Title

PRELIMINARY SURVEY ON THE NUTRITIONAL AND PALYNOLOGICAL TRAITS OF HONEY BEE-FORAGED POLLEN IN LIGURIA (ITALY)

Authors

IDA CONTI[1], PIOTR MEDRZYCKI[2], ANTONIO IANNONE[2], FRANCESCA VITTORIA GRILLENZONI[2], FRANCESCA CORVUCCI[2], DAVIDE DAGNINO[1], GABRIELE CASAZZA[1], CARLO MONTANARI[1], MAURO GIORGIO MARIOTTI[1]

Author details

[1]University of Genova – DiSTAV, Corso Europa 26, Genova, Italy

[2]Council for Agricultural Research and Economics, Honey Bee and Silkworm Research Unit, Via di Saliceto, 80, Bologna, Italy

ida.conti3@gmail.com

piotr.medrzycki@crea.gov.it

antonio.iannone@crea.gov.it

francesca.grillenzoni@crea.gov.it

francesca.corvucci@crea.gov.it

davide.dagnino@fastwebnet.it

Gabriele.Casazza@unige.it

Carlo.Montanari@unige.it

M.Mariotti@unige.it

Introduction

During their activity honey bees (*Apis mellifera* L.) explore the environment surrounding the hive and collect pollen from available sources. The palynological spectrum of the corbiculae reflects thus the bee-interesting flora at any time of the year (Diaz-Losada, Ricciardelli d'Albore, & Saa-Otero, 1998).

In many studies the seasonal variation of the palynological spectra of the honey bee-foraged pollen has been investigated, allowing thus to elaborate local calendars (e.g. Andrada & Telleria, 2005; Bhusari, Mate, & Made, 2005; Bilisik, Cakmak, Bicakci, & Malyer, 2008; de Sa-Otero, Armesto-Baztan, & Diaz-Losada, 2009; Dimou & Thrasyvoulou, 2007; Forcone, Aloisi, Ruppel, & Munoz, 2011; Fujiwara & Washitani, 2017; Sajwani, Farooq, & Bryant, 2014; Vergara Lopez, Gil Vives, & Boi, 2013; Webby, 2004). This tool can be used by the beekeepers to identify most advantageous periods and locations for the collection of corbicular pollen, e.g. when its features (yield, nutritional quality, taste, eventual residues of xenobiotics or undesirable natural compounds, even colour) assure high commercial value.

Beside of the economic aspects, also the impact on bee health status should be taken into account when programming pollen collection and the other beekeeping practices (like supplementary feeding or artificial swarming). It is known that bee health is influenced by many parameters of the nutritional quality of pollen diet, among which: protein content, digestibility, amino-acid profile, presence of essential amino-acids, lipids, vitamins, minerals (Brodschneider & Crailsheim). In the present study we focused on the protein concentration in bee-collected pollen, because it was shown that this parameter of nutritional quality of diet is often associated to many aspects of honey bee health (Schmidt, Thoenes, & Levin, 1987; Hoover, Higo, & Winston, 2006; Di Pasquale et al., 2013)

The protein content in pollen is determined by the floral origin (Roulston, Cane, & Buchmann, 2000) When mainly barely proteic pollen is available in the environment, a larger amount is necessary to satisfy the colony requirements (Somerville, 2001). In these conditions the colonies increase the number of pollen-foragers (Pernal & Currie, 2001). Therefore the low food quality is compensated, but this involves a higher energetic effort, both in foraging and digestion (Roulston & Cane 2000), and the subsequent decrease in honey production (Keller *et al*., 2005b). In particular cases, to assure the correct proteic supply, the colonies may also excessively consume hive stores. In the subsequent eventuality of adverse weather or pollen shortage in the environment, the colony development may be affected.

Thus, the collection of pollen by beekeepers, being potentially a stress factor for hives (Keller, Fluri, & Imdorf, 2005), should be performed with caution in these periods. Considering also the physiological state of the colony, in some cases it might be recommended to avoid pollen collection, or even to supplement bee nutrition. Therefore it would be very useful for beekeepers to

know how the protein content in pollen loads varies in time and space, and to be able to identify eventual periods/sites of high and low levels, according to plant phenology.

