

Executive summary:

The BEE DOC comprised a network of eleven partners from honeybee pathology, chemistry, genetics and apicultural extension aiming to improve colony health of honeybees. The BEE DOC filled empirically and experimentally knowledge gaps in honeybee pests and diseases¹ and quantified the impact of interactions between parasites, pathogens and pesticides on honeybee mortality. Specifically BEE DOC showed for two model parasites (Nosema and Varroa mites), two model viruses (Deformed Wing Virus, Black Queen Cell Virus) and two model pesticides (thiacloprid, τ -fluvalinate) how interactions affect individual honeybees and colonies. The BEE DOC analysed the transcriptome of honeybees to explore host-pathogen-pesticide interactions and identified novel genes for resistance against Nosema. The BEE DOC specifically addressed sublethal and chronic exposures to pesticides. Whereas interaction effects between the tested pathogens and pesticides could be identified at the level of the individual honeybee, these were strongly buffered at the colony level where no such effects could be found. Although this does not exclude potential interactions for other pathogens and pesticides in general, it did show that the colony provides a system that is better buffered against environmental changes than is the individual.

The BEE DOC developed various novel diagnostic screening methods including the BEE DOCTOR tool for multiple pathogen diagnostics in one test assay. The BEE DOC identified novel compounds in bee products with relevance for honeybee health and developed concepts for disease prevention using novel treatments with probiotic bacteria. The BEE DOC was linked to various national and international ongoing European, North- and South-American colony health monitoring and research programs, which not only ensured a pan-European but also a global visibility. This greatly facilitated the transfer of results not only within the scientific community but also into apicultural practice in the world community of beekeepers and the general public.

The BEE DOC's broad dissemination strategy succeeded in enhancing the public appreciation for the role of honeybees not only for honey production but also for its pollination services. The significance of honeybee colony losses for ecology, economy and society has been recognized at the level of policy makers and BEE DOC could substantially contribute to this raised awareness.

Project Context and Objectives:

The honeybee decline

Unfortunately, beekeeping has been a declining industry in the past decades with a constant drop in both managed honeybee colonies and beekeeping activities in most EU member states. The colony declining was due to human land use, poisoning, diseases and parasites. The BEE DOC aimed to address the health risks from interactions among parasites, pathogens, and pesticides. Typically, the apiculturist identifies symptoms at the colony level, and then starts diagnostic procedures to identify the disease and initiate a treatment. Yet, when clinical symptoms appear at the colony level, diagnosis often comes too late to save or cure the colony. Consequently, there is a clear need for fast, reliable, sensitive and cheap diagnostic tools that alert the beekeeper to potential problems before colony level symptoms appear. Treatments typically rely on chemicals, which are administered into the colony to target pathogens before colony collapse is inescapable.

The development of such treatments is based on searching for chemicals that are toxic to the pathogen, but harmless to the honeybee. However, so far, any chemical treatment of a honeybee disease, even if successful at the colony level in the short term, has not eradicated diseases at the population level, particularly if the pathogen has a high transmission rate and a high infectivity. As illustrated by present apicultural reality, any chemotherapy of honeybee colonies immediately leads to an obligate contamination of honey and, ultimately more worrying, to resistant pathogens. Moreover, the dramatic colony losses of the past decade suggest that treatments aiming at a single pathogen only, may in principle fall short in curing colonies altogether if the interactions between various pathogens are the main drivers of colony death. A major objective of the BEE DOC consortium was the development of novel diagnostic tools, allowing for a fast, inexpensive and reliable detection of pathogens and pesticides.

The parasite-virus-pesticide meltdown

In the aftermath of the dramatic colony losses in the US (Colony Collapse Disorder CCD) the enormous research efforts invested in the USA, failed to identify a single agent or factor as the definitive cause of the phenomenon. Instead, it seemed that CCD resulted from particular virulent combinations of parasites and pathogens, rather than a classical monocausal disease. Moreover, chronic exposures to pesticides that cause no problems for healthy colonies are suspected to interact with pathogens to produce lethal consequences for colonies already weakened by disease. The BEEDOC aimed at understanding such interactions among parasites, pathogens and pesticides. Since the large number of pathogens and pesticides affecting honeybee health] made it impossible to experiment with all possible combinations of pests, pathogens and pesticides in a rigorous, controlled manner

The BEE DOC focused on two major parasites of honeybees: *Varroa destructor* mites and *Nosema* spp. microsporidians.

The aim was to quantify potential interactions of these parasites with closely associated viruses and two model pesticides. The BEE DOC took a dual-track approach to study these interactions. On the experimental side, the BEE DOC concentrated on selected model systems to maximise the chance for identifying the general principles of such interactions. On the comprehensive side, the BEE DOC looked for correlations between primary factors in existing national monitoring surveys and through comprehensive additional assays of the experimental samples produced.

Parasites

V. destructor is the main obstacle to profitable beekeeping worldwide. Adequate and timely mite control is essential for apiculture in Europe and most other regions of the world. *Varroa* mite control is overwhelmingly based on chemicals that typically end up as residues in honey and other bee products. Even so, several tiny populations of European honeybees coexist sustainably with *Varroa* mites, and do not require chemical control for colony survival. The BEE DOC took advantage of these and similar populations in Australia (*Varroa*-free) and in Africa (*Varroa*-tolerant populations) through its global research network. The BEE DOC consortium aimed to identify traits allowing honeybees to survive *V. destructor* infestations without treatment by screening honeybee populations that are free of *V. destructor* and assessing the impact of apiculture on colony health.

Nosema spp. are wide-spread microsporidian gut parasites of adult honeybees. They infect host mid-gut epithelial cells and deteriorate the metabolic processes of infected bees. Two different *Nosema* species have been reported in *A. mellifera*. *N. apis*, a well established pathogen of *A. mellifera* with moderate virulence that does not usually cause lethal infections, and *N. ceranae*, originally a parasite of the Asian honeybee *A. cerana* now distributed by apicultural trade across the globe. It is now present in all continents except Africa and Antarctica. In Europe, *N. ceranae* had been claimed to cause the sudden collapse of *A. mellifera* colonies in Spain. *N. ceranae* most likely has been present in *A. mellifera* populations for at least a decade and honeybees can be co-infected with both *Nosema* species. *N. ceranae* seems to replace *N. apis* world-wide and to out-compete *N. apis* within a host bee. Due to the similarity in life histories of the two *Nosema* spp., it is likely that the interactions with other factors may be similar for *N. apis* and *N. ceranae*.

Virus infections

At least 18 viruses have been identified affecting brood and/or adult honeybees. *V. destructor* has been shown to be an important vector for several of these. Likewise, a number of viruses seem to be closely linked to *Nosema* infections. Since the large number of viruses affecting bees renders it impossible to run full factorial experimental designs on all possible interactions, BEE DOC focused on the best established and potentially most dramatic virus - parasite interactions. Data on the other viruses were only included from correlational evidence from large scale field data.

