Validated models for bees exposed to stressors I

Deliverable D9.5

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PoshBee

Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

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Preface

MALDI-BioTyping is a technique used in clinical microbiology for the bacteria identification. From this method, and from past experiences on MALDI Profiling on insect haemolymph a novel analytical method of analysis defined as MALDI BeeTyping was inspired and developed by BioPark since 2013. MALDI BeeTyping will be applied on bee's haemolymph and used for methodically improving the monitoring of bee health based on haemolymph analyses. This procedure was established based on previous competencies acquired on the fruit fly (*Drosophila melanogaster*) haemolymph (Uttenweiler *et al*., 1998) by Dr Philippe Bulet (WP9 Leader, CNRS Partner) in his former laboratory (IBMC, Strasbourg, France) and by the BioPark Team (Drs Karim Arafah and Sébastien Voisin) on the project HematoBeeTest® (HBT®, FEAGA 2013-2016). It is based on the comparison of the molecular mass fingerprints (MFPs) of peptides and proteins (<18 kDa) circulating in the bee haemolymph. The data generated by MALDI BeeTyping, within the frame of the HBT® project, are published in the journal of Proteomics under the reference DOI: 10.1002/pmic.201900268.

Objective

This deliverable aims to provide a model of use of MALDI BeeTyping in pesticide research applied to the *Apis mellifera* biological model. It covers the three scales of experiments:

- **Field experiments**, where bees are geographically localised in different areas and countries, with numerous factors influencing their behaviour.
- **Semi-field experiments,** in enclosures, where bees are receiving field representative doses of pesticides, in small hives containing bees of different ages.
- **Laboratory conditions** where bees are submitted experimentally to pesticides and/or other stressors.

This model of bee haemolymph analysis will be applied to different experiments with pesticides and pesticides associated to other stressors.

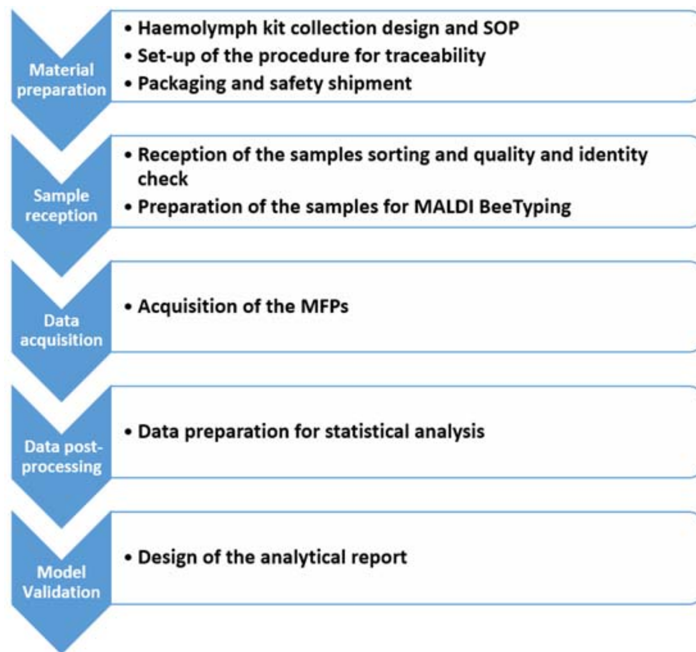
In this document, we present the general model and its workflow, and key points already observed during the elaboration of the model that required adjustments:

- Design of a specific haemolymph collecting kit and validation of the SOP for haemolymph collection (deliverable D9.1);
- Set-up of the procedure for traceability (barcode stickers, delivery form, sample database);
- Preparation of the packaging (coated and non-coated barcoded tubes, ice packs, collecting kits, delivery forms) and safety shipment requirements;
- Reception of the samples, sorting, quality and identity checks;
- Preparation of the samples for MALDI BeeTyping analysis according to SOP D9.2;
- Acquisition of the molecular mass fingerprints (MFPs) using an automatic mode and integration in a database merging the MFPs and the sample barcode;
- Data preparation (*e.g.* sample classification, spectra and peak settings, preparation of the MFP spectra) for statistical analysis (*e.g.* PCA analysis, automatic classification and machine learning);
- Design of the analytical report.