Beekeepers could determine with a good approximation the main botanical origins of the collected pollen basing on the palynological calendars of their area and the pellet colours. Then it would be possible to estimate the protein content of a pollen stock if the protein content (which is species-specific) of each pollen type was available. However, such database is incomplete at present (Conti et al., 2016; Roulston et al., 2000).

In this research we evaluated relationships between the palynological spectra and the protein content of the pollen loads collected in a preliminary monitoring campaign in the eastern province of Genova (Italy), where nowadays apiculture is in rapid development. The objectives of the study were to investigate:

1) which taxa are exploited by bees from early spring to late summer in different sites

2) which palynological types are significantly related to possible variations in protein content.

…

Materials and methods

Study area

Seven experimental apiaries (sites), coordinated by the beekeeper association Nuova Assoapi Ligure, were identified in Liguria (north-west of Italy), in the area between the hinterland of Genova and Sestri Levante (figure 1, annex 1). They were distributed within this macroarea to involve the main vegetation types in the basal and submontane belts. The climate in the study areas is mesomediterranean humid/perhumid (6 apiaries) and mesotemperate subhumid (1 apiary) (Rivas-Martínez, Saenz, & Penas, 2011).

The available Forest Cover Map (Regione Liguria, 2006) and Land Use Map (Regione Liguria, 2000) were integrated by field surveys covering the 1.5 km radius area surrounding each apiary, in order to obtain a description of the land cover (annex 2); this is the most probable area visited by bees for pollen foraging in complex landscapes (Steffan-Dewenter & Kuhn, 2003). The maps were elaborated using QGIS software (QGIS Geographic Information System). The environmental systems surrounding the hives are usually heterogeneous eco-mosaics characterized by several land cover types: urban areas (with private gardens and orchards, and public green), olive groves, Mediterranean scrub, mixed woodland dominated by *Pinus pinaster*, *Quercus pubescens* or *Castanea sativa*, submontane grassland and heath.

Sampling

Three healthy and queen-right hives were chosen in each apiary and equipped with front-mount

pollen collection traps (Lega srl) installed on the flight board. Corbicular pollen was collected separately from the trap of each hive, between April and September in 2014 and 2015 (annex 3), following the C.S.I. Pollen protocol (C.S.I. Pollen Manual, 2015). From each pollen pellet sample, a 20 g aliquot was randomly withdrawn and delivered to the CREA-API laboratory (Bologna, Italy). Subsequently, each sample was dried at 30°C for about 12 hours, then powdered and destined to the laboratory analyses.

Palynological analysis

The palynological analysis was carried out according to von der Ohe, Oddo, Piana, Morlot, and Martin (2004), properly adapted for pollen analysis. Two grams of pollen for each sample were dispersed in 50 cm^3 of distilled water and an aliquot of 0.01 cm^3 of this suspension was fixed on a microscopic slide. At least 1,000 grains for each slide were counted.

The raw palynological spectra, defined according to the nomenclature proposed by Persano Oddo and Ricciardelli d'Albore (1989), were then converted to the volumetric ones to reflect real pollen mass instead of grain counts (da Silveira, 1991). For this purpose, a database of average pollen grain's volumes was produced, based on the grain's dimensions reported by the Ponet database (AGES, 2016) and according to the procedure proposed in Conti, Medrzycki, Palmieri, Piana, and Mariotti (2018). In some cases the beekeepers provided one pooled sample instead of separate samples corresponding to the three hives. In order to uniform the dataset, per each sampling date the average palynological spectrum referred to the apiary was elaborated, and the successive analyses were conducted on the so obtained dataset. Moreover the palynological calendar, i.e. the chronological sequence of average palynological spectra, was built (annex 4).

