Changes in bioelement contents of summer and winter western honeybees (Apis mellifera) induced by Nosema ceranae infection

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Abstract

Citation: Aneta Ptaszynska Changes in bioelement contents of summer and winter western honeybees (Apis mellifera) induced by Nosema ceranae infection. **protocols.io**
https://www.protocols.io/view/changes-in-bioelement-contents-of-summer-and-winte-qwedxbe
Published: 11 Jun 2018

Protocol

Selection of honeybees colonies

Step 1.

The colonies originated from an apiary of 82 colonies at the Life Sciences University in Lublin, Poland (51°13'32.2'N 22°38'08.3'E). At the beginning of July 2015, 100 forager worker-bees (*Apis mellifera carnica*) from each colony were captured at the hive entrance. Forager bees were expected to have higher *Nosema* spp. infection level than younger bees [18] and were tested for *Nosema* infection (according to [19,20]). From the samples of 100-workers, 50 workers were removed from each and abdomens dissected, pooled, homogenized in distilled water and examined for the presence of *Nosema* spp. spores under an Olympus BX 61 light microscope. 10 of the remaining 50 workers were pooled, ground in distilled water and used for DNA analysis. If *Nosema* spp. spores were detected in a given sample, the colony was considered *Nosema*-infected (NI). In such cases, a heamocytometer was used to count the number of spores per worker bee. Colonies from which bees were found to contain no spores, were considered *Nosema*-free (NF). Consequently, five colonies which were *Nosema*-infected and five colonies which were *Nosema*-free were chosen as the experimental colonies and were subsequently kept in two locations 200 meters apart, separated by a hedge. This enabled the reduction of between-location drifting of forager-workers while at the same time, providing the colonies with access to the same food resources.

In mid-July 2015, two pooled samples of approximately 100 worker bees, were collected in the evenings from the outer combs of each experimental colony (five *Nosema*-infected and five *Nosema*-free), to determine the bioelement composition in summer bees. Then, at the end of March 2016, after the bee winter cleansing flight, this procedure was repeated to determine the bioelement composition in overwintered bees. Fifty worker bees out of each pool were then subjected to DNA analysis and microscopy in order to confirm the *N. ceranae* infection status of each sample.

The *Nosema*-free and infected colonies were kept in two locations 200 meters apart and separated by a hedge. All apiary work were carried out in the same manner at both locations. Colonies had access to the same food resources and in autumn were fed sugar-water (2:1) syrup in preparation for overwintering.

The soil sampling

Step 2.

Two soil samples (in triplicate) were also taken in the immediate vicinity of the *Nosema*-free and *Nosema*-infected colonies. Bioelement contents in the soil was expected to be related to the content in plants, and therefore also with the bee bioelement contents [10,12].

The honey sampling

Step 3.

In order to compare food resources, honey samples from *Nosema*-infected and *Nosema*-free colonies were analysed. Honey samples were taken during spring (May 2015), summer (August 2015) and winter (January 2016) and stored in sterile jars until further analysis.

Both spring and summer samples of honey and also winter-food samples were taken from the colonies and stored for further bioelement analysis. Bioelement contents in bee food is correlated with the bioelement bee-body concentrations [8-10] and therefore this, along with the soil analyses [12], confirms that both the *Nosema*-infected and the *Nosema*-free bees used very similar bioelement resources. While over winter, all colonies were supplemented with the same high quality food sugar (sucrose) syrup.

DNA analysis

Step 4.

DNA was extracted from each of the pooled samples of homogenized worker bees as follows: 100 µl of each homogenate was added to 180 µl of lysis buffer and 20 µl of proteinase K and total DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Subsequently, each of the DNA samples was used as a template for detection of *N. apis* and *N. ceranae* 16S rDNA by PCR with *Nosema*-specific primers: 321-APIS for *N. apis* and 218-MITOC for *N. ceranae* [21].

Analysis of bioelement composition

Step 5.

Honeybee samples. The bioelement composition in each of the pooled worker bee samples was determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, iCAP Series 6500, Thermo Scientific, USA). Two sets of pooled bees (10 workers each) were mineralized in a Microwave Digestion System (Bergh of Speedwave, Eningen, Germany) by using optical, temperature and pressure monitoring of each sample during acid digestion in teflon vials (type DAP 100). The mineralized worker bee bodies were digested with 7 ml HNO_3 (65% v/v) and 3 ml H_2O_2 (30% v/v). Each of the samples were performed in triplicate. Therefore 6 measurements were taken for each

experimental colony, giving a total of 30 measurements for each bioelement (5 colonies x 2 pooled samples x 3 replicates).

Honey samples. In total, 12 honey samples were analysed: four spring honey (two from NF and two from NI colonies), four summer honey (two from NF and two from NI colonies) and four winter stores (two from NF and two from NI – colonies). The mineralization of each 0.5g honey sample was conducted in a Microwave Digestion System (Bergh of Speedwave, Eningen, Germany) by using optical, temperature and pressure monitoring of each sample during acid digestion in teflon vials (type DAP 100). The mineralized honey samples were digested with 7 ml HNO_3 (65% v/v) and 3 ml H_2O_2 (30% v/v). Each of the samples were performed in triplicate.

