FINAL PROJECT REPORT

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List of Partners

Nr	short	Organisation
1	LIB	Institute for Bee Research Hohen Neuendorf (LIB)
2	ANSES	ANSES - Sophia-Antipolis Laboratory
3	AU	University Aarhus
4	AUA	Agricultural University of Athens
5	CT	ConsulTech GmbH (SME)
6	EHU	University of the Basque Country, UPV/EHU
7	FERA	The secretary of state for environment, food and rural affairs
8	GenoSK	GenoSkan (SME)
9	ICDA	Institute for Beekeeping , Bucharest (SME)
10	IO	The Research Institute of Horticulture
11	LLH	Hessische Ministerium für Umwelt, Energie, Landwirtschaft und Verbraucherschutz - Landesbetrieb Landwirtschaft Hessen, Bieneninstitut Kirchhain-
12	NBA	Norwegian Beekeeping Association
13	SLU	Swedish University of Agricultural Sciences, Uppsala
14	UABD	University of Aberdeen
15	UNINA	University of Napoli
16	UNIUD	University of Udine
17	FER	FERA Science LTD

Table of contents

1 Executive summary

Global apiculture is currently facing a deep crisis, characterized by increasing parasite and pathogen pressure in combination with a rapid loss of biodiversity.

Against this background, SmartBees has **assessed the state of European honeybee biodiversity,** based on a newly-established reference collection of >2.200 samples from all subspecies present in Europe. The DNA- analysis of these samples has permitted the identification of thousands of genetic markers that allow the differentiation of the four evolutionary lineages, of subspecies within each lineage, and partly even of subpopulations within subspecies. The most informative markers were integrated into a genotyping tool allowing breeders and researchers to assess the subspecies affiliation of any colony easily and affordably.

In order to **highlight the value of honeybee biodiversity and protect it**, we carried out a poll on European beekepers' attitudes towards local bees and their information needs, which was answered by almost 5.000 participants. An online toolbox was developed that addresses the issues brought up. To strengthen our knowledge base for the protection of honeybee diversity, we have estimated the minimum required population size required for conservation, and determined the levels of biodiversity in island populations of endangered subspecies. We have also inventoried the different conservation initiatives in place on our continent, and created a network between them.

An important step to **further the use of local bee populations** was the initiation of 23 groups of beebreeders in 15 European counties, working with 10 different subspecies. Intensive training was provided in the form of on-site courses and high-quality technical documents translated into up to 20 languages. Breeders of all subspecies were given access to modern quantitative genetics like the BLUP-based genetic evaluation. Nearly 1.900 breeding values of performance-tested queens were calculated and provided at the new designed website www.beebreed.eu.The network of breeders thus created led to the foundation, in 2018, of the "International Association for Honeybee Breeding".

A major goal of SMARTBEES has been to **identify and characterize genetic variants affecting varroa resistance**, notably the ability of bees to detect and remove infested brood. The results are truly impressive, and we have, across several subspecies, identified and validated a considerable number of genomic loci with highly significant effects upon the resistance trait. Genetic markers from this study have been included into the SmartBees SNP-panel/chip and can now be used to accelerate selection for Varroa resistance in European honeybees.

Our project also **revealed the complex interplay between varroa, honeybees, and viruses**, including the protein repertoire of varroa saliva and its role in immunosuppression of bees and virus transmission. Neonicotinoid insecticides and food deprivation accentuated the negative effects of varroa and viruses on bee health, but this could be mitigated by dietary supplements. The diversity of viral sequences accross the continent was characterized and we demonstrated how viral sequences adapt to either the mite or the bee host.

Overall, the project has led to 16 peer-reviewed publications, with many more still in the pipeline. Results have been disseminated to a wide audience through 48 publications in beekeeper and popular media, as well as through the 6 regional SmartBees-conferences and 6 project newsletters. Consequently, we are convinced that SmartBees will have a **lasting impact on the sustainability of European beekeeping**.

2 Summary description of the project context and the main objectives

The project is divided into 10 work packages (WPs)

WP1, Gene discovery of resistance traits, aimed to phenotypically characterize different European subspecies of *A. mellifera* regarding traits that confer resistance to parasites and viruses, and to elucidate the transcriptomic and genomic basis to these.

Varroa and its most frequently associated virus, deformed wing virus (DWV) have been responsible for millions of honeybee colony losses and the virtual eradication of feral honeybees from large parts of Europe. In recent years, it has become increasingly clear that resistance towards the Varroa mite can be heritable, meaning that genetic variation affecting the trait is present in the population. A major goal of SMARTBEES has been to identify and characterize such genetic variants affecting hygienic behaviour, i.e. the ability of bees to detect and destroy infested brood. With state-of-the-art sequencing methods to facilitate rapid screening of paired pools of individuals containing extreme examples of a trait e.g. resistance and susceptibility, we aimed to identify SNPs, InDels, and CNVs with differentially representation in phenotypic pools. Supported by transcriptional analysis and bioinformatic annotation, genetic markers were to be selected for supporting selection for increased Varroa resistance across multiple subspecies, using molecular tools.

In **WP2**, Tools and strategies for sustainable breeding, we aimed to create methods for the breeding of resistant and diverse bees, based on local stock. One of these tools is the web-based bee breeding service BeeBreed.eu which allows local breeders to benefit from the use of modern quantitative genetics. While existing before SmartBees, it had to be extended to be suitable for many countries, subspecies and the breeding situations in the participating countries. A second tool is the BLUP-based breeding model, the basis of breeding values. A third tool is a simulation software of bee breeding as an extremely valuable tool to develop sustainable breeding strategies.

Another aim of WP2 was to develop a DNA genotyping kit for honeybee breeders and producers. This genotyping kit could be used to improve selection results regarding hygienic traits towards the parasitic mite Varroa destructor (Varroa), heterozygosity at the CSD locus and subspecies specificity. In the course of the project, it was decided to split the DNA genotyping kit into three kits, to give breeders more flexibility in the use of the product.

The aim of **WP3** (Assessing honeybee biodiversity in Europe) was to create a unique and large-scale dataset to describe and characterize European honey bee diversity, using pool sequence data from a comprehensive and representative sampling effort of honey bees across all of Europe and adjacent regions. This collection represents all subspecies occurring in Europe and sufficiently characterizes the genetic diversity of *Apis mellifera* on the continent, at the same time providing valuable reference samples for future work.

By way of including data obtained with morphometric or microsatellite analysis, we planned to make it possible to link these newly generated genomic markers to known and published reference data and to historic subspecies descriptions. Based on the comprehensive genomic dataset, informative SNP makers were to be selected to allow assignment of honey bee samples to their subspecies of origin. The informative SNPs should be validated using advanced biostatistical techniques. Based on

these markers, we aimed to provide the means of rapid and cost-effective identification of honey bee samples.

WP4 - Promoting honeybee diversity in Europe: To raise awareness for honey bee diversity among beekeepers in Europe, their thoughts about honey bee subspecies and conservation was to be assessed on the European scale for the first time. The data generated were expected to provide the basis for future activities to promote conservation efforts.The presence and key management data of existing conservatories for indigenous populations of honey bees across Europe were to be reviewed and summarised. A network of conservation areas on the SmartBees website was planned, and guidelines for the size and definition were to be developed and published. To avoid negative effects of inbreeding and genetic drift, the minimum effective population size of honey bees in conservation areas had to be estimated via a comparison of several populations of *A. m. mellifera* across Europe. We also aimed to monitor several nature reserves and national parks in three European countries for the presence of wild colonies.

The main purpose of **WP5** (Development of new extension methods for sustainable apicultural production and maintained diversity) was to suggest new extension tools and communicative strategies for sustainable management of resilient bee populations on the European level. We aimed to achieve this by identifying today's information and learning needs across Europe, describe regional Knowledge and Innovation Systems within the beekeeping sector, and finally by developing a web-based tool-box for advisory services in apiculture and development extension. Through these activities, SmartBees should enable regional actors (within the knowledge and innovation system) to combine methods and tools into adapted regional strategies for knowledge development, involving beekeepers and breeders. WP5 was structured around three main tasks which create a strong foundation for an extension tool-box. The first task was to survey bee-keepers and bee-breeders in specific regions of Europe to identify similarities as well as differences in their perception of knowledge gaps, methods used and future information needs. The second task was to conceptually describe how different regions in Europe have chosen to organize the bee-keeping sector's knowledge and innovation system, which we call a B-KIS. As part of this work we aimed to adapt and test a methodology developed in other EU-projects (eg., PRO-AKIS). Finally, the third task was to analyze how the beekeepers' and advisors' needs could be met through a new web-based extension tool-box, also containing information to enable national and regional actors across Europe to adapt universal knowledge within extension and communication science to existing structures for knowledge development and dissemination. In total the ambition of WP5 was to support all actors involved in knowledge development, dissemination and transfer to improve their skills in enabling learning and to implement new knowledge, developed both within the SmartBees-project as well as from other sources.

The focal point of **WP6** (Field testing and selection on local bee populations) was to initiate and support breeding activities for the preservation of local honey bee populations across Europe by genetic improvement of commercially relevant traits with special attention on *Varroa* resistance. With the integration of elements of local adaptation and selection, the SmartBees breeding initiatives should further "Preservation via utilization" and encourage beekeepers across Europe to utilize and preserve

their local stock. Moreover, we aimed to further the integration of beekeepers into the genetic improvement of honey bee stocks through a "bottom up" extension and communication structure where beekeepers' awareness is of prime importance. Thus, the demonstration of the usefulness of modern cocepts such as the www.beebreed.eu database for estimation of breeding values, a sustainable and systematic long-term breeding concept should be established.