Nitrogen analysis

For the assessment of nitrogen content (indicator of proteins, Roulston et al., 2000), the Kjeldahl method (Bradstreet, 1954) was applied. A minimum quantity of 300 mg of pollen dust per sample was digested (400°C – four hours) in strong sulfuric acid in presence of sodium sulfate and copper sulfate as catalysts. Then, after alkalinization with 45% NaOH solution, the digest was steam-distilled and condensed in 4% boric acid solution. A direct titration with N/20 sulfuric acid was performed and the nitrogen percentage (%N) was calculated. For the previously mentioned reason (see 'Palynological analysis' in Materials and methods), per each sampling date the average %N referred to the apiary was calculated (annex 4) and the successive analyses were conducted on the so obtained dataset.

Statistical analysis

The multivariate statistical analyses were carried out on a reduced dataset, obtained by ignoring the palynological types less abundant than 10% in three samples. In fact they may add noise and

provide little additional information beyond more common types.
Principal component analysis (PCA) was used to describe and visualize the relationships among pollen samples on the basis of their palynological composition. PCA was carried out applying the vegan R package (Oksanen et al., 2017). %N was correlated with the principal components using the function envfit, in the vegan R package, with 999 permutations.
In order to identify the palynological types which are correlated with the nitrogen percentage in the pollen samples, the analysis of Multiple Regression was performed using Statistica software (StatSoft Italia srl., 2005).
The results of these analyses allowed to define %N homogenous groups of pollen samples. The %N of the so obtained groups were compared by the Kruskal-Wallis test, followed by the Conover post-hoc tests (p=0.05) for the separation of medians, using PMCMR R package (Pohlert, 2016).

Results and discussion

The dataset obtained with the monitoring campaign contained 70 samples (annex 4). Since the beekeepers often couldn't follow exactly the sampling protocol, 44% of the programmed samples are missing. This situation occurs commonly when the monitoring is based on a collaboration of citizen scientists.

Seasonal occurrence of main pollen types and identification of 'foraging seasons'
The reduced dataset (see 'Statistical analysis' in Materials and methods) contains 17 (out of the initial 138) palynological types. They constitute 80% of the total pollen mass sampled. They represent at least the 23% of the mass in all samples and at least the 69% of the mass in the 75% of samples.
In the PCA, the first two principal components explained the 52.22% of variance (i.e. 31.59% and 20.63% respectively for PC1 and PC2) of the entire palynological dataset. The PCA divided all the samples in three main groups characterized by four palynological types: 1) *Quercus ilex* gr., 2) *Castanea* and *Rubus* f., and 3) *Hedera* (figure 2). According to the palynological calendar (annex 4), these types indicate different periods: *Quercus ilex* gr. indicates late spring (mainly D3 and D4, see annex 3), *Castanea* and *Rubus* f. correspond to taxa flowering in early summer (mainly D4, D5 and D6), *Hedera* indicates late summer (D8 and D9).
While 'time' is the factor determining significantly the palynological composition of pollen samples, the factor 'site' seems to have minor influence, considering that all the four mentioned types are present in the pollen spectra of almost all the sites. This is in line with the projection of samples referred to different sites along the vectors corresponding to the four palynological types evidenced by PCA. This result is probably due to the common presence of the relative plants in the study

areas.

The experimental apiaries were located at different altitudes and in different micro-climatic conditions. For this reason, the blooming periods of the same species might not overlap in different sites. Our results allowed us to define five foraging seasons irrespectively to date, but based on the seasonal pattern of the abundance of the palynological types evidenced by PCA. Thus the samples, where possible, were assigned to one of the three main foraging seasons defined as follows:

- QU, when the abundance of *Quercus ilex* gr. in the sample was at least 20% and exceeded those of *Rubus* f., *Castanea* and *Hedera*
- RC, when the abundance of *Rubus* f. or *Castanea* was at least 20% and exceeded those of *Quercus ilex* gr. and *Hedera*
- HE, when the abundance of *Hedera* was at least 20% and exceeded those of *Rubus* f., *Castanea* and *Quercus ilex* gr.

