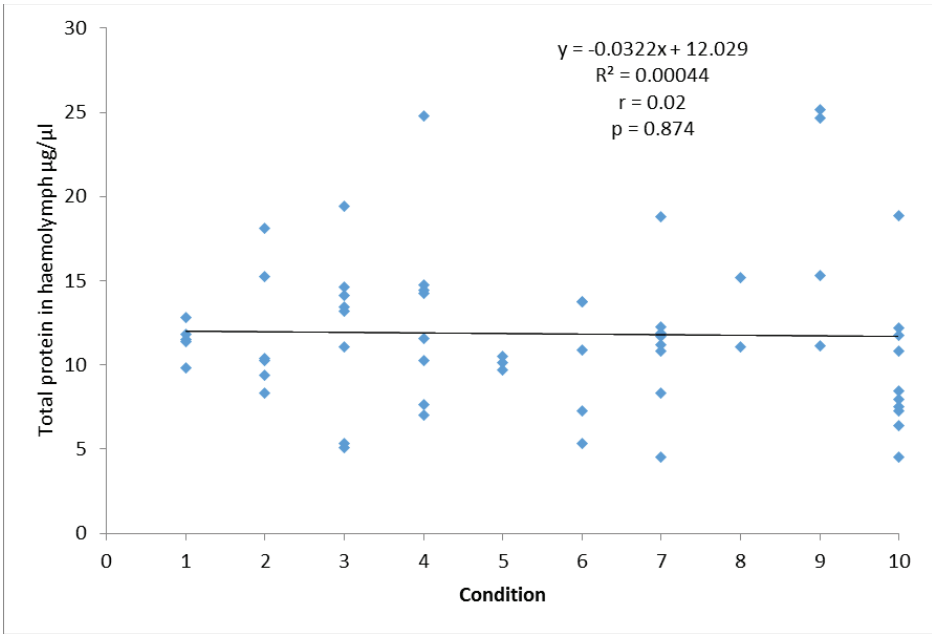
The average total haemolymph protein in the collapsed and the survived colonies differed insignificantly (Figure 2) and in the collapsed colonies was lower only by 9.1 µg/µl in comparison with the survived colonies.

Early spring

The apiary at Brno-city showed more than half winter losses in the early spring 2020. The Opava region apiary had only low losses about 15%. The best overwintering result showed colonies at Brno – countryside region. There were predominantly colonies with the excellent condition, without deaths or need to outlay any colony. There were only two colonies showing conspicuous weakening.

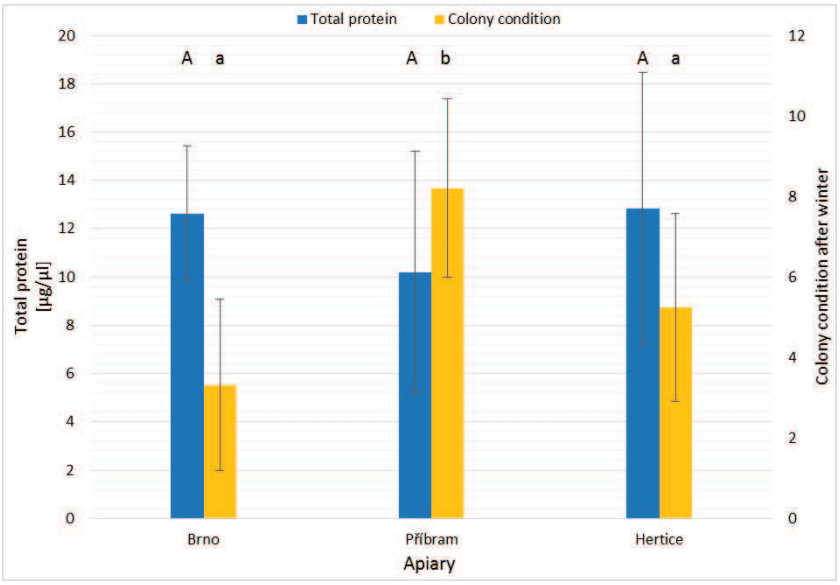
Regression analysis between the total haemolymph protein in early spring workers and the condition of overwintered colonies showed almost zero and negative dependence (Figure 3).

Figure 3 Regression analysis between of the total protein content and colony condition in early spring



Despite that the average total protein in the early spring workers differed among apiaries insignificantly ($F_{0.95}$ (2,56) = 2.085; p = 0.134) the average condition of the early spring colonies differed significantly ($F_{0.95}$ (2,56) = 25.620; p < 0.001) (Figure 4).