The work package's ultimate goal was to expand breeding programs for genetic improvement and preservation of local honey bee populations specially in those European regions where selective breeding practices were absent or neglected. To reach this goal, the SMARTBEES extension programs (joined forces with WP7) combined practical aspects (on-field training, testing protocols and kits etc.) and sophisticated interactive tools, such as smartphone apps and platforms, through which continuous communication and transfer of knowledge among all stockholders in the breeding structure was ensured.

An additional activity of WP6 was the determination of locally adapted *Varroa* infestation threshold values that are going to be incorporated in regular beekeeping practice in order to establish an integrated pest management model with restricted use of therapeutics. The threshold values strongly depend on population's specific features (colony development, resistance mechanisms etc.), environment and applied beekeeping practices and that is why it should be tested under different conditions and regions across Europe. Finally, the detected *Varroa* infestation threshold values should be approved and disseminated among the beekeepers and used as indicators for applying *Varroa* control measures.

The overall objective **in WP7 (Dissemination)** was to increase the awareness of the value of locally adapted bees and to combine and disseminate the results from the project to various user groups and stakeholders. The dissemination should be adapted and targeted to achieve cost- efficient transfer of knowledge and implementation of new strategies, tools and technologies as discovered in WP 5. Furthermore, WP7 participated in the effort to launch breeding programmes in WP 6 by establishing networks of breeders to run performance tests. It also aimed to promote participation in other project activities.

WP8 (Elucidating and enhancing honeybee resistance mechanisms to parasitic diseases) centres on understanding the interplay between the honey bee's immune response and the Varroa or DWV (deformed wing virus) that would give insight into potential susceptible and resistance mechanisms that may be exploited in some breeding programme. The immune genes and pathways involved in DWV-resistance were to be investigated by extensive comparative transcriptomic analysis of experimentally DWV-infected honey bees. The key genes involved in the innate immunity and pathways involved in DWV-resistance were to be revealed and then provided to WP2 for inclusion in the genetic selection for bees with increased resistance. Another aim was to test the hypothesis that the saliva of the varroa contains bioactive factors able to subvert the bee's immune system. To this end, the protein repertoire (proteome) of varroa saliva (132 proteins) was to be determined. Finally, the WP aimed at determining how honeybee nutrition interacts with parasites and pathogens.

The study of the DWV-mutants is at the core of **WP9 (Determining present and future pathogen threats).** DWV is a honeybee virus which generally persists in honeybees as a covert, non-pathogenic state. However, in the presence of the *V. destructor* mite, DWV become virulent notably by increasing in viral load. We hypothesized that changes in genome sequence are caused by the bee-adapted DWV-variants also becoming mite-adapted. Genomic and phenotypic changes of DWV were studied *in vitro* and *in vivo* by alternative change of host between honeybee and *V. destructor*. Moreover, the population structure and virulence of DWV viral strains collected from different European countries was monitored. Altogether, WP9 better characterized the changes in the genome of DWV that impact its virulence.

WP10 (Project management) finally encompassed the management of the project, facilitation of interactions between partners and the fulfilment of obligations towards the European Commission. The objective of this work package was to ensure that all working steps and the planned objectives could be achieved, through an effective management structure with clear responsibilities.

Furthermore, the project manager ensured a productive collaboration between the partners, monitored the project progress and reporting, filed amendments and supported partners in questions concerning legal changes, financial and scientific reporting.

3 Description of the main S & T results/foregrounds

WP1 Gene discovery of resistance traits

In order to identify honey bees with enhanced Varroa resistance/tolerance we have phenotyped individual workers for their ability to uncap *Varroa*-parasitized brood using an established bioassay. For each family tested, we have marked approximately 2,000 worker bees after hatching, and transferred them onto an infrared illuminated observation comb where their behavior against artificially infested brood cells was video-observed. We have carried out these observations on 4 different subspecies of *A. mellifera* (mellifera, carnica, caucasica, and macedonica) and subspecies hybrids, as well as on *A. cerana*, which is known to be resistant to *Varroa.* For each queen/family analyzed, we have sampled hygienic bees (=bees showing removal of infested brood), and non-hygienic (control) sister individuals. Altogether more than 120.000 bees have been tagged and individually evaluated for hygienic behavior. For all families producing a sufficient number of hygienic bees, we extracted DNA and performed largescale sequencing of the corresponding pools. We have thus sequenced more than 75 pools with a maximum of 100 workers per pool. Each pool was sequenced to a coverage of approximately 1x/bee except for queens which were sequenced to approximately 30X coverage to robustly identify genetic variants, thereby allowing for separation of maternal and paternal effects in the subsequent SNP-analysis. Across all families, more than 2 million SNPs were evaluated for significant association with a particular hygienic group. An example of the data is shown in Figure 1.1, where Fet values (Fisher exact test) are plotted against the genomic position (x-axis). We identified 93 peaks across all families, altogether harboring 2017 SNPs.

Figure 1.1 shows an example of selected peaks.

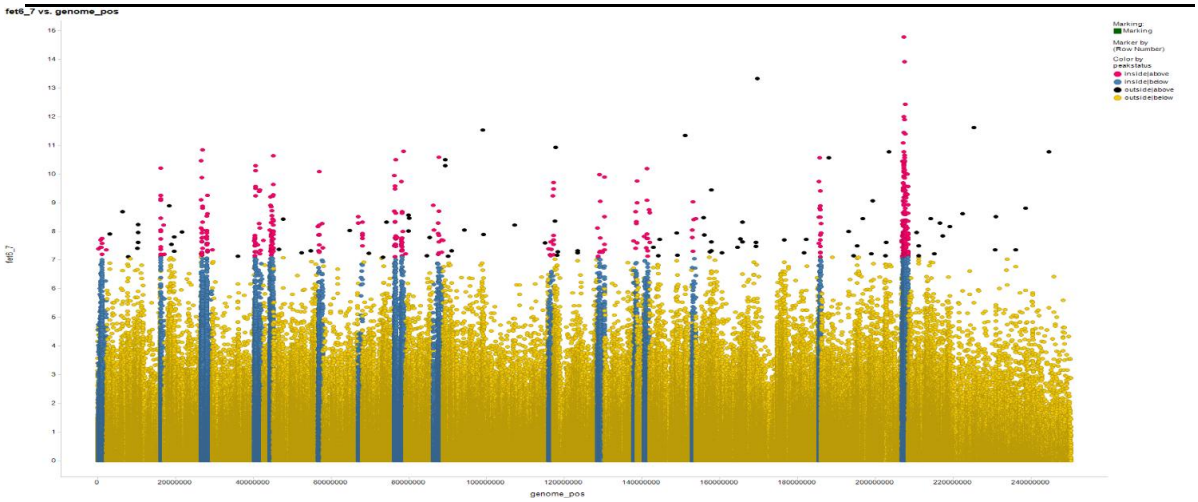

Figure 1.1: The selected peak intervals. The red color shows the SNPs above the significance threshold.

Interestingly, several families had overlapping peaks and 440 SNPS were common between four families. We subsequently annotated the SNPs for changes in protein coding potential and potential amino acids changes. These SNPs are of particular interest as being potentially causative of differences in hygienic behavior. The filtered and prioritized list of SNPs was used in Workpackage 2 to

establish a SNP genotyping panel, supporting efforts to enhance disease resistance through marker-assisted breeding.

Moreover, to support the annotation and interpretation of the results from the pool-seq association study, gene expression profiling using RNA-seq was performed. Across multiple families, more than 1362 genes and 199 non-coding RNAs were found to be significantly differentially expressed in a least one family while a smaller set of genes (106) were significantly expressed in 2 or more families.

Apart from identifying SNPs, Pool sequencing also lead to the identification of different structural variants such as insertions and deletion (INDELs) and copy number variants (CNVs). Although less useful as genetic markers, these types of genetic variants are able to affect gene expression and function and are as such candidates for being causative relative to the hygienic trait. Altogether, we identified 629 INDELs harboured within the "QTL-peak regions" defined by PoolSeq. The list of SNPs in addition to the list of INDELs and CNVs was used in WP2 efforts to establish a SNP genotyping panel.

In order to validate results from the pool-seq analysis, we performed a separate study in which 3450 individual bees (phenotyped for the behavioral trait) were genotyped using the SmartBees-chip developed in WP2. This study included bees from families who had undergone pool-seq analysis as well as new families. We used Transmission Disequilibrium Testing (TDT) which measures association of genetic markers in nuclear families by transmission from parent to offspring. If an allele increases the risk of having a disease then the allele is expected to be transmitted from parent to offspring more often in populations with the trait. The analysis was made both within queen family as well as for all the families combined. In our preliminary analysis of these data, we found that at least 37 of the genomic regions identified in the pool-seq analysis are validated by one or more significant SNPs in the marker-based association study. This is an extraordinary number compared to similar studies. When performing a combined analysis of bees from all phenotyped families (also families without pool-seq data) and applying the highly conservative Bonferroni correction, we identified 10 regions which are associated with hygienic behavior. Further analysis of the data will be performed, and the inclusion of queen genotype data will lead to an even more accurate finemapping and haplotype definition within significant loci.

The validation results thus confirm our findings from the pool-seq analysis, presenting strong evidence of association with hygienic traits in multiple genomic positions. Furthermore, they might suggest a mainly oligo-genic basis for this particular behavioral trait, making it highly amenable to marker-based selection.