Subsequently, other two periods were automatically individuated for the attribution of the remaining samples:

- I1, the foraging season preceding QU
- I2, the foraging season between RC and HE.

The five so obtained foraging seasons (in chronological sequence) include 12 (I1), 14 (QU), 24 (RC), 10 (I2) and 10 (HE) samples. The average palynological spectrum was elaborated per each foraging season (figure 3). As expected, the QU, RC and HE spectra appeared dominated by a few palynological types, differently from the I1 and I2 spectra.

Relationship between the foraging season and the nitrogen content

The high value of the coefficient of determination of Multiple Regression (R^2=0.83; Adjusted R^2=0.77), indicates that the 17 selected palynological types (see 'Statistical analysis' in Materials and methods) accurately predicted %N. Six of them were significantly correlated with the %N (table 1). The positive values of the partial regression coefficients (β) of *Hedera*, *Rubus* f. and *Castanea* are in line with the high specific nitrogen percentage of these pollen types compared to the other main types of the study (Conti et al., 2016). In the same framework pollen of *Plantago*, *Erica arborea* gr. and *Quercus ilex* gr., whose frequencies are negatively correlated with %N (table 1), are known for their barely or medium protein content (Conti et al., 2016).

Considering the specific %N of the main palynological types (Conti et al., 2016, Roulston et al., 2000), it is evident that the foraging seasons RC (dominated by *Rubus* f. and *Castanea* pollen) and HE (*Hedera*) are characterized by abundance of highly proteic pollen, differently from I1, QU (*Quercus ilex* gr.) and I2 (figure 3). Moreover, the envfit analysis showed that %N was significantly correlated with PCA (R^2=0.534, p=0.001) and particularly associated with PC2, being related positively to *Hedera*, *Castanea* and *Rubus* f. and negatively to *Quercus ilex* gr. Thus the foraging

seasons HE and RC are expected to be characterized by higher %N in pollen samples than the others.

The Kruskal-Wallis test (chi-squared=34.902, df=4, p=0.000) confirmed statistically the differences, among foraging seasons, in the median %N in pollen samples. The Conover post-hoc test evidenced that RC and HE were characterized by significantly higher values of %N than the remaining ones (figure 4).

This study is the first example of monitoring research where the relationships between the palynological spectra and the nitrogen percentage in pollen samples were explored. This approach allows to apply the palynological analysis of pollen samples as a tool for their separation in homogenous groups in terms of %N.

Conclusions

This biennial study consisted in a local monitoring of the pollen loads foraged by bees in the eastern province of Genova between early April and mid-September in 2014 and 2015. Notwithstanding the preliminary character of the investigation, interesting results and indications were obtained.

Even though the vegetation contexts of the experimental apiaries were different, in some periods similarities between different sites were evidenced from a palynological point of view. These similarities were driven by four palynological types (corresponding to taxa well-known by beekeepers) which allowed us to define five foraging seasons. Since it was discovered that %N in pollen samples varied significantly between the foraging seasons, we proposed a tool useful to distinguish periods of barely and highly proteic pollen, based on palynological traits of the samples.

The results of the study may be useful for the beekeepers to improve the apiary management and to enhance the yield. Thus it would be recommended to carry out similar monitoring studies, focused on the investigation of the relationships between palynological spectra and nutritional variables of pollen (e.g. %N, essential amino-acids). It needs to be stressed that for more reliable conclusions, similar researches should be set on at least a three-year period.

Aknowledgements

The authors would like to thank the beekeeper association Nuova Assoapi Ligure and all the beekeepers who volunteered to participate in this project. A special thank is due to Dr Monica Vercelli (University of Turin, Department of Agricultural, Forest and Food Sciences, Italy), Dr Marino Quaranta (Council for Agricultural Research and Economics, Agrobiology and Pedology

Research Centre, Florence, Italy), and Dr Bruna Ilde Menozzi for valuable comments and suggestions.