Summary

The haemolymph organ is the circulating body fluid in invertebrates, analogous to the human blood. The aim of the MALDI BeeTyping process (scheme below) is to analyse the haemolymph for bee health monitoring, in the same way that human blood analysis is used for human health monitoring.

Haemolymph samples were collected according to the SOP delivered within D1.1 Protocols for methods of field sampling and adapted for semi-field and laboratory samplings through the specific kit delivered to the experimenters. Each individual haemolymph sample collected from bees coming from field, semi-field and laboratory conditions has been analysed using MALDI Beetyping to record its MFPs. This "blood test" based on MALDI profiling will serve to improve the monitoring of bee health. The present deliverable details a validated model for the application of the MALDI BioTyping method on *Apis mellifera* haemolymph aiming to evaluate the impact of agrochemicals on the health status of honey bees. This model includes the different steps listed above to acquire individual MFPs from (i) haemolymph collection, (ii) sampling delivery and reception, (iii) sample preparation for analysis, (iv) data acquisition, processing and reporting. This model was validated on the field and semi-field samples.



This validated model for bees exposed to stressors I concerns Apis mellifera*. Two more models will be built later according to the project timeline, one for* Bombus terrestris *(validated models for bees exposed to stressors II), and one for* Osmia bicornis *as validated models for bees exposed to stressors III.*

1. Preparation of the material for the haemolymph collection and delivery

The traceability is a prerequisite for sample analysis, data management and their merging in an integrative data base, one final objective of the project. To ensure the identity of each individual sample within the circuit of project partners, we used the barcoding label for each individual bee sample (same bar-code for haemolymph and body, but with a different colour, red for haemolymph and black for the body). For each partner involved in the sampling, we provide on request kits for haemolymph collection and an appropriate number of collecting pre-treated tubes (coating to prevent proteolysis and melanisation). As a precaution, we have provided an additional set of tubes (approx. 5%). Each order includes a delivery form with the listed material present in the package and a detailed Standard Operating Procedure (SOP) of haemolymph collection for each category of experiments (field, semi-field and laboratory) (Deliverable D9.1 of WP9 sent on 16/11/2018). The sending of the coated tubes (for haemolymph) and classic tubes (for body) was in cold conditions (ice packs) and we informed the partners before the shipment.

2. Reception of the samples, sorting and preparation

Haemolymph samples were obtained from the different partners, according to the D1.1 Protocol provided to them by the BioPark partner for the field sampling. A training and a demonstration, regarding haemolymph sampling accordingly to the SOP, were delivered to the different partners engaged in the bee sampling in Bologna Workshop of WP1 (February 2019). A video and a notice were made available to help the operators (available on PoshBee website, WP01 Workshop Bologna/

Workshop presentations/ Haemolymph collection WP9). Conditions for keeping a safety storage and sending of the haemolymph samples have been recommended: freezer at -20°C and dry ice for sample keeping and sending, respectively.

2.1. Sample sending

This issue is of importance as this will condition in a large part the quality of MFP data. The different partners sent the parcels in dry ice, allowing to preserve the integrity of the haemolymph samples. A good communication is a prerequisite to secure the samples (exact dates of sending, safety form and sampling list and observations).

Comments and suggestions: We encountered an issue regarding the delivery of one pack of samples as the parcel has been blocked by the French customs at arrival to France on the base of the annotation on the parcel. The samples spent several days (8 days) at ambient temperature before being delivered to BioPark. Following this negative "episode", BioPark revised its SOP and provided to the PoshBee partners an additional official document that is sent by email to each partner to secure importation of the bee samples (haemolymph and body) whatever the country considered.

