Validated models for bees exposed to stressors II

Deliverable D9.6

9th November 2020

Author(s): Dalel ASKRI[1)], Karim ARAFAH[1)], Sébastien VOISIN[1)] & Philippe BULET[2)]

[1)]Plateforme BioPark d'Archamps, Archamps, France
[2)]CR, University Grenoble Alpes, IAB Inserm 1209, CNRS UMR5309, Grenoble, France

PoshBee

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

Prepared under contract from the European Commission

Grant agreement No. 773921
EU Horizon 2020 Research and Innovation action

Project acronym:	**PoshBee**
Project full title:	**Pan-european assessment, monitoring, and mitigation of stressors on the health of bee**
Start of the project:	June 2018
Duration:	60 months
Project coordinator:	Professor Mark Brown Royal Holloway University of London www.poshbee.eu

Deliverable title:	Validated models for bees exposed to stressors II
Deliverable n°:	D9.6
Nature of the deliverable:	Other
Dissemination level:	Public

WP responsible:	WP9
Lead beneficiaries:	CNRS, BIOPARK

Citation: Askri, D., Arafah, K., Voisin, S. & Bulet, P. (2020). *Validated models for bees exposed to stressors II*. Deliverable D9.6 EU Horizon 2020 PoshBee Project, Grant agreement No. 773921.

Due date of deliverable:	Month n° 30
Actual submission date:	Month n° 30

Deliverable status:

Version	Status	Date	Author(s)
2.0	Draft	09/11/2020	Dalel ASKRI[1)], Karim ARAFAH[1)], Sébastien VOISIN[1)] & Philippe BULET[2)] [1)]*Plateforme BioPark d'Archamps, Archamps, France. BIOPARK partner* [2)]*CR, University Grenoble Alpes, IAB Inserm 1209, CNRS UMR5309, Grenoble, France. CNRS Partner*

Table of contents

Preface

MALDI BeeTyping was developed by BioPark, starting in 2013, and is based on using honeybee haemolymph for monitoring bee health (Arafah et al. 2019). This "blood test" was inspired by MALDI-BioTyping, a technique that is used routinely in clinical microbiology for the identification of bacteria. In fact, the idea emerged from previous work by Dr Philippe Bulet (CNRS partner, WP9 leader) on MALDI Profiling on fruit fly (*Drosophila melanogaster*) haemolymph (Uttenweiler-Joseph et al. 1998). He developed this innovative mass spectrometry approach in his former laboratory (IBMC, Strasbourg, France) in the 90's and transferred it to the BioPark Team (Drs Karim Arafah and Sébastien Voisin) through the project HematoBeeTest® (HBT®, FEAGA 2013-2016). In brief, MALDI-BeeTyping is based on the analysis of the molecular mass fingerprints (MFPs) of peptides and proteins (<18 kDa) circulating in the bee haemolymph (Arafah et al. 2019).

Objective

This deliverable aims to provide a model for use of MALDI BeeTyping applied to the *Bombus terrestris* biological model. It covers three categories of experiments:

- **Field experiments**: The haemolymph of bumblebees living in their natural environment are collected in different countries and field areas;
- **Semi-field experiments**: In this case, bumblebees, which are maintained in enclosures, are receiving field representative doses of pesticides;
- **Laboratory conditions**: Bumblebees are submitted experimentally to pesticides and/or other stressors under controlled conditions.

This model of "blood test" on bumblebee haemolymph will be applied to the different samples collected from field to laboratory experiments with pesticides or pathogens and pesticides associated with other stressors.

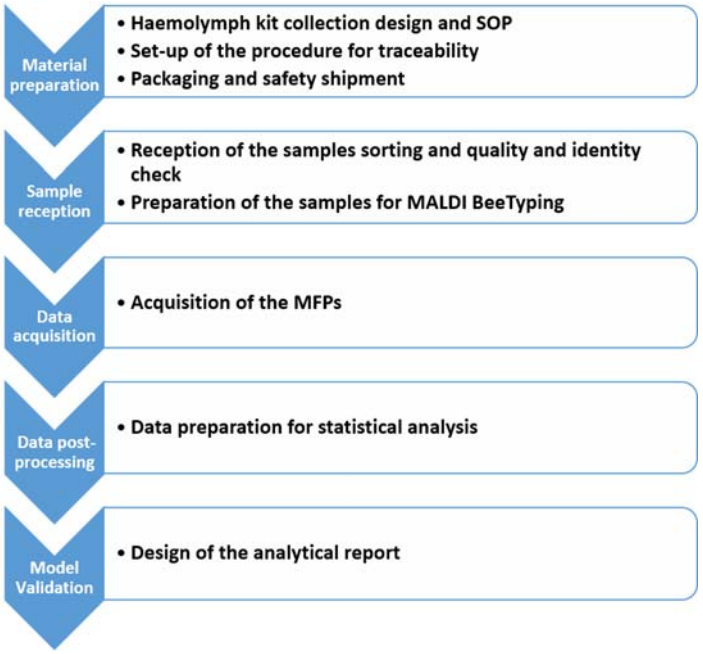
In this document, we report the general model and its workflow:

- Design of a specific bumblebee haemolymph collecting kit and validation of the SOP for bumblebee 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 bumblebee samples for molecular mass fingerprints (MFPs) by MALDI BeeTyping according to the SOP D9.3;
- Acquisition of the MFPs using an automatic mode, and integration in a bumblebee database that merges the MFPs and the sample barcode, including a sub-classification according to the sample origin (field, semi-field and laboratory);
- 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

Briefly, the haemolymph tissue collected from bumblebees is the circulating body fluid in invertebrates, analogous to the human blood. The aim of this "blood test" by MALDI BeeTyping (scheme below) is to analyse bumblebee haemolymph for bee health monitoring, in the same way that we already performed for honeybee haemolymph, and in parallel with tests on human blood that have been proposed by practitioners for human health monitoring.