Deformed Wing Virus (DWV) is closely associated with *V. destructor* and is by far the most widespread virus of honeybees. DWV became almost ubiquitous throughout Europe after the spread of *V. destructor*. It has been shown to be transmitted by, and to replicate inside *V. destructor*. Furthermore, DWV symptoms in bees appear to be related to whether or not replication occurred in the infesting mites. It is possible that DWV replication in both mites and bees may ratchet the overall virus titres beyond the levels possible through non-replicative mite transmission, or natural bee-bee transmission. Because DWV is closely linked to *V. destructor*, and because of its dramatic epidemiology, this will be a focal virus - ectoparasite system for the BEE DOC.

Black Queen Cell Virus (BQCV) is not known to cause large scale colony collapses. However, epidemiological data suggest a close link with *Nosema* infections, making the latter more harmful. Recent surveys show it to be present in about 30% of colonies in France and central Europe making it a significant virus. Although BQCV derives its name from its effect on developing queen pupae, it is naturally primarily distributed in adult bees which is also the (only) stage affected by *Nosema*. BEE DOC will focus on this virus - endoparasite combination to test for interaction mechanisms occurring at the adult stages of the bee.

1.1.5 Pesticides - The honeybee is unusually sensitive to a range of chemical insecticides, most likely due to a deficit of detoxification enzymes compared to other insects. Foraging bees can encounter lethal pesticide levels when foraging but they can also bring back contaminated nectar and pollen to the hive. Acute mortality can occur and its diagnosis is usually easily established by many dead bees at the front of the hive. The EU directive 91/414 section 2.5.3 regulates the use of pesticides in the context of apiculture. ...'no authorization will be granted if the hazard quotients for oral or contact exposure of honeybees are greater than 50, unless it is clearly established through appropriate risk assessment that under field conditions there are no unacceptable effects on honeybee larvae, honeybee behaviour, or colony survival and development after the use of plant protection products according to the proposed conditions of use'. However, honeybees can also encounter sub-lethal effects of pesticides that are much more difficult to detect since there are no obvious symptoms, as bees gradually die away from the hive. Moreover,

sublethal effects on behaviour can cause disruptions in social interactions which are essential for colony function. In addition to the pesticides the bees are exposed to during foraging, apicultural practice adds to pesticide load of honeybees. For example, beekeepers use various acaricides to control for mite infections, particularly Varroa destructor. Acaricides are supposed to be harmless for the bees, but since most of them are lipophilic they accumulate in the wax, increasingly contaminating the combs where the brood develops. Probably more important, nothing is known about the effects of interactions between agricultural pesticides foraged by the bees, the acaricides applied by the beekeeper and pest and pathogens.

In light of the huge suite of agro-chemicals used in agriculture and it was clearly impossible to run full factorial design experiments testing the effects and interactions of all of these compounds among each other, with the parasites, and the pathogens. The BEE DOC therefore focussed on the effects of two major compounds, one agro-pesticide, thiacloprid, and one acaricide, τ -fluvalinate. Thiacloprid, a pesticide of the neonicotinoid group with low acute bee toxicity, is an active, broad-spectrum insecticide used against major lepidopteran, coleopteran and orthopteran pests on a wide range of field and horticultural crops. This pesticide is widely used on oil seed rape and orchards which represent the field crops intensively used by honeybees. Consequently, the active ingredient is commonly found in the hive and in pollen pellets of foragers. τ -fluvalinate from the pyrethroid family is used to control a broad range of pests including moths, aphids, thrips and leafhoppers, and also in the honeybee hive to control for V. destructor. As it is used to control the mites in beekeeping, it accumulates in the wax of the comb at high concentrations and is one of the most common pesticides found in honeybee colonies. Thiacloprid and τ -fluvalinate represent active ingredients of the two most important and common pesticide groups (pyrethroids and neonicotinoids) with different mode of action in the target organisms. The BEEDOC objectives were to deal with the interactions of pests and pesticides at both the colony (WP2) and the individual level (WP1) and identify interactions of Varroa with DWV and selected pesticides; between N. apis and N. cerana ; of Nosema with BQCV, IAPV and the two pesticides

Disease resistance genetics and genomics

In BEE DOC, the immune system of honeybees was at the centre of interest because the responses of the bees towards virus infections need to be addressed. Although A. mellifera lacks about 30% of immune system genes that are known from different dipteran species (e.g. Drosophila melanogaster, Anopheles gambiae) the three basic immune pathways - the Toll-, IMD- and JAK/STAT-pathway have also been identified in the honeybee genome. One argument for the deficit of immune genes was that colony level pathogen resistance mechanisms would be more important in social insects compared to solitary insects. However, individual resistance mechanisms are equally essential in a social context as in the solitary one. It was therefore likely that the gene cascades in these pathways are differently regulated compared to the other insect model systems, yielding similar results yet in a

colony context. We expected to see very specific modifications of the already known immune gene cascades for evolutionary stable host parasite systems but also highly species specific yet unknown mechanisms for controlling the pathogen resistance of bees. Transcriptomic analyses should reveal differential genome responses to specific infections. Although such studies cannot identify the function of novel honeybee specific genes, they can reveal the function of gene cascades in response to various environmental conditions including bee health stressors, which are essential to understand gene control of the phenotype. The BEE DOC dealt with the genomic responses to multiple infections and environmental factors to quantify host - parasite - pesticide interactions at the transcriptome level aiming to identify gene cascades and novel candidate genes relevant for honeybee health.

Most phenotypes with relevance to disease resistance will be controlled by several so called quantitative trait loci (QTL). If there are only few major loci, these can be mapped by testing linkage with segregation of a large number of variable markers (e.g. microsatellite markers) which saturate the genome. Because the recombination rate of the honeybee genome is 19 cM/Mb, an order of magnitude higher than in *Drosophila*, honeybee mapping studies require a large number of marker loci to saturate the genome. For gene identification, the high recombination rate is however an advantage, because linked markers can be physically very close to a target gene. Once a genomic region with a QTL has been identified, the sequence allows for saturating this target region with a large number of novel microsatellite markers for fine mapping. Taking advantage of haploid males for mapping resistance traits The BEE DOC aimed to identify novel genes for resistance to *Nosema* by mapping and identifying potential major genes (QTL), which control the *Nosema* resistant phenotypes identified by the extension partners and comparing phenotypes in knock out mutants in *Drosophila*

Probiotic bacteria and honeybee health

Lactic acid bacteria (LAB) from the genus *Lactobacillus* were known as favourable bacterial species, commonly found in healthy individuals and commercially important through their use in probiotics (live micro-organisms, which confer health benefits on the host). *Bifidobacterium* is by definition not a 'true' LAB member, but because of its lactic acid production and its known positive effects on human and animal gastrointestinal flora, it is commonly placed within this group. The honey stomach of the honeybee has a unique probiotic gut flora with *Lactobacillus* and *Bifidobacterium* bacteria that have evolved in mutual dependence on one another. The LAB obtains a nutrient rich niche whereas the honeybees are protected by the LAB from harmful micro-organisms. LAB produces such antibacterial compounds as organic acids, hydrogen peroxide, diacetyl, benzoate, and bacteriocins, all of which are beneficial for humans and animals and presumably for honeybees as well. This new discovery warranted attention when honeybee health was to be considered and, focus should not only be on the pathogens, but also beneficial probiotics. BEE DOC investigated the potential benefit of probiotic bacteria to counteract effects from

pathogens. In addition plant compounds collected and metabolized by the bees had shown to have antibiotic and antiviral potential. Their function in concert with the other innate or acquired pathogen defence system was not well explored hampering the development of novel treatment based on using natural compounds. Hence the BEE Doc embarked to identify probiotic bacteria with a positive effect on honeybee health, to identify honeybee produced or foraged compounds with positive effects of honeybee health and to develop application methods for plant derived compounds. In synthesis, the BEE DOC planned to transfer these results into novel strategies for bee health, such that the apiculturist can be timely assisted in preventing colony losses. Proactive prevention, selection for resistance combined with efficient diagnostics should enable the beekeeper to take timely measures avoiding diseases before large scale colony collapses occur.