Figure 4 The average total protein content and average colony condition by apiaries in early spring



Legend: capitals letters = total protein; small letters = colony condition; different letters = p < 0.05 (Tukey's test)

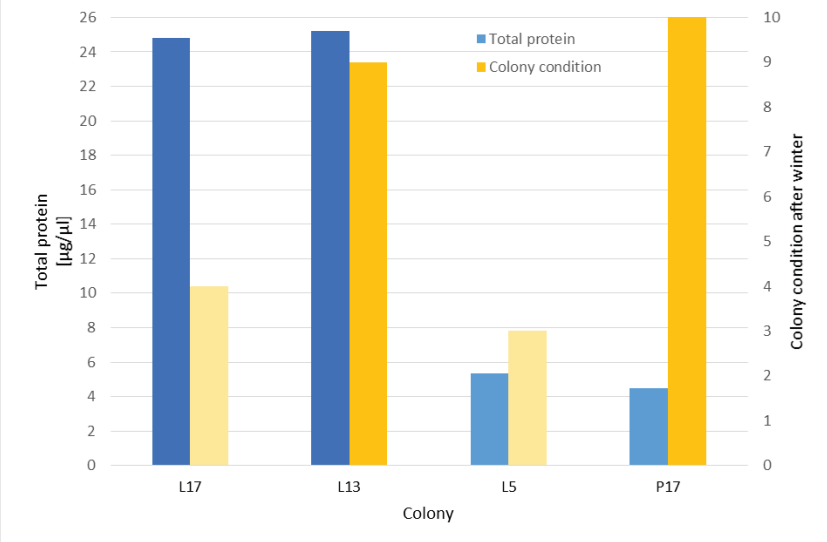
The haemolymph protein content in the early spring workers did not correspond with the colony condition in any way. This result is supported at example of four selected colonies with extreme combinations of both values (Figure 5). These examples show that there were colonies with

excellent/insufficient condition and at the same time with very low/very high level of the protein in haemolymph.

DISCUSSION

The colony No 19 with the highest level of protein in haemolymph in October 2019 died within the winter 2019/2020. Moreover, the weak colonies (No 2, 3 and 16) had the total haemolymph protein content higher than average in comparison with the rest of the experimental colonies. Although, the total protein was somewhat lower in the collapsed colonies, the difference with the survived colonies was not significant. The results of overwintering did not correspond with autumnal levels of protein in the worker bee haemolymph. Therefore, we decided to assay the protein level in many colonies located in several apiaries also at the first inspection of colonies in early spring 2020. Even in this case, it was not possible to find any significant differences. There was no correlation between the overall colony condition and the protein level. Even differences in the protein level among apiaries in the early spring were insignificant in spite of the strikingly different rate of collapses among apiaries.

Figure 5 The total protein content and condition colonies with extreme values in early spring



Legend: lighter shade of colour = low value, darker shade of colour = high value

Content of the total haemolymph protein from the pooled sample of haemolymph of honey bee workers did not correlate with the overall condition of a colony either in autumn or in spring. This result is not in conflict with results by Fluri et al. (1982) or Kunc et al. (2019). However, the total haemolymph protein content is not a predictive marker for 'potential overwintering problems' with regard to the applicated sampling of bees as it was proposed by Kunc et al. (2019). Besides other, they proposed sampling in October and compare measured results with their data, since, this approach might provide useful insight into the readiness of long-living bees which are necessary for successful overwintering and beekeepers could estimate proportion of long-living workers in the colony and so predict potential overwintering problems. However, their data did not involve estimation of proportion of long-living workers in the colony. Kunc et al. (2019) sampled worker bees from the comb without brood and adjacent to brood chamber to avoid the sampling of freshly emerged adults. There were sampled probably no freshly emerged imagoes, however, workers of all ages. The following question arises: Where or how were the workers sampled in the winter period without brood?

It is known that physiology in honeybee workers is age-related (Crailsheim 1990, Huang and Robinson 1995, Crailsheim and Leonhard 1997) and interpretation of physiological parameters without known age of a bee is rather complicated. Therefore, it can be presumed that a physiological parameter, e.g. total haemolymph protein, should be compared only in the same age colony cohorts. A simple average value of any physiological parameter obtained from several workers has lower importance for an explanation than values for each worker bee separately, and knowledge about the structure of workers in a colony as it was analogically proposed e.g. by Oliver (2012).

CONCLUSION

The total protein assayed from the pooled sample of haemolymph collected from workers of unknown age is not appropriate predictive maker for a capability of the honeybee colony to overwinter or evaluation of its overall condition. However, the total haemolymph protein is not disqualified as an applicable physiological parameter in the case of haemolymph collection from workers of known age with respect to the complex structure of the honeybee colony.

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