The mineralisation of honybees and honey process was as follows: 15 mins from room temperature to 140°C, 5 mins at 140°C, 15 mins from 140°C to 185°C, 10 mins at 185°C and then cooling down to room temperature. The pressure did not exceed 20 bars during mineralisation. After the mineralisation, the clear solution was cooled to room temperature and then transferred to 50 ml graduated flasks and filled with deionized water (ELGA Pure Lab Classic).

Soil samples. Six soil samples were also taken. Three were taken in the immediate vicinity of the colonies which were *Nosema*-free and similarly, the three from the *Nosema*-infected colonies. 0.5 grams of soil from each sample was digested in 8 ml of aqua regia, with 2 ml hydrofluoric acid in a high-pressure microwave digestion system (Berghof Speedwave, Eningen, Germany). Then the digested samples were made up to 50 ml with deionized water.

The operating conditions of the ICP-OES equipment were as follows: RF generator power of 1150 W, RF generator frequency of 27.12 MHz, coolant gas flow rate of 16 $L \cdot min^{-1}$, carrier gas flow rate of 0.65 $L \cdot min^{-1}$, auxiliary gas flow rate of 0.4 $L \cdot min^{-1}$, maximum integration time of 15 s, pump rate of 50 rpm, viewing configuration – axial, replicate – 3, flush time of 20 s.

The following multi-element stock solutions from Inorganic Ventures were used to prepare standards for all the analyses described above:

1. A) Analityk-46 for Cu, Fe, Mg, P, K, Na in 5% HNO_3 (1000 µg/mL)
2. B) Analityk-47 for Al, As, Cd, Cr, Pb, Mn, Hg, Ni, Sc, Se, Sr, V, Zn in 10% HNO_3 (100 µg/mL)

1. C) Analityk-83 for Ca, K, Mg, Na, P, S in 2% HNO_3 (1000 mg/L)
2. D) Analityk prepared from single-element stock solutions for B, S, Si in 5% HNO_3 (1000 mg/L)
3. E) CGMO1-1: Mo in H_2O/tr. NH_4OH (1000 µg/mL).

The bioelement symbols are compliant with the standards of the International Union of Pure and Applied Chemistry (IUPAC).

Statistical analyses

Step 6.

Four pooled worker bee groups were analysed: summer *Nosema*-free (S-NF), summer *Nosema*-infected (S-NI), winter *Nosema*-free (W-NF) and winter *Nosema*-infected (W-NI). Tukey tests (one-way ANOVA, Statistica version 12.0, StatSoft Inc., USA) at the significance level of $\alpha = 0.05$ were used to prepare the results presented in Table 1.

Principal component analysis (PCA) was performed using the software package, Statistica (version 12.0, StatSoft Inc., USA), at the significance level of $\alpha = 0.05$. The data were log-transformed, centred and standardised by bee group (i.e. S-NF, S-NI, W-NF, W-NI) but not by sample; thus, PCA was performed on the correlation matrix. The data matrix for PCA had four columns and 22 rows and the influences of two factors were considered: *Nosema* infection status (*Nosema*-infected or *Nosema*-free bees) and worker bee type (summer or winter). Consequently, the analysis was used to compare the multi-elemental stoichiometric relationships between bioelements and in this context, among worker bee groups: winter bees, summer bees, *Nosema*-free bees, and *N. ceranae* infected bees. The interactions between these factors were evaluated by two-way ANOVAs (Statistica version 12.0, StatSoft Inc., USA) performed separately for each bioelement. Correlations (interrelations) between respective bioelement content in bees were assayed on the correlations vectors at the PCA graph.

Differences in bioelement contents in the winter stores, spring and summer honeys were assayed both by comparing two one-way ANOVAs ($p \leq 0.005$; honeys produced by *Nosema*-infected and *Nosema*-free bees) and two-way ANOVA ($p \leq 0.005$ winter bee food, summer and spring honeys produced by *Nosema*-infected and *Nosema*-free bees) (Statistica version 12.0, StatSoft Inc., USA). These data were presented in Supplementary Materials Table 1. Additionally, principal component analysis (PCA) was performed using the software package, Statistica (version 12.0, StatSoft Inc., USA), at the significance level of $\alpha = 0.05$. The data were log-transformed, centred and standardised by honey or food group (i.e. SpH-NI spring honey made by *Nosema* infected bees, SpH-NF spring honey made by *Nosema* free bees, SH-NF summer honey made by *Nosema* infected bees, SH-NI summer honey made by *Nosema* free bees, WF-NF winter food stored by *Nosema* infected bees, WF-NI winter food stored by *Nosema* free bees) but not by sample; thus, PCA was performed on the correlation matrix. The data matrix for PCA had four columns and 22 rows and the influences of two factors were considered: *Nosema* infection status (*Nosema* infected or *Nosema* free bees) and spring or summer honey compared to winter stores. These data were presented in Supplementary Materials Fig 1 and Fig 2.