Project Results:

Final Report Summary for BeeDoc (244956 CP-FP) for the period 1.3.2010 - 28.2.2013

OVERALL CONCEPT AND PROJECT OBJECTIVES

Unfortunately, beekeeping is a declining industry and the past decades saw a constant drop in both managed honeybee colonies and beekeeping activities in most EU member states. Most beekeepers are either hobbyists or part-time operators, with a rapidly ageing demography. On top of this, wild or feral bee colonies are also rapidly declining due to human land use, poisoning, diseases and parasites. In monetary terms, these losses of honeybee colonies in the EU alone result in a significant direct damage exceeding 400 million EUROS per year for the apicultural industry. One of the principal reasons for the decline in managed honeybee colonies, and of beekeepers, is extensive and unpredictable colony death. While for small-scale hobbyist beekeepers this can be discouraging enough to abandon the hobby, for (semi)-professional operators this is a crucial limitation to business planning and expansion. Moderate and predictable losses can be accommodated and planned for. However, extensive and uncontrollable losses make beekeeping as a profession, with heavy investment in material and equipment, an enterprise at permanent risk of bankruptcy. This financial uncertainty also limits recruitment of a new generation of beekeepers, especially to the professional ranks. It is these colony losses that this project aims to address.

The combination of pests, parasites and pesticides results in an inadvertent 'meltdown' with one negative factor enhancing the negative impacts on honeybee health of the others. Unfortunately, the large number of pathogens and pesticides affecting honeybee health] makes it impossible to experiment with all possible combinations of pests, pathogens and pesticides in a rigorous, controlled manner. Since the call text explicitly referred to the two major parasites of honeybees: Varroa destructor mites and Nosema spp. microsporidia. The BEE DOC therefore focused on the interactions of these parasites with closely associated viruses and selected pesticides. On the experimental side the BEE DOC concentrated on selected model systems to identify the principle interactions with the greatest significance for colony health throughout Europe, and the world. On the comprehensive side the BEE DOC looked for correlations between primary factors in existing national monitoring surveys and through comprehensive additional assays of the experimental samples produced.

In order to efficiently control diseases with novel, sustainable strategies, the BEE DOC aimed at understanding the infection processes at all relevant levels: from the apiary, via the

colony and individual bee, down to the molecular immune mechanisms at the genome level. The interactions among pathogens driving virulence and transmission of diseases were comprehensively studied to eventually design more efficient treatments that are also effective at the population level. The BEE DOC worked out strategies that not only increased bee tolerance to specific diseases, but also showed how pathogen virulence can be reduced by jamming critical mechanisms in the infection pathways.

The BEE DOC worked with seven tightly interconnected work packages run by eleven partners coming from honeybee extension, pathology, genetics, genomics and chemistry. The work packages combine empirical evidence from large scale field data sets, field experiments, controlled laboratory experiments, development of diagnostic tools and transfer into actual extension.

WORK PACKAGE 1

INTERACTIONS AMONG PARASITES, PATHOGENS AND PESTICIDES IN INDIVIDUAL BEES

The overall objective of WP1 was to identify interactions between Varroa, DWV, other viruses and selected pesticides at the level of the individual honeybee (larval/pupal and adult bees) through quantification of experimentally induced interactions within the laboratory and using standardised protocols. The rationale for WP1 was that, fundamental to determining the role of multiple factors on honeybee colony demise is an understanding of their impacts at an individual bee level. The purpose of WP1 was therefore to address the intricate mechanisms and interactions of multiple factors detrimental to honeybee health at the level of the individual honey bee using highly controlled experimental paradigms.

The first set of observations and experiments (Tasks 1.1 to 1.3) dealt with honey bee larvae/pupae. Under natural settings in the hive, a close relation was found between the DWV titre of parasitizing Varroa mites and the DWV pathogen load of resultant honey bee pupae that develop from parasitized prepupae. Additional observations and experiments showed that DWV is directly responsible for symptomatic DWV infections in adult honey bees and that, in nature, this is caused primarily by quantitative transmission of the virus by Varroa during parasitism of honeybee prepupae. Initial experiments were undertaken with honey bee prepupae that were removed from their cells, experimentally treated topically with pesticides, returned to cells, and then Varroa mites added to them as a virus vector. Experimental manipulations of prepupae proved intractable (high mortality) and so a revised experimental paradigm (standardised protocol) was developed to investigate the impact of sublethal doses of pesticides and viruses on honey bee larvae/pupae. This involved rearing juvenile stages entirely in vitro from egg through to adult in microtitre plates, then

administering sublethal doses of pesticides in larval food and pathogens either in larval food or by injection.

Our experiments showed that viruses transmitted by Varroa mites and pesticides had a detrimental effect on honey bee brood development and survival. When administered in combination and at sub-lethal doses, there was an additive - and occasionally a synergistic/multiplicative - effect between pesticides and viruses in hindering larval development and increasing larval/pupal mortality. Specifically:

- BQCV interacted additively with either t-fluvalinate (a synthetic pyrethroid pesticide) or thiacloprid (a neonicotinoid pesticide) to reduce larval survival; and
- DWV interacted synergistically (multiplicatively) with either t-fluvalinate or thiacloprid to reduce larval survival.

When parasitizing juvenile stages of honey bees, BQCV and DWV have profound effects on their hosts, developing honey bee larvae, causing developmental abnormalities and mortality with increasing pathogen loads. Pesticides (t-fluvalinate and thiacloprid), when experimentally administered at sub-lethal doses to larvae or pupae, generally interacted additively with these two viruses, DWV and BQCV, to elevate mortality and developmental abnormalities. There was even a potentially synergistic interaction between DWV and pesticides when the virus was fed at high but biologically realistic doses to larvae. In summary, pathogens had a major impact on larval/pupal health, pesticides had a subtle effect on larval/pupal health but interacted additively or even synergistically with pathogens.

The second set of observations and experiments (Tasks 1.4 to 1.7) dealt with honey bee adults as hosts and the pathogens DWV, BQCV, Nosema spp. and the pesticides as used in larval/pupal experiments. We developed standardised protocols for experimentally treating caged honey bees with DWV, BQCV, Nosema spp. and pesticides, including either feeding or injecting DWV into adult honey bees and either feeding (thiacloprid) or allowing surface contact (t-fluvalinate) of pesticides.