2.2. Reception of the samples, sorting and traceability

At the arrival, the samples are checked to control the integrity of the sending in terms of information forms/sample lists and sample number.
In parallel, when necessary we request additional observations or modification in the established sample list by the experimenters to integrate it in our general Poshbee sample database.

At reception of this supplementary file, the traceability barcode stickers are checked and the tubes (haemolymph and body) sorted in order and classified to facilitate any further operation. At this stage, the samples are checked individually in order to detect any abnormality (*e.g*. colour, viscosity, presence of physical contaminants).

In case of necessity, a feedback including any observation that may request clarification is transmitted to the partner providing the samples to get additional information prior analysis.

All samples are tracked by WP9 team using their barcode. All samples belonging to a same experimental condition (or country for field and semi-field work) are listed and classified in a dedicated Excel datasheet.

Comment & Suggestions: In general, the quality of the haemolymph samples was good. Sometimes, abnormalities were detected in the volume collected, in the viscosity and the haemolymph colour. We mentioned the abnormalities in the Excel file in order to facilitate the further interpretation of data. BioPark may also request if needed clarification on the procedure used by the partner for haemolymph collection. BioPark recommends to pay utter attention to follow the validated SOP. Considering the semi-field and the laboratory samplings, the SOP has been adjusted as mentioned above due to alterations observed within the field samplings.

3. MALDI BeeTyping: Data acquisition

Following the receipt of the package, the establishment of the sample list for traceability, and the keeping of the samples at-20°C, the samples were thawed at the time of MALDI BeeTyping analysis. Sample preparation, as described by the specific SOP designed (D9.2), consisted on the dilution of the haemolymph by a factor of 100. All analyses are done on an AutoFlex III Smartbeam® MALDI-TOF-MS (Bruker GmbH, Germany) with the FlexControl 3.4 and FlexAnalysis 3.4 software, for spectrum

acquisition and data analysis, respectively. Analyses are performed in a linear/positive mode. The linear mode allows to acquire ions in the mass range selected (*m/z* 1,000 to 18,000). The positive mode is recommended for MFPs recordings.

Each individual diluted haemolymph sample is spotted 3 times on a reusable MALDI plate (MTP 384 target plate polished steel BC) and data are acquired at once 3 times for each spot (total 9 technical replicates) in automatic mode. We designed our own calibration kit composed of *Apis mellifera* peptides supplemented with ProtMix® from Bruker (this calibration set is defined as Apiscal) and checked to evaluate the performance and optimum operative condition of the mass spectrometer (spectral resolution and reproducibility, analytical sensitivity, mass accuracy) using specific ions from the Apiscal mixture prior every acquisition of MFPs. The MALDI MS equipment is calibrated with fresh Apiscal according to the SOP (D9.2). The sampling size allows us to use a single MALDI plate to analyse several conditions at once.

4. MALDI BeeTyping: Data preparation and post-processing for statistical analysis

The raw data obtained by MALDI BeeTyping are classified according to the different experimental conditions and proceeded using appropriate settings (*e.g.* spectra and peak settings) using the ClinProtools Software 2.2 from Bruker (Germany). A post-processing step involving spectral normalization of all calculated peak area is also performed prior to statistical analysis. From the nine (9) replicates per sample, an average spectrum is generated and used for statistical analysis. Following this treatment, the average spectra are used to build Principal Component Analysis (PCA). The PCA can generate 3D score plots, variance per PCA component and a peaklist sorted according to the normality of the distribution and the appropriate statistical test of significance to discriminate experimental sample populations (namely experimental classes) using supervised/unsupervised PCA. **In addition**, machine learning-based algorithm are used to build computational model of spectral recognition and classify the samples according to different parameters (e.g. stressor type, intensity of bee exposure to agrochemicals).