In our attempts to identify potential causal genetic variants, we have performed functional clustering, annotation and gene-enrichment tests of the genes harboring the most highly significant SNPs. Out of 133 SNPs within the validated regions, 78 SNPs could be annotated to gene IDs of which 9 were non-coding RNAs (Lnc RNAs) and the rest protein coding genes. Our gene ontology reveals, among our candidate genes, several genes involved in neural development/function and olfactory pathways. Several studies have suggested that that hygienic behavior is mediated by olfactory cues. More than 150 olfactory genes were located in the candidate peak intervals from the pool-seq analysis. These genes are now being studied in more detail and the SNPs annotation within them is ongoing.

To sum up, the validated SNPs, InDels and CNVs in genomic regions significantly associated with hygienic behavior offer insight into the genetic basis of hygienic behavior and will definitely improve our knowledge of the molecular basis of honeybee hygienic behavior and varroa resistance.

Another objective of work package one was to identify and compare resistance factors unrelated to hygienic behavior. One such potential resistance factor is the inhibition of varroa reproduction by characteristics of the honeybee brood. We compared different European subspecies of *A. mellifera* for this trait (in the absence of worker bees). More than 110 pieces of brood from four subspecies were used for these tests. No difference in mite reproductive success was found between the subspecies. Another trait potentially conferring resistance to varroa is grooming behavior. Through a cooperation with partners in Nepal, we compared different types of grooming of European (*Apis mellifera*) and Asian (*A. cerana*) bees, in order to see whether grooming could explain the known difference in mite resistance between the two species of bees. We also included mites of the genus Tropilaelaps in this study, which are currently absent from Europe but which will possibly become a problem to European beekeeping in the future. These studies confirmed the fact that Asian bees more strongly express grooming, but they also showed that resistance behavior to Tropilaelaps-mites does exist in European bees.

Taken together, work package one has significantly advanced our understanding of honeybee resistance to varroa mites.

WP2 Tools and strategies for sustainable breeding

The service BeeBreed.eu provided by LIB is a comprehensive system which covers the collection and refinement of performance data through a web interface, the calculation of breeding values with a BLUP-based model, and the distribution of breeding values and supporting documents as well as tools to aid the selection process through the web interface. The first aim of Task 2.1 was the adaptation of the existing service BeeBreed.eu to more countries, subspecies, and bee populations and the extension of the performance testing protocol. The main accomplishments are (i) the multi-language support of BeeBreed.eu, (ii) the implementation of an international bee nomenclature, (iii) the integration of an extended test protocol, (iv) the restructuring of the data model together with the necessary adaptation of the calculation software and web interface, and finally (v) various breeding model developments for the situations in new breeding programs.

One of the distinctive cornerstones of BeeBreed.eu is that the individual breeder can enter the breeding and performance data himself, which has big advantages for data quality and the identification of breeders as part of a breeding program. As often breeders are not sufficiently able to read English or German, the multi-language support is essential to expand this concept to more countries. As the best experts for the correct translation of specific beekeeping terms are the national coordinators (often scientists and beekeepers at the same time), a powerful tool was implemented to put the coordinators in the position to translate the web site in context. The coordinators translated BeeBreed.eu which is now available in 14 languages.

The basic idea of the structured nomenclature od breeding queens, originating early in the 20th century, is that it should not only unambiguously identify a queen but also inform about the birth year, the breeder, and the association. With the internationalization of bee breeding, a straightforward continuation is that the country should be apparent as well. This has many advantages, not the least political, as often the national governments or beekeeping associations are the main sponsors of bee breeding.

Before SmartBees, BeeBreed collected only the final evaluation score for each property which required that each individual breeder must have a separate system of hive recording. In WP6, a performance testing protocol was developed on the basis of traditional test parameters with several extensions and BeeBreed.eu now implements the input of the full protocol, including measurements of individual beehive inspections. This has many advantages not only for the new breeding groups. The breeder is relieved from the task to have a separate recording system, the breeding administrator has a better insight into the testing process of the individual breeder which creates a new tool for efficient collaboration.

Among the many changes to the data model, the new layer of "bee population" has the most far-reaching consequences. Among the breeding queens of a subspecies there are isolated subpopulations, between which no comparison can be drawn because there is no comparative testing. Here, the breeding values would pretend a false comparability. This is highly relevant in SmartBees, where there are several separate breeding groups within *A.m.carnica, A.m.mellifera* and *A.m.macedonica*. Thus, for any breeding value to be displayed, the population has to be selected first – and among the queens in a population, breeding values are comparable. This also solved the problem that certain bee populations are (naturally) hybridized or simply unknown.

Assumptions valid for the Central European breeding population for which BeeBreed.eu was developed can no longer be taken for granted and had to be modified for the new breeding programs. Mainly, the marked winter period with no brood, where the number of bees, and critically, of Varroa mites is reduced, does not exist in Mediterranean Europe. Normally, swarmed colonies can be discarded from further breeding, but in some SmartBees breeding groups they were used as their loss could not be compensated. This prompted developments of the breeding model to cope with these situations. In the SmartBees testing protocol colony strength is assessed by counting the number of frames with bees, the number of frames with brood, and brood density while previously only evaluation marks for overall colony strength, spring development, and overwintering were recorded. To relate the quantitative assessment of the SmartBees protocol to final evaluation marks, a mathematical model was developed for the breeding value estimation of colony strength. The installation of mating control is difficult for a starting breeding group, therefore a model was developed for unknown paternal ancestry which considers regional bee pools from which drones are taken. The publication of the model in comparison to the current approach is under preparation.

Stochastic simulation studies are a valuable tool in predicting the potentials and risks of different animal breeding schemes. We have developed software to perform such studies while taking the biological and genetic peculiarities of the honeybee into account. Doing so, we succeeded to define new standards for reliable long-term simulation studies. In particular, we established that long-term studies should be based on genetic models with finite numbers of loci rather than Fisher's infinitesimal genetic model as only the finite locus models show a realistic decay of genetic variance over time. A scientific paper on these results is submitted.

Our studies further underlined that an introduction of mating control is crucial to generate genetic gain over more than just one generation and that breeding schemes without mating control are always ineffective in the medium term. Especially, when a small breeding population faces a large unselected rest population, close to no genetic response could be generated with uncontrolled matings. We could also show that in areas where there is no danger of hybridization, insecure mating stations

that cannot fully guarantee for the descent of present drones can nevertheless achieve good breeding success.

We performed various series of simulations to find the optimal selection strategy for honeybee breeding schemes. For a small population we established an optimal sister group size of four queens and that the number of mating stations should be between 5% and 10% of the number of breeding colonies. For larger population sizes more validations will have to be carried out but the existing data suggests that similar numbers will hold. As the establishment of secure mating stations is not possible everywhere, artificial insemination should be used in areas where the geographic conditions do not allow for isolated mating stations.

In task 2.2 of WP2, three completely new honeybee genotyping kits were designed. The aim of this part of the SmartBees-project was to develop a genotyping tool for honeybee breeders. The tool should provide information about the presence or absence of genetic factors leading to hygienic behavior against the parasitic mite *Varroa destructor* (Varroa), information about the allelic status of the CSD locus, and subspecies specific markers for European honeybees.

Through collaboration with SmartBees-partners in WP1, we received a list containing 2325 SNPs for hygienic behavior against varroa. In our own WP, 2134 specific probes were identified in the hyper variable region (HVR) of the CSD locus. Through collaboration with SmartBees-partners in WP3 a list of 4197 SNPs was created representing 12 different subspecies found in Europe. A total of 8656 SNPs was used in the design. The Infinium XT beadchip genotyping platform designed by Illumina Inc. was selected. This is a very robust genotyping platform, often used for genotyping of large numbers of samples in the Agricultural segment.

After designing the genotyping panels, the genotyping results from the InfiniumXT beadchip were validated by genotyping more than 10.000 honeybees collected by SmartBees-partners. For validation of the selected SNPs a Machine Learning Algorithm was employed. In order to build a model to classify and predict assignment of out-of-sample data, a LinearSVC model was created. Cross-validation was performed to estimate the accuracy of the model. This method was used for the WP1 and WP3 data. The HVR is a very complex region, so for the validation of the WP2 data, raw data was analyzed. Using this method, it was possible to determine unique allele probes within the drones.

Simultaneously a sampling method was developed. This method should fit into the daily management of the bee hives, as sampling should be easy to handle. Historically honeybees have been sampled using small tubes with ethanol. In this new system, the thorax of the honeybee is cut out and transferred directly into a small tube containing a conservation buffer. This method will always give the same size of sample material, and it is very easy to handle on robotics in the laboratory.

WP3 Assessing honeybee biodiversity in Europe

Reference collection of European honey bees

We compiled a comprehensive collection of honey bees from locations across Europe that comprises more than 2200 samples, representing all subspecies present on the European continent. It sufficiently characterizes the genetic diversity of *Apis mellifera* on the continent and provides valuable reference samples for future work.

Population genetic structure of European honey bee subspecies

Based on whole genome sequence data of 22 pools representing 12 subspecies, the population genetic structure of European honey bees was investigated using average genome-wide population differentiation (pairwise F_{ST}). Overall, the 22 pools grouped into four main branches corresponding to the well-known evolutionary lineages (M, C, O and A) (Figure 3.1). While F_{ST} values between subspecies of different evolutionary lineages averaged at 0.333, F_{ST} within the same lineage reached 0.063. The lowest F_{ST} was found between two *A. m. iberiensis* pools (F_{ST} = 0.016), while the highest value was observed between the *A. m. mellifera* pool from Ireland and the *A. m. caucasica* population (F_{ST} = 0.534).