In our experiments we found that viruses transmitted by Varroa mites, Nosema microsporidia and pesticides all have a detrimental effect on honey bee adult survival. When pathogens were administered orally to host honey bees in cages, they occasionally interacted synergistically to elevate host mortality. When a viral pathogen (DWV) was administered by injection into host honey bees in cages (simulating the natural route of infection via Varroa), it interacted synergistically with a common pesticide used to control

Varroa, greatly enhancing adult honey bee mortality. Synergistic interactions occur between pathogens and pesticides to enhance adult honey bee mortality.

Specifically:

- Nosema apis and Nosema ceranae exist in European populations and, when fed simultaneously to adult honey bees, grow at similar rates within their host;
- BQCV interacted synergistically (multiplicatively) with Nosema to reduce adult survival; thiacloprid interacted additively with BQCV-Nosema treatments to reduce survival further;
- Nosema reduced DWV growth in host honey bee midguts when the latter was administered orally but DWV did not reduce Nosema growth;
- DWV injected into adult honey bees caused very high mortality, more than observed when honey bees were treated with Nosema; DWV interacted additively with Nosema to enhance mortality; and
- DWV injected into adult honey bees and t-fluvalinate were additive in reducing adult honey bee survival even further.

Nosema, BQCV and DWV had profound effects on adult host honey bees, often interacting additively or synergistically to enhance host mortality. There is a causal relationship between Nosema and DWV replication in the host (in its midgut/ventriculus). The acaricide t-fluvalinate does not interact markedly with tested pathogens but neonicotinoid thiacloprid does with Nosema. Interactions between pathogens and pesticides, whether accidentally acquired by foraging honey bees or purposefully administered to colonies by beekeepers to control Varroa mites, need to be considered when revising beekeeping management practices to reduce honey bee mortality. Overall, pathogens had a profound impact on adult honey bee survival whereas pesticides had a subtle, additional and occasionally synergistic impact on adult survival.

WORK PACKAGE 2

INTERACTIONS AMONG PARASITES, PATHOGENS AND PESTICIDES AT THE COLONY LEVEL

The overall objective of WP2 was to identify interactions between Varroa, BQCV, Nosema and selected pesticides at the level of the honey bee colony. The purpose of WP2 was therefore to investigate to what extent sublethal and/or synergistic effects of pesticides and pathogens on the individual bee have an impact on the honey bee colony as a 'superorganism'.

Specific objectives of WP2 had been to:

- identify interactions of Varroa with DWV and pesticides,
- identify interactions of Nosema with BQCV and pesticides and
- identify interactions of viruses with Varroa/Nosema and pesticides at the colony level

Effects of a chronic exposure of full sized honey bee colonies to different combinations of pesticides over a one year period (task 2.1)

We established colonies from artificial swarms of uniform size at three locations at Hohenheim (UHOH, Germany; 2010 and 2011), Liebefeld (ALP, Switzerland 2011) and Avignon (INRA, France, 2010). Colonies were then randomly assigned to one of four pesticide treatment groups (N=4-5 per group), and continuously exposed to the following treatment regimes:

- 1) thiacloprid feeding (approx. 1 kg sugar syrup solution with 1,000 ppb per week),
- 2) 0.4 g τ -fluvalinate applied as Apistan® strip (one per brood chamber, replaced after 6 weeks),
- 3) thiacloprid plus τ -fluvalinate treatment and
- 4) untreated control.

These compounds were chosen because they are widely used in plant protection and beekeeping practice and are commonly detected as residues in nectar and pollen.

We could not detect any group specific differences in population dynamic or winter mortality of the colonies. However, at one location (ALP) all colonies not treated with τ -fluvalinate died in late autumn due to an exceptional high infestation with Varroa mites.

From these experiments we can therefore summarize that:

- (i) a chronic contamination with sublethal doses of thiacloprid and/ or τ -fluvalinate does not have a measurable effect on population dynamics of treated colonies and
- (ii) Varroa infestation is a crucial factor for the survival of honey bee colonies independent from the pesticide application.

Effects of combinations of pesticides and pathogens on the longevity, social behavior and flight activity of worker bees on the colony level (task 2.2 and 2.3)

We here established a new test system consisting of observation hives and mating nucleus colonies. In contrast to the hoarding cages these entities represent a functional colony with a limited number of bees facilitating the quantification of longevity and behaviour of treated bees. We compared the life span, social behaviour and flight activity of individually marked bees that have been chronically (thiacloprid and τ -fluvalinate) or once-in-a-lifetime (clothianidin) exposed to pesticides and pathogens (BQCV, *Nosema ceranae*). Pesticides and pathogens were applied as single treatments and in different combinations. In total, data over the lifetime of more than 5,000 bees were recorded.

From these results we can clearly state that effects proven at the individual level not necessarily appear on the colony level. We could confirm a striking effect of BQCV on the longevity and, depending on the duration of the experimental periods, a somewhat lower effect of *Nosema* infection on both, life-time and flight activity. The chronic exposure of thiacloprid and τ -fluvalinate had only in one replicate a slight but significant effect on the longevity while a single application of about 0.25 ng clothianidin (corresponding to approximately 7% of the LD50) did not reveal any effect. A remarkable result is that at the colony level no synergistic effects of the applied pesticides on the longevity and flight activity could be detected.

A conclusion from these experiments is that tests on individual bees or bees in hoarding cages (see results of WP1) are not sufficient for a final evaluation of the effects of pesticides and pathogens on the colony level. Our approach with free flying small colonies represents an additional tool for such evaluations.

Prevalence of six honey bee viruses over the season in treated and control colonies (task 2.4)

We here performed four sampling dates in 16 colonies kept under a defined regime in task 2.1. Therefore, we could not only record the prevalence, but also potential seasonality of the different viruses over time. By use of the MLPA screening method four out of the 6 viruses were detected at least once in the investigated samples, namely the SBV, BQCV, DWV and CBPV. In contrast to those, neither SBPV, nor ABPV-JAPV-KBV was found in the samples. Our qualitative results showed that the colonies are exposed to a certain range of honey bee viruses. There seem to be seasonal variations, most obvious for the DWV, which is known to be closely linked to the mite *Varroa destructor*.

These colonies were also used for propolis sampling in autumn. The analysis of the typical constituents of propolis did not reveal specific patterns in relation to the pesticide application.

Pan-European Nosema surveillance (task 2.5)

Our data clearly show that Nosema spores are present in almost every honey bee colony in Europe. Surprisingly, we could only find *N. ceranae* in German samples and probably in French samples too. Obviously, this new parasite has - at least in Southern Germany - completely replaced the original parasite *N. apis*. In Northern Europe it seems that *N. apis* is still dominant for unknown reasons. Environmental factors like temperature might contribute to these differences in species distribution.

The number of infected colonies and the infection rates of all partners revealed a slight seasonality with a higher prevalence in spring and autumn. However, this seasonality seems to be less pronounced, compared to *N. apis*.