References

AGES. (2016). Ponet database. Retrieved from http://ponetweb.ages.at/pls/pollen/pollen_suche

Andrada, A. C., & Telleria, M. C. (2005). Pollen collected by honey bees (*Apis mellifera* L.) from south of Caldén district (Argentina): Botanical origin and protein content. *Grana, 44*, 1–8. doi:10.1080/00173130510010459

Bhusari, N. V., Mate, D. M., & Made, K. H. (2005). Pollen of *Apis* honey from Maharashtra. *Grana, 44*, 216–224. doi:10.1080/00173130510010585

Bilisik, A., Cakmak, I., Bicakci, A., & Malyer, H. (2008). Seasonal variation of collected pollen loads of honeybees (*Apis mellifera* L. *anatoliaca*). *Grana, 47*(1), 70–77. doi:10.1080/00173130801923976

Bradstreet, R. B. (1954). Kjeldahl Method for Organic Nitrogen. *Analytical Chemistry*, *26*(1): 185–187. doi:10.1021/ac60085a028

Brodschneider, R., Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie*, *41*, 278–294. doi:10.1051/apido/2010012

C.S.I. Pollen manual. (2015). C.S.I. Pollen: How to investigate pollen diversity available for honey bees. Retrieved from http://www.irishbeekeeping.ie/index.php/csi-pollen-project

Conti, I., Medrzycki, P., Argenti, C., Meloni, M., Vecchione, V., Boi, M., & Mariotti, M. G. (2016). Sugar and protein content in different monofloral pollens - building a database. *Bulletin of Insectology*, *69*, 318–320.

Conti, I., Medrzycki, P., Palmieri, N., Piana, M. L., & Mariotti, M. G. (2018). V*olumentric expression of palynological spectra for nutritional studies*. Manuscript submitted for publication.

Da Silveira, F. (1991). Influence of pollen grain volume on the estimation of the relative importance of its source to bees. *Apidologie*, *22*, 495–502.

de Sa-Otero, M. P., Armesto-Baztan, S., & Diaz-Losada, E. (2009). Analysis of protein content in pollen loads produced in north-west Spain. *Grana*, *48*, 290–296. doi:10.1080/00173130903149140

Di Pasquale, G., Salignon, M., Le Conte, Y., Belyunces, L. P., Decourtye, A., Kretzschmar, A., … Alaux, C. (2013). Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? *PLOS ONE*, *8*(8). doi:10.1371/journal.pone.0072016

Diaz-Losada, E., Ricciardelli d'Albore, G., & Saa-Otero, M. P. (1998). The possible use of honeybee pollen loads in characterising vegetation. *Grana*, *37*, 155–163. doi:10.1080/00173139809362660

Dimou, M., & Thrasyvoulou, A. (2007). Seasonal variation in vegetation and pollen collected by honeybees in Thessaloniki (Greece). *Grana*, *46*, 292–299. doi:10.1080/00173130701760718

Forcone, A., Aloisi, P. V., Ruppel, S., & Munoz, M. (2011). Botanical composition and protein content of pollen collected by *Apis mellifera* L. in the north-west of Santa Cruz (Argentinean Patagonia). *Grana*, *50*(1), 30–39. doi:10.1080/00173134.2011.552191

Fujiwara, A., & Washitani, I. (2017). Dependence of Asian honeybee on deciduous woody plants for pollen resource during spring to mid-summer in northern Japan. *Entomological Science, 20,* 96–99. doi:10.1111/ens.12228

Hoover, S. E. R., Higo, H. A., & Winston, M. L. (2006). Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. *Journal of Comparative Physiology B*, *176*, 55–63. doi:10.1007/s00360-005-0032-0

Keller, I., Fluri, P., & Imdorf, A. (2005). Pollen nutrition and colony development in honey bees - Part II. *Bee World*, *86*, 27–34.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., … Wagner, H. (2017). Package 'vegan'. https://cran.r-project.org/package=vegan

Persano Oddo, L., & Ricciardelli d'Albore, G. (1989). Nomenclatura melissopalinologica. *Apicoltura, 5*, 63–72.