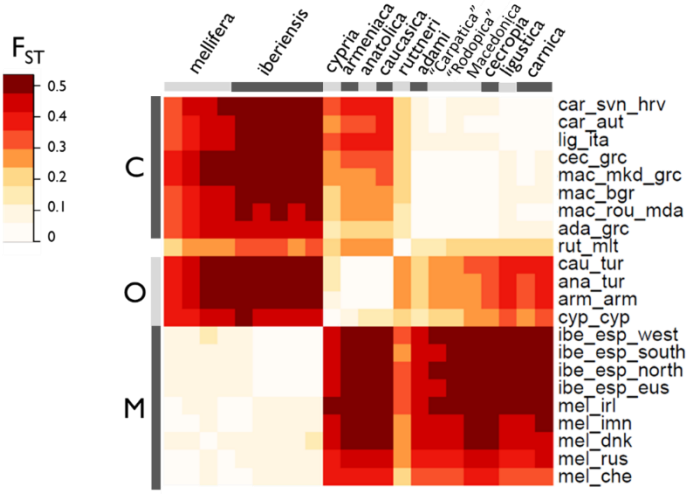

Figure 3.1. Population structure of European honey bees shown as heatmap of pairwise F_{ST} between each pool.

Selection of ancestry-informative SNPs

In addition to F_{ST}, Principal Component Analyses (PCA) were used to select ancestry informative markers (AIMs) based on the allele frequencies for each SNP in each pool and subsequently selecting the top SNPs with the highest contributions to the significant principal components (PCs). For a visual representation of the method, 100 individual genotypes were simulated using the allele frequencies of the selected SNPs in a PCA. Using this approach, the evolutionary lineages M, C and O were well separated with the first two PCs (Figure 3.2A), while lineage A can be differentiated with the third component (not shown). Based on a sufficient number of specific SNPs, resolution could be improved, and it was also possible to separate subspecies within the same lineage (Figures 3.2B-D).

Together with markers that were developed in WP1 and WP2, an Illumina Infinium XT BeadChip was designed for genotyping. The final number of SNPs from WP3 contributing to this genotyping panel was 4165 SNPs.

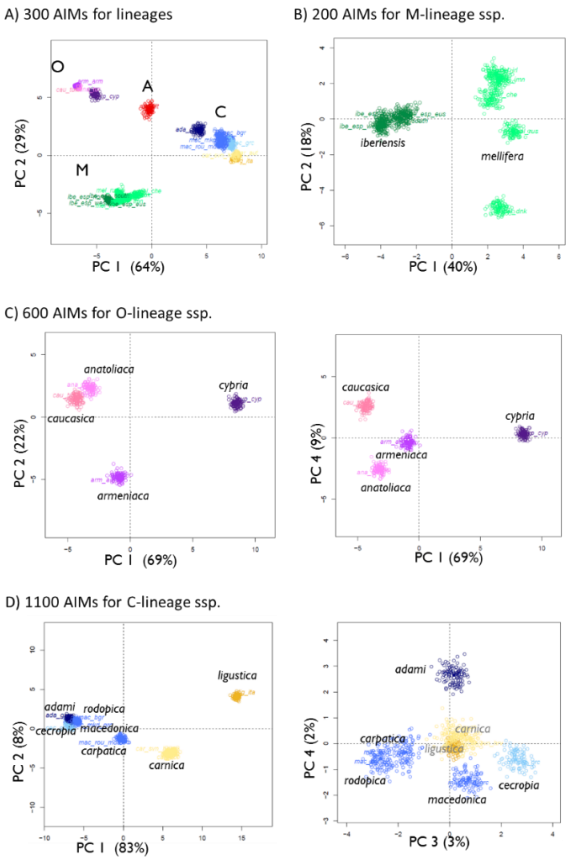

Figure 3. 2. PCA-Plots with the PCA-selected SNPs.

Validation of selected markers

The selected AIM SNPs were validated in a two-step system employing machine-learning algorithms and involving both samples with known origin and new and independent ones. Based on the predictions of the selected validation model, the Linear Support Vector Classification (LinearSVC), it could be shown that the subspecies assignment of the unknown samples could be predicted on average 98% correctly (Figure 3.3).

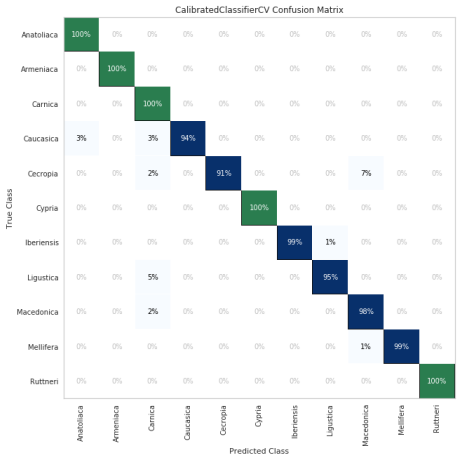

Figure 3.3 Confusion Matrix plot to predict subspecies allocation of unknown samples.

Selective Sweeps due to Varroa introduction

Our sampling contained several populations that have been exposed to the parasitic mite *Varroa destructor* for different time periods during the expansion of mite range from east to west after shifting hosts from *A. cerana* to *A. mellifera* in far eastern Asia, more than one hundred years ago. We compared SNP variation in the *A. m. mellifera* population from Russia, where Varroa had been present at last since the 1940's (that is for more than 70 years) to variation in populations where the parasite had arrived more recently – *A. m. mellifera* from Denmark and from Switzerland. In addition, we also acquired samples from the Isle of Man in Great Britain that is even today still Varroa-free. Two concurring peaks were found in all these comparisons, located on chromosomes 7 and 8. However, no correlation was found between these results and markers obtained from WP1 in association to Varroa sensitive hygienic behavior. Further investigations are necessary to understand the functional role of the genes found within the sweep regions and to investigate what other factors could lead to the identified signals.

WP4 Promoting honeybee diversity in Europe

Stengthen beekeepers' involvement in conservation activities

Via a questionnaire, beekeepers' knowledge and their thoughts about honey bee subspecies and conservation were assessed on the European scale for the first time. The results show a huge diversity of knowledge and interest in conservation issues and provide the basis for future activities to promote conservation efforts.

Conservation areas

Hybridisation due to increased movement of bees for honey production, pollination and overwintering in more favourable regions as well as trade with honeybee queens are currently the main threats to the diversity and conservation of locally adapted populations. We have reviewed and summarised the presence and key points about the number of colonies and size of the protected area of existing conservatories for indigenous populations of honey bees across Europe and created a network on the SmartBees website. In addition, guidelines for the size and definition of such areas were developed. The results are currently being processed and prepared for publication.

Effective population size

The effective population size is a critical measure relevant to conservation efforts, allowing detecting signs of genetic drift in small populations and determining minimum population size necessary for effective conservation and avoiding inbreeding. A comparison of several *A. m. mellifera* populations of varying size from across Europe, based on genome-wide SNP variation (figure 4.1), appears to indicate that a population of 100 colonies or more may be sufficient for sustaining the necessary genetic diversity within a population, but this number requires validation. Additional data are expected to be available soon to further clarify this question.

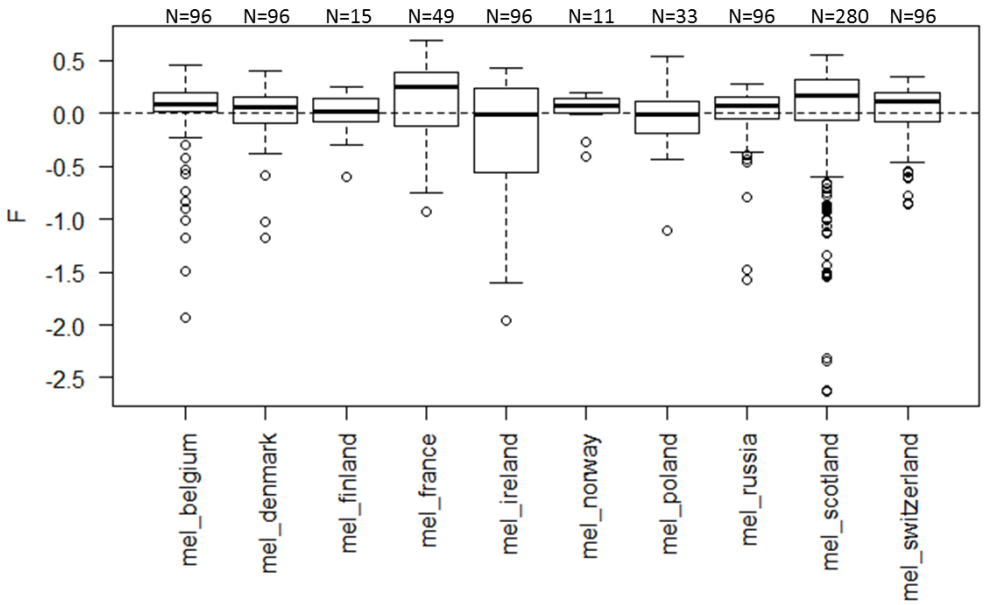

Figure 4.1: Boxplot of individual inbreeding coefficients (F) estimated for each *A. m. mellifera* population. The box denotes the upper and lower quartiles, and the median is represented by a solid black line within each box. Numbers above boxplots designate sample size in each group.

Status of feral or wild populations in Europe.