Our data clearly contradict reports from Southern Spain indicating tremendous damages of infected colonies. We did not see any clinical symptoms and none of the participating beekeepers complained about damages of his or her infected colonies in terms of the new parasite *N. ceranae*. No winter colony losses could be confirmed due to Nosemosis. These results are verified by the surveys from all partners and by additional analysis of artificially infected bees in observation hives.

For all our data compiled over a 2-3 year period, no infection course for *N. ceranae* could be clearly determined. Therefore long term monitoring projects are needed, which focus on prevalence, infection course and infection intensity of Nosema spp. in honeybee colonies throughout Europe.

WORK PACKAGE 3

The overall objective of WP3 was to measure transcriptomic response in honey bees to factors tested in WP1 and WP2 (Varroa, Nosema, viruses, selected pesticides) using microarray and RNA sequencing techniques in order to understand the genomics of pathology at the individual and colony levels.

Specific objectives of WP2 had been to:

- quantify host - parasite - pesticide interactions at the transcriptome level
- identify gene cascades and novel candidate genes relevant for honeybee health

Task 3.1 Effect of pathogenic stressors on transcriptome with microarrays.

We completed a digital gene expression analysis for abdomens of Varroa-parasitized adult bees (Alaux et al. BMC Genomics 2011). We investigated the transcriptome of 10 day-old worker bees parasitized in the brood cell by the mite Varroa destructor, in the presence and absence of pollen diet. The 4 experimental groups (control bees without a pollen diet, control bees fed with pollen, Varroa-parasitized bees without a pollen diet and Varroa-parasitized bees fed with pollen) were analyzed by performing a digital gene expression (DGE) analysis on bee abdomens. Around 36,000 unique tags were generated per DGE-tag library, which matched about 8,000 genes (60% of the genes in the genome). Comparing the transcriptome of bees fed with pollen and sugar and bees restricted to a sugar diet, we found that pollen activates nutrient-sensing and metabolic pathways. In addition, those nutrients had a positive influence on genes affecting longevity and the production of some antimicrobial peptides. However, Varroa parasitism caused the development of viral populations (Deformed wing virus, Varroa destructor virus, Kakugo virus) and a decrease in metabolism, specifically by inhibiting protein metabolism essential to bee health. This harmful effect was not reversed by pollen intake.

Task 3.2 Test interactions parasite - pesticides on transcriptome with microarrays at the individual level

We have completed RNAseq analysis for brains of Nosema ceranae-, BQCV- and N.ceranae/BQCV-parasitized adult bees. The brain transcriptomes of 15 day-old bees infected with BQCV, Nosema ceranae or a combination of BQCV/N. ceranae were analysed. As part of the results from WP1, a higher mortality was observed for co-infected bees, suggesting a synergistic interaction between the two pathogens. Brains were analysed by Illumina mRNA sequencing to determine if viral and N. ceranae infections produced similar response patterns and if a combination of pathogens produced synergistic or additive patterns of expression. Nosema infection produced very few changes in the brain compared to controls (13 genes). BQCV (compared to controls) resulted in 146 genes changing of which only 1 is shared with Nosema infection. In contrast the combination of BQCV/Nosema changed 71 genes, of which 6 were shared with Nosema infection, 32 shared with BQCV infection, and 32 genes were unique. Genes involved in immune and defense response (abaecin, apidaecin, apidaecin 14, hymenoptaecin) were significantly represented in both BQCV and BQCV/Nosema-induced brain transcriptomes. The brains of bees seem to be little affected by or able to cope with Nosema infection. In addition, Nosema infection combined

with BQCV reversed the expression of the 113 genes induced by BQCV, suggesting a normalizing effect of Nosema on the BQCV-infected honey bee brain.

Task 3.3 Test interactions parasite - pesticides on transcriptome with microarrays at social level

We have completed digital gene expression analysis for brains of Varroa destructor- or Nosema ceranae-parasitized adult bees. We compared the transcriptomes of the brains of adult workers parasitized by Varroa destructor or Nosema ceranae. Given that the stress of parasitism induces behavioural changes in adult honey bees, we hypothesized that the brain transcriptomic profiles of Varroa- or Nosema-parasitized bees would show similar expression changes, which also were marked by characteristics of forager behavioural profile. We compared the transcriptome of the brains of 10 day-old bees parasitized by Varroa destructor in the larval cell or fed with Nosema ceranae spores as one-day-old bees to control bees using digital gene expression. In Nosema-parasitized brains, few gene changes (57 genes) were observed compared to Varroa-parasitized brains (455 genes). There was significant overlap between gene expression profiles of brains from Varroa and Nosema-parasitized bees, but no significant overlap with forager/nurse brain gene expression profiles. Two viruses, deformed wing virus and Varroa destructor virus were identified in the brain, and deformed wing virus was significantly higher in Varroa and Nosema-parasitized brains. Expression changes in Varroa-parasitized brains were enriched for functions related to glutamate/GABA receptors and neurotransmitter metabolism. Thus, the shared responses to parasitism by Varroa or Nosema in the brains of honey bees reveal a general response in the brain that may relate to subsequent behavioral changes, such as early departure from the hive. Varroa-parasitized bees exhibited additional gene expression changes that may reflect the disruption of learning and memory development by the mite.

WORK PACKAGE 4

IDENTIFICATION OF NOVEL GENES FOR RESISTANCE TO NOSEMA

The overall objective of WP4 aimed at prevention of diseases by searching for novel honeybee specific genes that convey resistance towards diseases. The BEE DOC took advantage of the haploid drone genome which greatly accelerated the mapping of such genes compared to diploid organisms. The function of the identified candidate genes was tested and verified using gene expression studies in honeybees, and also by using knock out mutants in Drosophila.

Specific objectives of WP4 had been to:

- conduct the mapping and identification of potential major genes (QTL), which control the Nosema resistant phenotypes.
- identify these QTL by taking advantage of the haploid genotypes of males and the genome
- test gene function in the honeybee
- knock out genes in Drosophila

Task 4.1 Mapping population

We identified a honeybee population resistant towards Nosema in Denmark and established a mapping population by producing hybrid queens of a susceptible and this resistant population to identify major QTLs for the resistance trait using drone offspring of these hybrid queens.

Task 4.2 Bulk segregant analysis.

In a first mapping step we used "bulk segregant analysis" with over 400 microsatellite marker loci to test pooled DNA from many drones with extreme phenotypes.

Task 4.3 Fine mapping. In a second step, we narrowed down potential target regions within these linkage groups and selected 20 novel microsatellite markers in the region of interest directly from the genome sequence. We could identify one specific region comprising the gene *aubergine*, a transcription factor from the *piwi* family as a prime candidate gene determining the resistance phenotype.

Task 4.4 Expression of candidate genes.