Bumblebee haemolymph samples were collected according to the SOP delivered within D1.1 Protocol for field sampling, and adapted for semi-field and laboratory sampling through the specific bumblebee kit delivered to the experimenters. The bumblebee kit is derived from the one developed for honeybee haemolymph samples. As a general procedure, each individual bumblebee haemolymph sample collected from the field, semi-field and laboratory conditions has been analysed using MALDI BeeTyping to record MFPs. This "blood test" based on MALDI profiling will serve to improve the monitoring of bumblebee health as we reported already for the honeybee. The present deliverable details a validated model for the application of the MALDI BioTyping method on *Bombus terrestris* haemolymph, aiming to evaluate the impact of agrochemicals on the health status of bumblebees. 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, and (iv) data acquisition, processing and reporting. This model was applied and validated on laboratory, semi-field and field samples.



This validated model for bees exposed to stressors II concerns Bombus terrestris*.* One model was built last year for *Apis mellifera.* The present model may be applied to other bumblebee species without any adjustment.

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

Traceability is a prerequisite for sample analysis, data management, and merging these in an integrative database, one of the project objectives. To ensure the identity of each individual sample collected by project partners, we use a barcoding label for each individual bee sample (the same barcode for haemolymph and body, but with a different colour, red for haemolymph and black for the body). For each partner involved in the field, semi-field and laboratory sampling, we provide on request the bumblebee kits for haemolymph collection. In addition, an adjusted number of collecting pre-coated tubes to prevent proteolysis and melanisation are added to the parcel. As a precaution, we provide an additional set of tubes (approx. 5%). Each parcel includes a proforma form and a detailed Standard Operating Procedure (SOP) to collect bumblebee haemolymph for field, semi-field and laboratory experiments (Deliverable D9.1 of WP9). The coated tubes (for haemolymph) and classic tubes (for body) are delivered in cold conditions (ice packs) and the partners are informed by mail before any shipment.

2. Reception of the samples, sorting, and preparation

The bumblebee haemolymph samples were obtained from the different partners, in accordance to the D1.1 protocol provided to them by the BioPark partner for the field sampling. Training and demonstration, regarding haemolymph sampling according to the SOP, were delivered to the different

partners engaged in bee sampling in the Bologna Workshop of WP1 (Task 1.3.1, MS2). A video and a notice were made available to help support partners (available on the PoshBee members website, WP1 Workshop Bologna/Workshop presentations/Haemolymph collection WP9). Conditions for effective storage and delivery of the bee haemolymph samples are recommended: freezer at -20°C and dry ice for sample storage and delivery, respectively.

2.1. Sample delivery

This issue is important as this will strongly determine the quality of MFP data. The different partners sent the parcels either in dry ice or ice packs, enabling preservation of the integrity of the haemolymph samples. Good communication is a prerequisite to secure the samples (exact dates of sending, safety form, sampling list and observations) and their traceability.

2.2. Reception of the samples, sorting, and traceability

On arrival, the parcels are checked to assess the integrity of the delivery in terms of information forms/sample lists and the precise number of samples.
When necessary, we request clarification and additional information linked to the established sample list provided by the experimenters to integrate them (if any) in our general Poshbee sample database.

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

Where necessary, feedback, including any observation that may require clarification, is delivered to the partner who provided the samples to get any additional information prior to analysis.

Sample information is tracked by the WP9 team using the individual sample barcodes. All samples are classified in a dedicated “Sample 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 recorded the abnormalities in the “Sample Excel datasheet” to facilitate the data interpretation. 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 Excel datasheet”* for traceability, and preservation 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 (D9.3), consisted of a ten-fold dilution of the bumblebee haemolymph. 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 enables the capture of ions in the mass range selected (*m/z* 600 to 18,000). The positive mode is used for MFP recordings.

Each individual diluted haemolymph sample is spotted three times on a reusable MALDI plate (MTP 384 target plate polished steel BC) and data are acquired at once three times for each spot (N = nine technical replicates) in automatic mode. The calibration is performed using a mixture of Pepmix II and

Protmix I from Bruker to evaluate the performance and optimum operative condition of the mass spectrometer (spectral resolution and reproducibility, analytical sensitivity, mass accuracy). The calibration procedure follows the SOP (D9.3). The sample 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 processed 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 areas 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 peak list 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 algorithms are used to build a computational model of spectral recognition and classify the samples according to different parameters (e.g. stressor type, intensity of bee exposure to agrochemicals).

5. Conclusions

In this deliverable (D9.6), a clear scenario for generating 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 as MALDI BeeTyping and is usable on *Bombus terrestris* 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).

6. References

Arafah, Karim et al. 2019. «MALDI–MS profiling to address honey bee health status under bacterial challenge through computational modeling». *PROTEOMICS* 19(23): 1900268.

Uttenweiler-Joseph, Sandrine et al. 1998. «Differential display of peptides induced during the immune response of Drosophila: A matrix-assisted laser desorption ionization time-of-flight mass spectrometry study». *Proceedings of the National Academy of Sciences of the United States of America* 95(19): 11342–11347.