A search for feral honeybees yielded a few colonies that were sampled with support from managers in nature reserves and national parks and scientific colleagues from Greece and Germany, in the last year of the collection period. In spite of many contacts to protected areas, no samples could be obtained from Spain.

Analysis of the Greek samples revealed high similarity to samples representing *A. m. macedonica* from Bulgaria and samples collected on the island of Crete, that appear to be hybridised with *A. m. macedonica* to some extent (figure 4.2). Thus, feral and managed honey bees appear to freely exchange genes with each other, enhanced by the fact that in all three countries surveyed (Greece, Germany and Spain), there are no legal restrictions against beekeeping activities inside the protected areas. In conclusion, there is no reason to distinguish the two populations as separate units, the honeybees kept by beekeepers are representing the local population of honeybees.

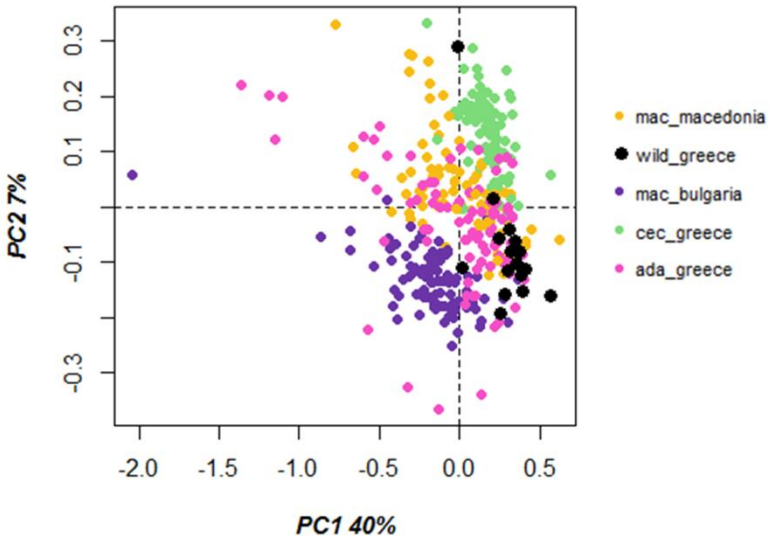

Figure 4.2. Principal component analysis with feral Greek samples as well as A. m. macedonica from Macedonia and Bulgaria, A. m. cecropia, and A. m. adami.

WP5 Development of new extension methods for sustainable apicultural production and maintained diversity

Sustainable bee-keeping involves both biological, technical and economic challenges. Nonetheless, it is not until people change or implement best practices and new knowledge that real improvements can be measured. In the diverse processes of knowledge development and transfer within the bee-keeping sector in Europe, extensionists and advisors play a central role. Strategies to develop bee-keeping in Europe must therefore include issues related to communication, learning and behavioral change. In the SmartBees project this has been done in an integrated way; through training and dissemination activities, but also by a specific work package aiming to develop a web-based resource for communicators and advisors within the bee-keeping sector. This has been the main purpose of WP5 and the new homepage was launched at the EURBEE-conference in Ghent in September 2018; http://www.bee-extension.org

But an extension tool-box must be adapted to the specific needs and pre-conditions within the bee-keeping sector. Consequently WP5 started with a survey of bee-keepers and bee-breeders in several regions of Europe, focusing on their information needs as well as preferences regarding learning methods and tools. Understanding the target group is important but not enough. We also need to know more about how different parts of Europe have chosen (consciously or not) to organise their knowledge and innovation system within the bee-keeping sector (what we call a regional B-KIS). We approached this issue in a conceptual way, describing eight regions in Europe using the same method. This resulted in a report and a better understanding of what specific challenges different regions have if and when developing regionally adapted strategies to improve extension and advisory services.

The ongoing professionalization of the beekeeping sector in Europe will force us to focus more on the quality of the services provided to the beekeepers. New findings, higher competence, societal

demands and new technologies will put pressure on trainers and educators to work more consciously with communication planning. It is within this context that one should understand the aim of WP5 in SmartBees. Bee-extentionists and advisors will be able to use the web-based extension tool-box as a resource when planning and evaluating educational and communicative activities. By this we aim to strengthen the sector by bridging the so called implementation gap; moving from theory to practice more effectively.

The final result, the URL: http://www.bee-extension.org, will be administrated by the National Competence Centre for Advisory Services at the Swedish University of Agricultural Sciences (SLU). On the new homepage communicators and educators will find guidelines, helpdesk and best practices, but also possibilities to deepen their theoretical knowledge on extension through in-depth texts. The models presented are adapted to the specific challenges within bee-keeping, with a large number of bee-keepers not used to pay for advisory services, and building their competence development on informal learning among peers. The aim has been to tap into existing pre-conditions, while at the same time adding insights from extension theory and advisory services from other sectors in agriculture.

Lack of effective communicative skills and strategies might become a threshold for not only a sustainable and increased production from honey bees, but also society's ability to strengthen and develop new ecosystem services based on pollinators. WP5 within SmartBees has created the preconditions to make such thresholds as low as possible.

WP6 Field testing and selection on local bee populations

The implementation of WP6, "Field testing and selection of local bee populations", entirely achieved the objectives set in the SmartBees project proposal. Thus, during the period of 48 months, a systematic sequence of actions was taken towards the establishment of a self-sustainable breeding structure and practice, relevant for different regions in Europe. In some aspects, we went even beyond the objectives set out in the proposal. Our report is structured into three main activities undertaken during the project duration:

I. Breeders' identification, dissemination and capacity building

Identification of the target group (beekeepers, breeders and relevant specialists) was a crucial step in initiating activities and ensuring sustainable project development. Several steps were taken for the identification of competent and enthusiastic breeders, beekeepers and specialists from regions with endangered and/or neglected European honey bee populations. In total, we published 46 peer-reviewed and popular articles, abstracts, book chapters and posters that contained relevant information concerning project aims, activities, timeframe, the possibilities of involvement of breeders as well as results and achievements. To boost the accessibility, the materials were translated into up to 13 languages and published in 38 proceedings and beekeeping journals across Europe. In addition, the overall communication and dissemination of information was enhanced by giving talks and participating in more than 130 events (seminars, workshops, local training, conferences, symposia etc.) as well as by contacting the already established and well-recognized international and regional beekeeping and academic networks and initiatives.

The competences of the engaged partners are a fundamental standing point for ensuring adequate implementation and realization of breeding initiatives. To improve the overall understanding concerning honey bee breeding and also to improve breeders' technical skills for the application of the recommended methods for testing, an extended capacity building program was implemented all over Europe. The first step was the preparation of the tailor-made **"Performance Testing Protocol, A guide for European honey bee breeders",** in cooperation with the project partner LIB-B (Annex 6.1, English version). The protocol describes the basic requirements, recommendations and timeframe for setting up and maintaining testing stations and development of regional and/or national breeding structure. In addition, this document contains detailed and step-by-step descriptions of twelve (12) methods and tests for assessment of traditional and *Varroa* related traits. The document was translated into 20 languages and published online (http://SmartBees.eu/Extension/Performance/).

The above-mentioned protocol and method descriptions were visualized (videos, gif animations) and made more accessible to the beekeepers by the development of smartphone and tablet application **"Virtual Testing Apiary"**. This freeware application, available on www.testbees.eu, provides main information and details for standardized assessment and test of the traits of interests as well as calculators that assist breeders to improve the quality of data collection. In addition, the standardization of the methodology for testing colonies under different environmental and beekeeping conditions and consequent adequate estimation of the breeding values improved with the development and distribution to SmartBees breeders of an innovative "**Performance Testing Kit"** that contains tools and equipment for correct application of the testing methods.

The success and sustainability of the breeding activities significantly depend on the technical knowledge and experience as well as the organizational capacity. That is why in WP6 we devoted significant efforts towards the enhancement of beekeepers' understanding of breeding and improvement of their management and communication capacity. During the project period, we organized and conducted 21 on-field practical and theoretical training sessions entitled **"Contemporary honey bee breeding"** in 17 countries (Figure 6.1), in which more than 400 beekeepers actively participated. During the aforementioned trainings, the breeders were instructed how to use the mentioned methods and kit.

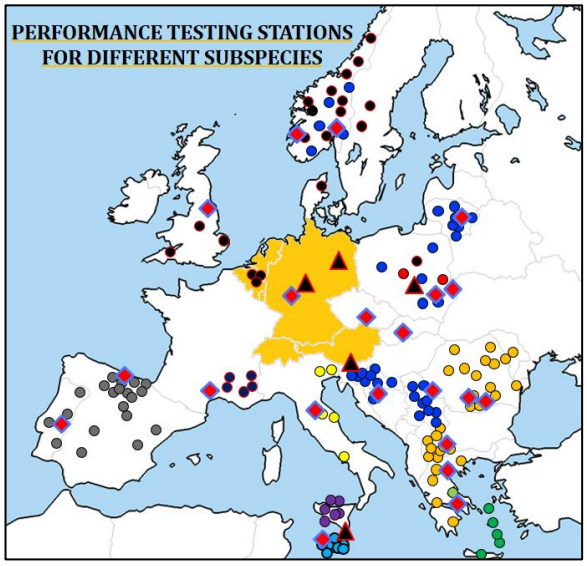

Figure 6. 1. Map of Europe with locations of the testing apiaries ( ), training (and seminars) organized during the project duration. Different colours of the circles indicate different subspecies. The orange coloured regions are countries with longstanding systematic breeding practices.