Pohlert, T. (2016). Package 'PMCMR'. http://CRAN.R-project.org/package=PMCMR

QGIS Geographic Information System (Version 2.14.7) [Computer software]. http://www.qgis.org/

Regione Liguria. (2006). Carta dei Popolamenti Forestali 1:25000. [Map]. Retrieved from http://cartodownloadpubb.regione.liguria.it/webpubb/geoservices/download/web_pubb/index.html?id=360

Regione Liguria. (2000). Carta Uso del Suolo 1:25000. [Map]. Retrieved from http://cartodownloadpubb.regione.liguria.it/webpubb/geoservices/download/web_pubb/index.html?id=39

Rivas-Martinez, S., Saenz, S. R., & Penas, A. (2011). Worldwide bioclimatic classification system. *Global Geobotany*, *1*, 1–634.

Roulston, T. H., Cane, J. H., & Buchmann, S. L. (2000). What governs protein content of pollen: pollinator preferences, pollen pistil interactions, or phylogeny? *Ecological Monographs*, *70*, 617–643.

Sajwani, A., Farooq, S. A., & Bryant, V. M. (2014). Studies of bee foraging plants and analysis of pollen pellets from hives in Oman. *Palynology, 38,* 207–223. doi:10.1080/01916122.2013.871652

Schmidt, J. O., Thoenes, S. C., & Levin, M. D. (1987). Survival of honey bees, Apis mellifera (Hymenoptera: Apidae), fed various pollen sources. Annals of the Entomological Society of America,. *80*(2), 176–183.Somerville, D. C. (2001). Nutritional value of bee collected pollens. A report for the rural industries research and development corporation. *Rural Industries Research and Development Corporation,* 01/047

Statistica (Version 7.1.) [Computer software]. http://statistica.io/

Steffan-Dewenter, I., & Kuhn, A. (2003). Honeybee foraging in differentially structured landscapes. *Proceedings of the Royal Society B, 270*, 569–575. doi:10.1098/rspb.2002.2292

Vergara Lopez, J. M., Gil Vives, L., & Boi, M. (2013). Estimation of the polleniferous potential of a Mediterranean landscape through the analysis of pollen harvested by Apis mellifera L. *Grana*, *52*, 147–159. doi:10.1080/00173134.2012.758775

von Der Ohe, W., Oddo, L., Piana, M., Morlot, M., & Martin, P. (2004). Harmonized methods of melissopalynology. *Apidologie*, *35*(Suppl. 1), S18–S25. doi:10.1051/apido:2004050

Webby, R. (2004). Floral origin and seasonal variation of bee collected pollens from individual colonies in New Zealand. *Journal of Apicultural Research*, *43*, 83–92. doi:10.1080/00218839.2004.11101116

Captions

Table 1. Result of the Multiple Regression analysis (independent variables=palynological types; dependent variable=nitrogen percentage; method: all effects) of the dataset (annex 4, gray rows). The palynological types are listed in decreasing order of |β|.
* significant correlation ($p<0.05$)

Figure 1. Geographical location of the study area (see annex 1 for GPS coordinates).

Figure 2. Projection of the palynological types and %N (vectors), as well as the samples (points) on the Cartesian system of the first two principal components, resulting from the PCA applied to the palynological spectra of the dataset (annex 4, gray columns). Different symbols indicate different experimental apiaries (abbreviations in annex 1).

Figure 3. Average palynological spectra of the samples according to the foraging season. “Others” corresponds to palynological types with negligible contributes (average seasonal abundances <3%). Specific %N of anther pollen (from Conti et al., 2016 and Roulston et al., 2000) are indicated with colours.
* in Conti et al., 2016, *Cistus salvifolius* and *Helianthemum* are reported as *Cistus monspeliensis* gr. and *Cistus incanus* gr. respectively. Being unavailable the %N of *Asparagus acutifolius*, the value of *Asparagus officinalis* was used.

Figure 4. %N in pollen samples depending on the foraging season. Different letters correspond to significantly different %N ($p<0.05$ according to Conover post-hoc test).