Since we detected epistatic interactions among loci in the QTL analyses it seemed prudent to first concentrate on the genetically simple haploid drones before embarking on the analyses of diploid genomes of workers. In order to correlate the gene expression with phenotypic data we used the same phenotypes as in the QTL study (survival and spore number) to compare the gene expression with the phenotypes. Hence we compared the immune response of the tolerant and the susceptible honeybee lineage, taking advantage of the haploid males to study its potential impact on the tolerance towards *Nosema ceranae*. After artificial infections of the *N. ceranae* spores, the lineage selected for *Nosema* tolerance showed a higher *N. ceranae* spore load, but a lower mortality and an up-regulated immune response. The differences in the response of the innate immune system between the selected and unselected lineage were strongest at day six post infection. In particular genes of the Toll-pathway were up-regulated in the selected strain, probably is the main immune pathway involved in *N. ceranae* infection response. After decades of selective breeding for

Nosema tolerance in the Danish strain, it appears these bees are tolerant to *N. ceranae* infections.

Task 4.5 ds RNAi of candidate genes.

Due to unforeseen toxicity of the RNAi treatment, we could not knock down the candidate genes in the drones within the foreseen timeframe. We therefore chose alternative routes to independently verify the importance of the detected QTL for resistance. The tolerant honeybee colonies in Denmark had been selectively bred for Nosema free over decades. As the tolerance towards the Nosema infection had been a result of artificial selection, we screened chromosome 14 in the resistant population in Denmark for a selective sweep with microsatellite markers. By comparing the genetic variability of ten colonies of the selected honeybee strain with a population sample from 22 unselected colonies, a selective sweep was revealed within the previously identified QTL region. The genetic variability of the swept region was not only reduced in relation to the flanking markers on chromosome 14 within the selected strain, but also significantly reduced compared to the same region in the unselected honeybees. This confirmed the results of the previous QTL mapping for reduced Nosema infections.

In addition and based upon the results in the fruit fly *Drosophila melanogaster* (Task 4.6) we studied the changes in the gut epithelium of infected drones with histological tools. There were profound differences in the cellular immune response. The drones from the resistance strain showed a much stronger cellular response than the susceptible bees.

Task 4.6 Role of candidate genes in *Drosophila*.

Since we failed to block genes in the honeybee, the experiments in *Drosophila* became a major backbone to understand the molecular mechanisms involved in the Nosema resistance. We analysed knock out mutants in *Drosophila* homologous to aubergine and tested the function of these genes in the fruit fly by comparing wild type with mutant flies. For this purpose we used previously generated *Drosophila* mutants as well as hairpin knock-down stocks from the VDRC (22,247 stocks, 12,251 genes) and NIG-Fly (11,386 stocks, 6,030 genes) stock centres. The *Drosophila* mutants were tested for their effects on humoral (Toll or Imd signalling) or cellular (haemocyte mobilization, lamellocyte formation, phagocytosis) immune responses.

To investigate the role of aubergine and piwi in the intestine, we crossed aub- or piwi-RNAi stocks to the GAL4 line NP3064, which drives expression in the larval intestine. In the offspring we found a substantial number of third instar larvae with melanotic spots in the abdomen, a phenotype that is rare in the controls. These discolorations, known as nodules,

usually appear at wound sides, in necrotic tissue or when blood cells encapsulate foreign objects such as bacteria or parasitic wasp eggs and they are regarded as a manifestation of a cellular immune response. Mutations that cause aberrant or constitutive activation of blood cells, such as the constitutively active Toll mutation Tl8, are also known to cause this defect [18]. Consequently, we found nodules in all offspring larvae coming from crosses of UAS-Tl8 to NP3064-GAL4. However, in contrast to crosses with aub- or piwi-RNAi, nodules in these larvae were not limited to single region of the body.

The fact that silencing piwi or aubergine expression in haemocytes enhances most hematopoietic phenotypes observed in the Tl8 mutant background, could indicate that piwi and aubergine may function directly in the Toll pathway. The melanotic phenotype we observe in the hindgut when we knock down aubergine or piwi with a general gut driver is consistent with a role for these genes in the local immune response.

WORK PACKAGE 5

DIAGNOSTIC TOOLS

The overall objective of WP5 was to development of three diagnostic tools at different levels of application.

Specific objectives of WP5 had been to:

- develop diagnostic tools for pathogens and pesticide exposure
- cover three levels of application: research grade, extension grade and field grade.
- develop an insect cell culture system for propagation of honeybee viruses

Task 5.1 Diagnostic DNA chip (Research grade)

The research grade diagnostic tool is a colorimetric DNA chip with 110 targets covering the immunity pathway, markers for pesticide exposure, Nosema infection, Varroa infestation and nutritional stress. This method is called 'BeeClinic'. This method was developed and optimized using E. coli challenged honey bees. A colorimetric development has as consequences that we should use nylon or nitrocellulose coated glass slides. In a first stage the coating of the slides was optimized to prevent leakage of the different conditions. After this problem was solved the hybridisation and development was optimized. The slides are then scanned using the ArrayIt® SpotWare™ colorimetric microarray scanner and 16-bit TIFF images were generated at 5 µm resolution. The TIFF images were processed with Mapix

(Innopsys) to ascribe a value to the spot intensity, which was then corrected by background intensity. The intensity data were standardized across different slides using the actine house keeping gene. Using this optimized method some data sets were generated for Nosema infected honey bees, BQCV infected and Nosema/BQCV infected honey bees. We could show that Nosema infected honey bees are immune repressed like shown before and that marker genes provided from WP3 show the same expression pattern as determined by Alaux et al. Another dataset was generated for chronic exposed honey bees to tau-flavinate and thiacloprid. The differently expressed genes were determined for just emerged honey bees and old honey bees. We could show that younger bees react more strongly compared to older bees. But generally the bees are immune repressed. The detoxification proteins belonging to the CYP6AS clade were upregulated upon exposure while the CYP9Q clade and the AChE were repressed.

Task 5.2 PCR based diagnostic tools (Extension grade).

For the extension grade an multiplex-ligation probe amplification (MLPA) approach was developed which makes it possible to screen for 10 different honey bee viruses in one reaction. This tool is called 'BeeDoctor'. For each virus, probes were developed to screen for the negative and positive strand from the positive stranded RNA honey bee viruses. Because the screened viruses are RNA viruses we used the RT-MLPA assay. qPCR positive samples for different viruses were used to validate the method. Because of its high sensitivity and specificity, the RT-MLPA assay was used in an epidemiological survey of honeybee viruses based on adult bee samples collected in Flanders during the summer of 2011. The 'BeeDoctor' assay was used to screen 363 apparently healthy colonies from randomly selected apiaries throughout Flanders. This survey showed that almost 80% of colonies are infected with at least one virus, and many with multiple infections, showing that virus infections in apiaries are quite common, even in the absence of clinical symptoms.

Task 5.3 Diagnostic Stick for virus infections (Field grade)

In a third tool, the field grade tool, we wanted to produce a prototype immunological Lateral Flow Device for rapid field-testing of six different honeybee virus infections. Antibodies were raised against synthetic peptides based on the capsid protein sequences of the different viruses rather than against natural virus isolates. The primary evaluation proved without doubt that the antisera contained highly specific antibodies that reacted exclusively and with high avidity with their corresponding antigens. However, the secondary evaluation proved that significant non-specific background problems existed within the antisera when using natural honeybee extracts, or virus preparations derived from these. Due to these problems we could not develop this field grade tool to completion until the issue with unspecific cross reactions has been resolved.