The central role in organizing the training events, data collection and management of the regional breeding groups was taken by 23 coordinators (http://SmartBees.eu/Extension/Performance/Breed-Coord/) who, through a series of five seminars (Figure 6. 1) had a chance to exchange knowledge and experience, gained during the testing under different environmental conditions, and to report about challenges. Towards the end of the project, as a consequence of such intensive communication and collaboration, the idea to establish an **"International Honey Bee Breeding Network"** was born. It was formalized during the last seminar on 19 October 2018 in Hohen Neuendorf. This achievement of WP6, beyond the project's objectives and expectations, may help to ensure sustainability and perpetuation of the breeding activities towards genetic improvement and preservation of the European honey bee populations.

II. Testing and data collection for propagation of locally adapted honey bee stock

The establishment of testing apiaries around Europe started in 2015, right after the first training events and publishing of the performance testing protocol. In total, more than 130 apiaries (beekeepers) from 19 countries (Figure 6.1) were involved in the testing and collection of data from more than 3000 colonies from different regions in Europe. Till the project's end date, in close cooperation with the project partner LIB, breeding values for 1895 queens from 15 countries and 10 European honey bee subspecies were estimated (Figures 6.2 & 3). The estimated breeding values are regularly published online on "Virtual Testing Apiary" for further propagation in the broader population as well as for the next testing generations. In the meantime, additional capacities across Europe are testing colonies from consecutive generations. For six subspecies, *A. m. iberiensis*, *A. m. macedonica/carpatica*, *A. m. ruttneri*, *A. m. adami*, *A. m. cecropia* and *A. m. caucasica*, the first-ever breeding values were estimated by the project.

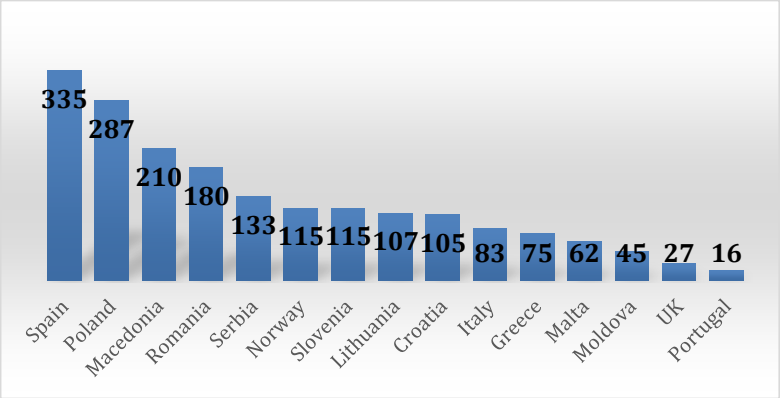

Figure 6.2. Estimated breeding values by countries from three testing generations (2015-2016, 2016-2017 and 2017-2018) during the project implementation.

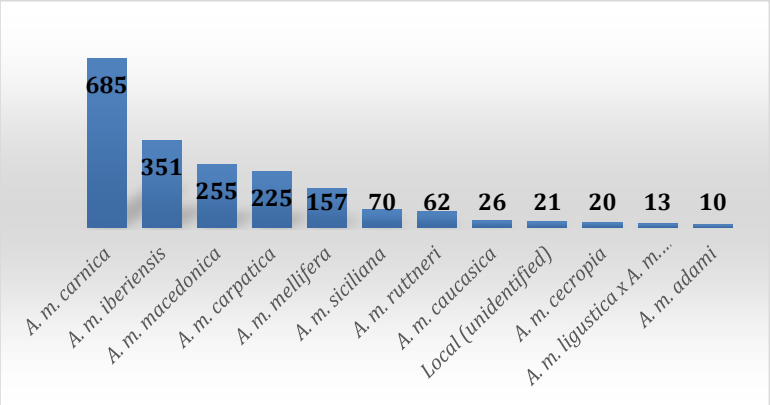

Figure 6.3. Estimated breeding values per populations/subspecies from three testing generations (2015-2016, 2016-2017 and 2017-2018) during the project implementation.

Special support was provided to breeding efforts for the preservation of the endemic honey bee subspecies *A. m. ruttneri* in Malta (**Malta Case Study**), including an extensive training program for breeders, laboratory support for identification of the best available genotypes (collaboration with WP3), research activities, communication with the authorities in Malta (meetings with the Ministry, the President of the country etc. in collaboration with WP3, WP4 and WP2) and raising public awareness through a serial of popular and scientific articles. As a consequence of these actions, the Ministry for Sustainable Development, Environment and Climate Change of Malta recently initiated actions for the conservation of *A. m. ruttneri*. Having in mind the urgency of protecting the population of Maltese honey bees, this is one of the foremost achievements of WP6.

During the implementation of WP6, we identified challenges related to the initiation and maintenance of honey bee breeding programs in different regions in Europe. Thus, besides the evident differences in the environmental conditions and variations in biological features of honey bee populations, the cultural and traditional differences among the European beekeepers play a significant role for the success of running breeding programs. The experience and the knowledge gained during the implementation of the activities certainly contributed to the adjustment of the methods and are incorporated into the second version of the improved “Performance Testing Protocol”.

III. Detection of locally adapted *Varroa* infestation threshold values

Significant research progress was done for the estimation of regional *Varroa* threshold levels. In collaboration with partners within and outside of SmartBees from Romania, Poland, Denmark, Moldova and Croatia data were collected from 217 colonies from summer 2015 to Early Spring 2017 for five parameters relevant for the estimation of the regionally adapted *Varroa* threshold levels. The evaluation of the different infestation parameters shows high correlations between the adult bee infestation and natural mite mortality during the entire period indicating that each parameter can be used as a good indicator of colony infestation level. The decision for one of those methods should be based upon beekeepers' experience and preferences. Our data show that the infestation of adult bees is sufficiently indicative of the infestation level of the whole colony and in the same time most convenient for the appliance. Furthermore, the results show that the differences in *Varroa* infestation among the colonies from different groups (threated/removed, lost in winter, overwintered below economic size and overwintered economic size) are getting more distinct as the season proceeds. Thus, in week 38 (mid-September) a biological threshold level (colonies survived until next spring) of 9.3 mites per 10 g of bees was found. However, the estimated economic threshold value (survivor colonies with economic size i.e. maximum capacity for honey production in the forthcoming season) was 3.2 indicating that lower infestation in week 38 might not affect colony performance for the forthcoming season. Based on the data from the parameter "Natural mite mortality" the biological threshold was 14.4 mites per day and the economic threshold below 5 mites per day. Finally, the brood infestation of 21.3 % in week 38 was identified as a biological threshold and the infestation of below 12.3 % as an economic threshold. All these outcomes were communicated to the beekeeping and scientific communities during the project closing seminars organized by WP7 and will be published in a scientific article that is under preparation.

WP7 Dissemination

In WP7 we established a website for the project where we presented the project aims and the consortium partners. The website has been used to inform about project achievements, to post a biannual newsletter and to establish contact with stakeholders.

Breeding and conservation networks and dissemination to governmental and non-governmental organisations: SmartBees established a network of beekeepers and bee breeders that were subcontracted to carry out performance testing of local honeybee populations. This network covered all subspecies of European honeybees. SmartBees has also supported conservation activities of various kind throughout Europe. For example, SMARTBEES partners have been strongly involved in the conservation efforts to save the honeybee subspecies *Apis mellifera ruttneri* on Malta. To facilitate the continuation of the selective breeding to improve the local honey bee populations after SmartBees came to an end, we initiated and supported the establishment of a new organisation: International Honey Bee Breeding Network (IHBBN). The aim of the organization is to aid to the genetic improvement of local honey bee stocks and the conservation of the different subspecies of the honey bee within their native range.

Newsletter: During the project period, we have published 6 newsletters. The newsletters have been posted at the project website as well as sent to subscribers that registered themselves at the project website.

Articles in beekeeping magazines: Beekeeper magazines were regarded to be a dissemination channel that would reach a large proportion of the beekeepers in Europe. We have written 5 manuscripts in English aimed for beekeeper magazines. The English manuscripts have been translated into many national languages by the SmartBees partners and the manuscripts have also been sent to the editors of beekeeper magazines in the European Beekeeper Associations with an invitation to translate and publish the manuscripts in their own language.

Scientific dissemination: SmartBees consortium partners have presented their work orally at many international congresses, conferences and symposia. Until now, 16 scientific papers have been published and a lot of manuscripts are under review in different scientific journals. As in most research projects data accumulates during the project period and numerous additional scientific publications based on the research in SmartBees are expected in the coming year.

WP8 Elucidating and enhancing honeybee resistance mechanisms to parasitic diseases

Honey bees are equipped with a highly efficient immune system capable of protecting themselves against a wide range of infectious agents. However, *Apis mellifera* is susceptible to varroa infestation, in which case the deformed wing virus (DWV) becomes a markedly more virulent infection. But, what is the underlying mechanism(s) that allow varroa to have such a long and intimate association with the bee without eliciting any robust and effective immune response and what are the immune genes and pathways involved in responses to DWV infection? Neither the viral immune resistance mechanisms nor how varroa infestation affects the bee/DWV equilibrium are understood. It is also not clear how stressors such as abnormal temperature, xenobiotics (like pesticides) and poor nutrition affect the bee's immunecompetence.