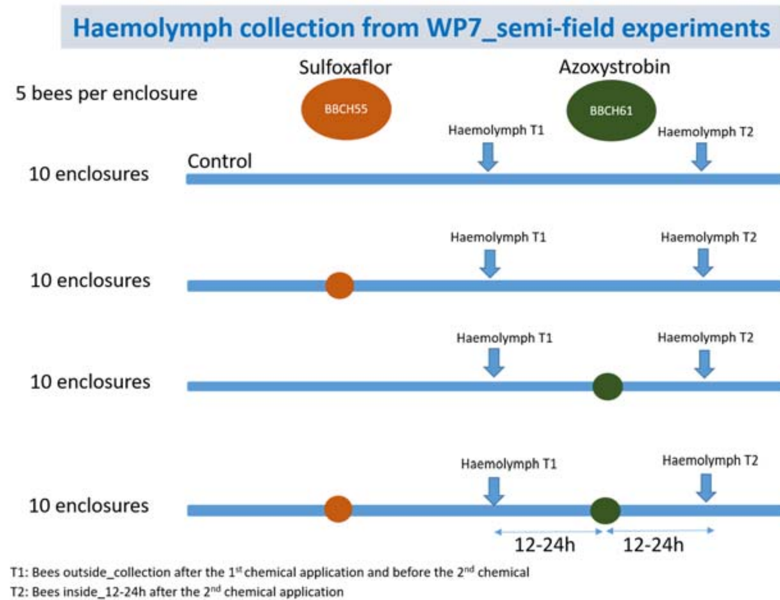
5. Validation of the *Apis mellifera* model on a case study

We validated the proposed model on the haemolymph samples from the semi-field experiment conducted in UK (ATLANTIC pollination as P37, RED BEEHIVE Company as P40) for the following reasons:

- the agrochemicals used in this semi-field experiment (WP7) are identical to those used in the lab experiments (WP3, 5 and 6);
- the sample sets (control, sulfoxaflor or azoxystrobin, alone or successively applied) were complete and satisfactory for all designed statistical analyses (200 samples per time point);
- the semi-field experiments are closer to real life than experimental conditions developed in a laboratory environment.

Briefly, from the semi-field work, WP9 team collected haemolymph at the UK site according to the experimental design reported on the scheme on the right. Haemolymph is collected at two time points (T1 and T2) in order to evaluate the impact of the treatment performed within the enclosures on two sets of bees: one set before the treatment when hives are outside the enclosure (T1) and a second set corresponding to bees collected within the enclosures (T2). The results and conclusions briefly listed in this section will be detailed in dedicated scientific communications (oral and scientific publications).

By analysing the PCA of the *Apis mellifera* haemolymph semi-field samples (T1 and T2) collected in UK (end of July 2019), we observed that:



- Within the T1 samples, the model proposed allows to identify individuals with specific MFPs.
- The MFPs generated on these samples can discriminate the T2 samples from the T1;
- A successive agrochemical treatment with first sulfoxaflor and azoxystrobin in second is easily discriminated from the controls, thanks to the T2 samples. This discrimination was not evidenced when only one agrochemical is applied.

Comments: After observation of the haemolymph during the collection or the sorting of the samples at BioPark, we visually observed the presence of white haemolymph in T1 and T2 (higher number). To avoid any effect of those samples on the PCA of the groups, we removed the white samples to analyse them as a different sample class.

6. Conclusions

In this deliverable (D9.5), a clear scenario for generating molecular mass fingerprints (MFPs) by MALDI profiling has been established. This approach is derived from the well-known MALDI BioTyping (FDA and EMA approved) used in clinical microbiology for microbiological identification. This approach is referred to MALDI BeeTyping and is usable on *Apis mellifera* haemolymph to monitor the impact of stressors through a simple "blood" analysis. The validation of this scenario was performed on a representative experimental case study.

This validated model of MALDI Beetyping is now applicable to laboratory, semi-field and field samples in different stress conditions (biotic and/or abiotic). In addition, it will serve as foundation for developing the *Bombus terrestris* and *Osmia bicornis* models.