Task 5.4 Insect cell culture for honeybee viruses

In a last task we wanted to develop an insect cell culture system for propagation of honey bee viruses. Therefore Sf9 cells were used and different virus preparations were added. We could detect the virus (positive strand) in the medium but we failed to detect the positive and negative strand of the virus inside the cells which suggests that the honeybee viruses are not able to enter the cells. We had been aware of the high risk this task would entail and the eventuality of failure had been included as an option in the Description of Work.

WORK PACKAGE 6

SURVEILLANCE OF PATHOGENS IN SELECTED HONEYBEE POPULATIONS

The overall objectives of WP6 were to investigate the mechanisms behind Varroa mite tolerance, to identify the importance and prevalence of virus infections associated with this mite and to evaluate the influence of apiculture on honeybee health.

Specific objectives of WP6 had been to:

- identify traits allowing honeybees to survive V. destructor infestations without treatment
- screen honeybee populations that are free of V. destructor
- assess the impact of husbandry on colony health
- data-mine large scale on-going colony health monitoring projects around the world

There had been four tasks to WP6, namely Task 6.1 - Task 6.4.

Task 6.1 Traits allowing honeybees to survive infestations of V. destructor mites

We have identified suppression of mite reproduction in two separate honey European honey bee populations as an important trait in colonies that survive Varroa infestations without mite control. The suppression of mite reproduction is probably achieved through different traits in the two populations. The collected data conclusively demonstrate that for these surviving populations, reduced mite fertility/fecundity is at the core of colony survival with mite infestations. There were significant differences between the surviving colonies and the control colonies in both Avignon and Gotland for the infertility rates, the proportion of dead progeny, the proportion with an absence of a male mite, the proportion of mother mites with delayed egg-laying, and the fecundity expressed as the mean number of eggs laid per reproducing mother mite. Although all the parameters rendered significant differences from

the control colonies in the statistical analysis, the most dramatic within location differences were seen between the mean fecundity for both locations, the infertility rates in Avignon, and the delayed egg-laying on Gotland. Mite reproductive success was also significantly different between the surviving population on Gotland and the surviving population in Avignon. Comparing the reproductive parameters indicates that the fecundity and the proportion of infertile mites were highly significantly different between the two mite tolerant populations.

We have screened Varroa resistant populations for virus infections and compared the infection prevalence and virus titres in samples from Sweden, Mexico and South Africa. The analyses of virus infections in bees on Gotland did not suggest DWV resistance helps these mite resistant colonies to survive. Likewise, both in Mexico and in South Africa, mite resistant colonies survive without being DWV resistant. Unexpected was the significantly lower titres of BQCV and SBV at the end of the season in the mite resistant colonies in Sweden compared to control colonies. This could indicate variations in virus infection susceptibility between mite resistant and non-mite resistant colonies for infections other than the mite associated DWV infections. A further conclusion we can draw is that, at least in South Africa, high density beekeeping might negatively impact on honey bees in terms of pathogen loads.

Task 6.2 Virus screening in honeybee populations with no Varroa

We have screened honeybee populations free of Varroa and compared with adjacent populations where Varroa is present for virus infections. The results show few major qualitative differences, but rather quantitative differences. The results highlight the dramatic impact for DWV prevalence and titers of mite infestations. There is a consistent pattern of lower overall pathogen and virus prevalence and loads in Varroa-free regions and colonies than in Varroa-infested regions and colonies. There are major differences between individual viruses in this regard: DWV for example, which is known to be actively transmitted by Varroa, is consistently more prevalent and abundant in regions with a history of Varroa infestation than in Varroa-free regions while BQCV is much less affected by current or historical Varroa infestation, as is CBPV, which was only detected with any frequency in Canada. SBV does not appear to be much affected by Varroa status in Canada, but more so in Sweden and was largely absent from both Varroa-infested and Varroa-free regions in Norway. This accurately reflects the current uncertainty concerning the nature of the relationship between SBV and Varroa found in other studies.

Task 6.3 Data mining selected on-going monitoring projects on bee health

We have studied data from monitoring projects in Germany, Sweden and the international COLOSS network. From the German data it is obvious that in Germany, the nosema parasites (and in particular *Nosema cerana*) is regularly found in many colonies without an obvious

link to colony mortality as reported from Spain. Of the virus infections present, it is by far DWV that is most common. When DWV is correlated with colony losses it appears that the probability of losses is slightly increased if DWV is diagnosed. However, DWV is closely associated with Varroa mites and in this case an effect from DWV independent from Varroa influence has not been carried out. Clearly, Varroa mite levels is closely linked with colony losses in the German data material and it can be concluded that variation in the mite infestation levels is the parameter that explains most of the losses experienced.

From the Swedish data material it is obvious that losses vary greatly between years and that also in the past large losses were encountered in individual years. There may be a tendency during the last decade with losses above average (12.9 %). The reasons behind losses appear to vary between years but according to web questionnaires organised during the last decade a main culprit in losses appears to be the Varroa mite. Although other parameters besides mite control seem to influence wintering success, such as time for winter feeding and amount of winter food, the mite problem with its associated virus infections is at the core of winter losses in Sweden.

Data from the COLOSS network illustrate that there is often no uniform trend but that local conditions vary and wintering results will deviate accordingly. However, all available information end up with the same fundamental conclusion: Although many different factors or pathogens may be associated with colony losses, the main culprit is still and with no doubt, the Varroa mite.

Task 6.4 Comparing colony health in wild and managed honeybees

We have investigated virus prevalence in managed, feral and wild honeybee colonies. The population genetic analysis showed a significantly higher heterozygosity and number of alleles for the managed populations compared to the feral and wild colonies. The comparison between feral and wild populations did not show significant differences. Prevalence for viruses was significantly higher in the managed colonies of South Africa compared to feral and wild ones in spite of the genetic diversity, probably because of effects from within apiary disease transmission. In Mexico, the disease burden of honey bees was similar in regions with high versus low densities of managed honey bees.

WORK PACKAGE 7

NOVEL TREATMENT AND CONTROL

The overall objectives of WP7 focused on prevention and control based on bacteria and compounds naturally occurring on honeybee colonies. The BEE DOC characterised specific probiotic bacterial flora linked to the honeybee and its interactions with pathogens. Furthermore, the potential of using plant and propolis compounds collected by honeybees and honeybee produced peptides in honeybee products, was studied for their potential use in controlling disease in honeybees.

Specific objectives of WP7 had been to:

- identify probiotic bacteria with a positive effect on honeybee health
- identify honeybee produced or foraged compounds with positive effects of honeybee health
- develop an interactive web based treatment tool 'The Bee Doc' for apiculturists

Task 7.1 Effect from LAB on *Melissococcus plutonius*.