To analyze immune genes and pathways involved in DWV-resistance, we performed an extensive comparative transcriptome analysis of experimentally infected honey bees. To this end, we infected honey bee pupae and drone larvae with DWV and analyzed the transcriptomes of infected and naïve bees with RNAseq to reveal the precise cellular mechanisms affected. The results from DWV-infected worker bees clearly showed that the key genes of the honey bee innate immune system (abaecin, apidaecin, hymenoptaecin defensin 1 and defensin 2) were significantly up-regulated. Furthermore, we identified genes of neuronal and muscular development which were significantly dis-regulated in infected honey bees in response to an infection with virulent DWV. We confirmed our RNAseq data by gene expression analysis of individual genes using RT-qPCR. Furthermore, we confirmed our molecular data by experimental infection of honey bee pupae. Thus, key genes involved in innate immunity and pathways involved in DWV-resistance were confirmed. The key genes were provided to WP1/2 for inclusion in the genetic selection for bees with increased resistance.

Varroa infestation is the trigger that changes DWV from a benign, covert viral infection to an overt, pathological state with disastrous consequences for the individual bee and the colony. One hypothesis is that the saliva of the varroa contains bioactive factors able to subvert the bee's immune system allowing both the prolonged feeding of the varroa and greatly increasing the virulence of DWV.

Varroa saliva micro-injected into honey bee pupae greatly increased the DWV titre far more than DWV injected with whole mite homogenate. This result supports the hypothesis that some factor in the saliva allows DWV to become established at a higher titre in naïve bee brood. The proteome of both the saliva (138 proteins) and salivary gland (1302 proteins) was determined by LC-MS/MS and bioinformatics analysis using transcriptome and genome databases of varroa, ticks, predatory mites and parasitioid wasps. Many putative bioactive factors in the saliva proteome were identified and several of them were demonstrated to be either exclusively or predominantly expressed in the salivary glands relative to other varroa tissues. The importance of six of these putative bioactive factors was investigated by creating varroa that were deficient in each of these bioactive factors by specific gene-knockdown and then assessing their phenotype after feeding on bee pupae. Several of these bioactive factors were demonstrated to be important in the bee-varroa relationship with increased varroa mortality and changes in bee immune gene expression when the bioactive factors were absent.

The ability of honey bees to adequately respond to varroa and DWV infestations is affected by various stressors which can compromise the immunocompetence of the bee. In order to pinpoint heritable traits influencing the vulnerability of the bee to different abiotic stresses we investigated the effect of food deprivation, pesticide contamination and other stressors including abnormal temperatures on relevant bee response genes. Food deprivation and sub-lethal doses of the neonicotinoid clothianidin appeared to trigger DWV replication by affecting the honey bee immune competence, whereas results on temperature were not conclusive. According to our results, both nutrition and certain pesticides (i.e. the neonicotinoid clothianidin) can affect immune-response and in turn viral proliferation and, thus, survival. Therefore, we propose that the external stressors affecting immune-competence toward DWV-infection are, in order of importance: the neonicotinoid clothianidin, and related pesticides impacting antiviral defence and poor nutrition.

Nutrition can play a key role in the immune response of honey bees. We examined the effect of supplementation with essential amino acids on the ability of adult worker bees to suppress amplification of deformed wing virus (DWV) in laboratory bioassays. The laboratory feeding bioassays demonstrated that there was no significant difference in the viral copy number between bees injected with DWV fed on sugar solution alone or sugar solution supplemented with essential amino acids. Therefore, under the conditions of the test the presence of essential amino acids did not boost the adult worker bee's ability to suppress levels of DWV. Investigations into the effect of dietary pollen on the survival of mite–infested bees were also performed in caged bee studies. Pollen supplementation had a positive effect on survival of mite-infested bees and reduced DWV titres. Further investigations demonstrated that the survival of mite-infested bees was enhanced by dietary supplementation of the apolar (lipid) fraction of pollen, whereas the polar fraction of pollen did not appear to be protective. Taking these findings out into the field, colonies supplemented with pollen at the end of the active season had reduced bee mortality and a reduced percentage of bees exhibiting the characteristic symptoms of overt DWV infections. Thus, our findings indicate that bee keepers should supplement their colonies with dietary pollen to mitigate the effects of varroa infestation and DWV infection to decrease their overwintering colony losses.

WP9: Determining present and future pathogen threats

Since the worldwide spread of *V. destructor*, deformed wing virus (DWV) has become one of the main threats to honeybee colonies. Several closely related variants of DWV have been characterized including DWV-A, DWV-B (also named Varroa destructor virus 1 - VDV-1), recombinant between DWV-A and DWV-B variants and the less documented DWV-C. The vectorization of DWV by *V. destructor* to the bees implies the selection of virulent variants. These modifications in virus population were studied in order to better anticipate the evolution of DWV and the threat it represents.

To understand the changes in DWV when it switches between Insecta and Acari it is not only desirable but also necessary to have access to cell culture systems from both the original and intermediate hosts for virus propagation. Unfortunately, there are no *V. destructor* cell lines, nor even protocols for obtaining *V. destructor* primary cell or tissue cultures, but more than 40 established cell lines from closely related ticks (Acari: Ixodidae and Argasidae) are available (Bell-Sakyi et al., 2012). We therefore infected a panel of 21 ixodid and argasid tick cell lines with purified DWV-isolates and screened them for replication of DWV. We also infected honeybee primary cells and heterologous insect cell lines with DWV. We found eight tick cell lines in which increasing or constant levels of DWV RNA were detected by RT-qPCR over a six-day period after infection. We also screened the cell lines for replication of DWV-A variants and for the DWV-B variants and found that the tick cells constitute better host cells for the latter *V. destructor*-adapted variants. However, further in vitro-infection experiments revealed that DWV cannot be propagated in tick cell lines, primary cells or lepidopteran cells in terms of generating high virus titres which can be purified quantitatively for NGS. Therefore, we could not use these cells for DWV-production or for analysis of variant clouds. To analyse the shift of the DWV-variant cloud when DWV switches from the honeybee to the *V. destructor* mite, we therefore focused on experimental infections of honeybees and honeybee pupae.

DWV-A and DWV-B both undergo a bidirectional passage between bees and *V. destructor*. To investigate how these two variants are transmitted between an insect (i.e. honeybee) and an acarine (i.e. V. destructor), we developed molecular diagnostics able to accurately differentiate both DWV-A and DWV-B. Using an artificial feeding system developed for *V. destructor*, we studied how these two DWV variants are transmitted from *V. destructor* to the “artificial bee”. Remarkably, there was only 1% of the original DWV–A remaining in the *V. destructor* within a 2 days but DWV-B levels greatly increased reflecting the much higher rate of replication of DWV-B than DWV-A in V. destructor tissues. It would seem most of the DWV-A reported in *V. destructor* in other studies is within its gut contents and is defaecated rather than transmitted into the bee. Overall, we take these results to indicate that DWV-B is better adapted to the Acari (i.e. V. destructor) environment than is DWV-A.

Next, we investigated the establishment and replication of DWV-A and DWV–B in bee pupae with extremely low levels of initial DWV. DWV-A infections went shooting up within a few hours and peaked by 60 hours with a 1 million-fold increase of DWV-A after *V. destructor* infestation, whereas DWV-A in pupae without *V. destructor* increased just 3½ -fold. DWV-B levels did not increase at all in the absence of *V. destructor*, but increased by 30-fold in the presence of *V. destructor* – which is massively different from the 1-million fold increase for DWV-A. Overall, we take these results to indicate that DWV-A is better adapted to the Insecta (i.e. honeybee) environment than is DWV-B.

We investigated if there were changes in DWV-A and DWV-B as they are transmitted between *V. destructor* and bees. The bee pupae and their haemolymph, the associated *V. destructor* and its salivary glands and saliva all from the same brood cell (i.e. a host-parasite pair) were studied. DWV-A was by far the predominant strain (>95%) within the whole pupal bee and its haemolymph. However, the *V. destructor* salivary gland and the saliva contained much higher proportions of DWV-B. Our results indicate that DWV-B is favoured and selected for within the V. destructor. Nucleotide sequence analysis of the DWV populations in each of the tissues showed that the DWV population within the bee pupae was markedly different from that of the V. destructor salivary glands and saliva suggesting some selection and filtering mechanism within the V. destructor before transmission to the bee.

In an alternative approach, *V. destructor* mites were allowed to feed on artificial feed packets for 5 days containing an isolated and specified DWV inoculum. The DWV populations in both the feed packets and the *V. destructor* were analysed. In particular, two viral genes were analysed in detail in the *V. destructor* and were determined to be more similar to the DWV-B than the DWV-A variant indicating that DWV-B is the more mite-adapted DWV variant.

Our hypothesis is that DWV exists as a quasispecies and DWV-isolates represent mutant clouds that change their relative sequence-space master sequence when moving between honeybees and *V. destructor* mites. The virus mutants are considered bee-adapted when they are circulating in the bee host only (DWV-A mutants) and mite-adapted when they are replicating in the mite (DWV-B mutants). Following this hypothesis, moving of DWV between the bee and the mite host might be accompanied by shifts in DWV sequence-space master sequence and virulence. Our results showed that DWV-B is favoured and selected for within the feeding V. destructor mites although they imbibed almost only DWV-A from bees, indicating a shift in the DWV mutant cloud. Further, DWV-B can subsequently be transmitted back to the bees because DWV-B was detected in the diet after the DWV-B replicating mites fed on the feed packets. Moreover, when we infected honeybee pupae with the mite adapted DWV-B and re-isolated the DWV particles therefrom we observed again a genetic shift in the DWV master sequences, namely the re-isolated DWV master sequence was closer related to the bee adapted DWV-A variant, than to the more virulent DWV-B variant. This genetic shift was mainly driven by the sequence change in the L-protein gene and the RdRp (RNA-dependent RNA-polymerase) gene as revealed by further analysis beyond the funding period. Furthermore, this genetic shift was indeed accompanied by a shift in virulence. DWV-B showed a significant higher virulence than DWV-A for honeybee pupae (read out: mortality) and adult bees (read out: neurotropism and cognitive impairment). These results clearly proved our hypothesis that DWV master sequences change when the virus moves between its hosts, the mite and the honeybee. Moreover, we identified the L-protein gene and the RdRp gene as a possible sequence signature of DWV virulence (Gisder et al., Environ Microbiol, manuscript accepted in Nov 2018).

V. destructor mite infestation (varroosis) of honeybee colonies is now worldwide spread including all over Europe. Little is known about the genomic and virulence diversity of the DWV closely related variants in Europe. We collected samples from 121 honeybee colonies across 15 European countries. DWV-B variants were detected in samples from all countries and exhibited a higher detection level than DWV-A variants. Particularly, DWV-B variants were detected in the samples from France, Spain, United Kingdom, Germany, Croatia, Macedonia, Greece and Serbia. DWV-A variants were detected in

samples from Italy, Moldova, Croatia and Romania and Serbia. Lastly, recombinant viruses were found in samples from Spain, France, Macedonia, United Kingdom and Italy. Among the DWV-A/DWV-B recombinant viruses detected, we observed new recombinant-genomes that will require further investigations about their virulence. The analysis of virulence of two variants on honeybee pupae showed no major differences between DWV-A and DWV-B suggesting that more than the variant-type, it is the injection of virus by the mite to the pupae that is responsible for wing deformities.

All together, we developed new methods and made significant observations that will contribute to better understand the evolution of DWV variants and the threat they represent.

4 Potential impact of the SmartBees project

Impact with regard to the selection of varroa-resistant lines of honeybees: Varroa destructor is seen as the most frequent cause of colony losses throughout Europe. Our project has led to the identification of thoroughly validated genetic markers for hygienic behavior, which is seen as maybe the most important trait conferring resistance against this parasite. These markers have been integrated into a genotyping tool that will allow breeders to assess the "hygienic potential" of prospective breeding queens much faster and with less effort than with traditional methods of measurement of the trait. This achievement can therefore be expected to greatly increase the speed of selection of bees resistant to varroa.

Another achievement that almost certainly will help to speed up the production of varroa-resistant bees is the identification of varroa threshold values for different European climates. This knowledge allows breeders to judge at which level of infestation colonies should be excluded from the breeding process but can still be saved by treatments in order to preserve their productive potential. Finally, the project has allowed to train numerous beekeepers, participating in the local breeding groups, to perform selection for resistance traits in their local populations. This puts varroa resistance breeding on a much broader level, and hopefully will help to avoid a scenario where a small number of highly-bred resistant lines from only one or two subspecies of *A. mellifera* are distributed all over the continent, thereby accelerating the loss of biodiversity.

The project has also led to a more comprehensive understanding of how varroosis and viruses damage honeybees. This encompasses the identification of genes involved in resistance towards the varroa mite and towards DWV, of mite-derived factors suppressing host immunity, and a wholistic view of mite-virus-bee interactions. It also includes an understanding of DWV-adaptability to its hosts. Some of this research is of broader importance for the understanding of host-pathogen/host-parasite relationships, also outside the apicultural sector. Description of the virus diversity in Europe can help to anticipate their evolution and estimate the emergence of new threats. The study on dietary influences of bee susceptibility is contributing to a scientifically-grounded understanding of optimum nutrition to enhance the resistance of bees to DWV. Our characterisation of molecular resistance mechanisms is a large step towards the selection of *V. destructor*- and/or virus-resistant bees. Ultimately, this research will contribute to reducing colony losses.

Impact with regard to the preservation of honeybee biodiversity: In order to preserve biodiversity, it first has to be assessed. The data collected by SMARTBEES permit a sound assessment of the present state of intraspecific diversity of European honey bees, which is a) based on the most comprehensive collection of bee samples existing to date, and b) an analysis of genome-wide genetic variation in populations across Europe. Based on these data, specific genetic markers for subspecies diagnosis of honey bee samples of unknown origin have been developed and a genotyping panel featuring Illumina Infinium technology has been designed. With this tool, an accurate, rapid and cost-efficient subspecies diagnosis of unknown samples of honey bees is now for the first time possible. Such a tool will be not only of high value for genetic monitoring programs in protection and conservation areas, but it will also enable breeders of endangered lines to have their breeding stock evalu-

ated and thus help to promote their conservation efforts. Breeders all over the continent can now benefit by verifying that the bees they are breeding are the bees that they want to breed.

A second precondition to preserving bee diversity is to give local breeders the means to effectively select their bees for the traits which render them amenable for "conservation through utilization". In this regard, an important achievement was the enlargement of the internationally-used breeding platform beebreed.eu to include bees of all European subspecies, and the fact that local breeders are now given the opportunity to benefit from modern quantitative genetics to calculate breeding values for their queens.

A third precondition to preserving biodiversity is to raise awareness of its value. In this field, the SmartBees-questionnaire on beekeepers' attitudes towards local bees has shown that much remains to be done. Still, we have been able to motivate and mobilize breeders from 17 European countries to actively get involved in the conservation of their own local bees, and this is a great success. The establishment of the "International Honeybee Breeding Network" by some of these breeders can be seen as an indication that this effort will have a lasting impact. Another most noteworthy and concrete result of these efforts is the involvement of beekeepers on the island of Malta in conserving the subspecies *A. m. ruttneri*. This subspecies is restricted to the Maltese archipelago and must be considered as critically endagered. SmartBees has taken part in meetings with local authorities to help local beekeepers to create a legal framework for the protection of their honeybees. The work to create a mating station on the smallest island Comino was started with the help of SmartBees, and in addition, much support from WP6 was provided to test their local bees and set up a breeding program. Across Europe, beekeepers are now expressing their desire to use locally adapted honey bees, frequently also to use the original subspecies. For instance, a new association is being formed in Denmark for the dark honeybees, in Italy the San Michele all'Adige declaration to protect the endemic honeybees has been published, and a book on protection of local bees has been published in the UK. Numerous European colleagues involved in protection and conservation activities have already indicated an interest to use the genetic tools developed in WP3, thereby demonstrating that the desire to protect local honey bees is on the rise.

Finally, SmartBees has also allowed for a greater level of coordiantion of public and private conservation efforts accross Europe. The network of initiatives inthis sector that was set up will hopefully llow to exchange experiences and tackle common problems like the lack of sufficiently efective ways of mating control. The project has also been ale to bring about a first estimation of the effective population size that is required for sustainably conserving a population, and this information is evidiently of great value for any activity in this field.

5 Dissemination of results and capacity building

Capacity building: An important part of SmartBees has been devoted to the training of beekeepers and –breeders, with the aim of enabling them to become involved in the conservation of their local bees, as well as in the selection of productive and disease-resistant stock. More than 30 on-site training events on breeding techniques were organized, and breeding groups initiated in 17 countries.

This has had a direct and lasting impact on the technicity of European beekeepers, as illustrated by the fact that some of them have decided to perpetuate the work initiated within the project through a new international association. Another part of project's legacy is the collection of high-quality technical documents, available online in up to 20 languages, which are distributed and used far beyond the frontiers of the EU.

Another important element of capacity building is the conceptualization of the ways in which information is produced and transmitted within the European beekeeping sector. The project has produced exemplary descriptions of the knowledge- and information-systems of European countries, which can now guide future efforts to bring about necessary reforms or efficiently spread technical progress. Through an understanding of bee-keepers and bee-breeders needs and existing knowledge and information systems within the beekeeping sector, it is now possible to suggest relevant extension tools and implementation strategies. A web-based extension tool-box is part of an effort to strengthen the knowledge and innovation system by supporting advisors and educators in implementing new knowledge and best practice.

Dissemination of results: The spreading of knowledge produced has been a priority throughout the implementation of the project. Four different channels were chosen, targeting the scientific community, the beekeeping sector, and the general public.

The *beekeeping community* was mainly addressed through articles in beekeeper journal, through the project website, and through the 7 regional conferences organized by the consortium. More than 48 articles appeared that explained e.g. the value of honeybee biodiversity, ways to effectively breed for varroa resistance, and the importance of bee nutrition for resilience. Members of the consortium collaboratively translated these manuscripts into many languages. The project website was created early on during the project phase and has been continually updated. It has been extended ever since and visited by >3.000 persons in the last year alone. Notably, it has served as download source for many of the technical documents produced. The regional conferences were held in different places accross the EU and were hosted by regional partners. The main effect here was to inform local multiplicators of our findings, such as the heads of breeding associations, but also members of the local administration and public service. Moreover, project representatives gave at least 50 presentations in various assemblies of beekeeper associations and other events. The six newsletters produced by the consortium also contributed to the dissemination of results.

The *scientific community* was and is being addressed through 17 peer-reviewed publications, with many more still to come. Until today, members of the consortium also contributed at least 60 SmartBees-related presentations to scientific events like Apimondia, EURBEE and other congresses. A book on honeybee conservation is at the final stage of editing.

From the beginning, SmartBees has enjoyed a great amount of interest from *popular media*. Dozens of articles have been written about the project, some of them appearing in major newspapers like "Die Zeit" (Germany), and radio stations have broadcasted reports about the project. SmartBees was also represented at the Shanghai Science Festival, and featured in TV-clips.

6 Project public website and contact details

Project logo

The project's website is available under www.SmartBees-fp7.eu/

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