The inhibitory effects of honeybee probiotics (LAB) on European Foulbrood (EFB) *Melissococcus plutonius* (provided by P4) have been investigated using both in vitro and in vivo tests. The LAB microbiota partly inhibited the bee pathogen *M. plutonius* in vivo and totally in vitro, *Lactobacillus kunkeei* and the thirteen identified LAB together showed the best inhibition properties. The overall effect of adding the LAB mixture to bee larval food was a significant reduction in the number of dead larvae. The results demonstrate that addition of LAB to the food of young honeybee larvae exposed to *M. plutonius* decreases the number of larvae succumbing to EFB infection.

Effect from LAB on *Nosema ceranae*.

Bees were put in cages, with 15 bees in each cage, and the cages treated with four different treatments. Each treatment was replicated in four different cages for a total of 16 cages and 240 bees. Ten bees from three cages of each treatment were infected by 10 000 fresh *N. ceranae* spores in 10 µL sugar solution, using individual feeding of each bee with a constriction micropipette. The results obtained cannot demonstrate an effect from the LAB microbiota on *N. ceranae* development. However, such an influence cannot be ruled out because of the failure to produce different LAB levels in the different groups.

Probiotic bacteria and the immune system.

A total of 36 samples have been extracted and are delivered for gene expression analysis. The results will not be delivered within the time frame of BEE DOC but will be available in April 2013.

Characterisation and naming of the honey bee LAB microbiota.

Eleven probiotic bacterial species were found in the honey bee stomach. Eight of these bacterial spp. proved to be novel spp. where the rest could be included under previously described bacteria. The microbiota is composed of several phylotypes within Bifidobacterium and Lactobacillus. 16S rRNA gene analyses, phenotypic and genetic characteristics revealed that the Lactobacillus phylotypes represent eight novel species.

Task 7.2 Biological activity of plant secondary metabolites

A total of 9 plant extracts were tested to determine their MIC against two strains of Paenibacillus larvae AFB (Provided by P3). The most efficient extracts were pomegranate, rosemary and ginseng extracts which showed MIC against P. larvae subsp Erick I of 200, 25 and 200 µg/mL, respectively and MIC against P. larvae subsp Erick II of 450, 25 and 100 µg/mL, respectively. Thus the most active phytochemicals were those of rosemary extract (carnosic acid, carnosol, rosmanol).

Twelve compounds extracted from different plant were also tested: ellagic, gallic, acids, resveratrol, kinurenic acid, naringin, hesperidin, neohesperidin, rutin, chlorogenic acid, epigallocatechin gallate, cinnamaldehyde; baicalein and vanillic acid. The most efficient phytochemicals inhibiting microbial growth were resveratrol, ellagic acid and epigallocatechin-gallate, which showed MIC against P. larvae subsp Erick I of 15, 50 and 150 µg/mL, and MIC against P. larvae subsp Erick II of 15, 150 and 200 µg/mL, respectively.

Once the antimicrobial capacity of these plant extracts and compounds was determined, studies on antipathogenic (or anti-Quorum Sensing) activity against different bacterial strains were performed. At least five of the tested extracts/compounds (cinnamaldehyde, pomegranate extracts, ellagic acid resveratrol and rutin) showed anti-QS activity against the biosensor strain and the pathogenic bacteria.

The chemical composition of the essential oils with acaricidal activity was determined by GC-MS.

Development of extracts formulations for colony applications

Most extracts were easily applied by solution or suspension in water at the active concentrations using ultrasound baths and heating to increase the solubility. Application by spraying is a suitable method. In the case of essential oils, a deposit of a volume of the oil in a filter paper, and location of the paper in the environment of the bees is sufficient as they are volatile compounds.

Development of acaricidal test model and evaluation of the effect on Varroa mites.

The toxic effects of different concentrations of the three selected essential oils on the honeybee and on Varroa mites were evaluated. The effects were tested on infested and non-infested honeybees. At concentrations that were not harmful for the honeybee, some essential oils showed some effects on the mites, and particularly relevant was the modification of their behavior as mites did not hook on bees in the presence of the essential oils, while they hooked the bees in the controls.

We found some difficulties to complete the assays as the colonies in our apiary died or were without a useful number of mites. We obtained colonies from other regions in Spain (Córdoba) in order to have colonies with Varroa mites, and this allowed reproducing the studies with other bees and mites. A more complete application of these treatments will be tested in spring to prove their efficacy on Varroa.

Task 7.3 Biological activity of propolis compounds

In order to identify propolis constituents with potential to be used against bee pathogens, we first performed a screening for their activity against honeybee pathogen *Paenibacillus* larvae. Propolis was extracted with 70% ethanol and the obtained extract was successively re-extracted with petrol ether and then with ethyl acetate. These two fractions were tested for their activity against *P. larvae*. The results were promising and the petrol ether fraction was more active, so we decided to concentrate on this less polar extract and isolate individual compounds from it.

The results for MIC after 24h show that the strain *P. larvae* ERIC I (swe 159/97) is the most susceptible one to propolis constituents. In general, *P. larvae* subsp. *pulvificans* seems to be less susceptible to propolis constituents than *P. larvae* subsp. *larvae*. The most active compounds were the flavonoids pinocembrin and 3-O-acetyl pinobanksin, and the caffeate mixture. MICs are among 30-125 µg/mL for the most active compounds.

Although the Minimal Inhibitory Concentrations (MICs) for the tested compounds, even for the most active of them, are much higher than the MICs of the antibiotics active against American foulbrood, propolis constituents have a very important advantage. They are of plant origin and are naturally present in the hive.

A further study was performed to clarify if there were any differences in propolis and plant metabolite usage in propolis between colonies with clinical symptoms of American Foulbrood (AFB) and healthy colonies from Sweden. Propolis from healthy colonies contained much higher levels of ferulic and caffeic acid and the benzoic acid ester coniferyl benzoate, than the propolis from colonies with AFB. Especially the concentration of coniferyl benzoate was 3 - 4 times higher in healthy colonies. This compound deserves further attention with respect to its possible action against *Paenibacillus* larvae.

Two of the most promising substances, the caffeate mixture and the flavanone pinocembrin, were sent for anti-*Nosema* tests to Avignon, together with ethyl acetate fraction. The two fractions (ethyl acetate and petrol ether) were sent also to Hohenheim for tests against *Varroa*. No significant results were observed.

Analysis of the chemical composition of propolis from hives treated with pesticides.

No significant differences could be detected in the amount of different compound classes, as well as in the balsam amount, between propolis from differently treated and from untreated hives. The application of chemometric approaches to the data (Principle Component Analysis, Cluster Analysis) also failed to indicate any detectable changes in propolis derived compounds with respect to pesticide treatment.

Plant secondary metabolites in propolis and *Varroa* resistance

We studied the chemical composition of the propolis from resistant and from susceptible colonies from Avignon and from Gotland in order to clarify if there are any chemical differences in this defensive material, which might contribute to the resistance.

The analyses showed that the most substantial difference between the two groups (resistant and susceptible) was the content of several individual propolis constituents, caffeic acid, and its prenyl esters: 3-methyl-3-butenyl caffeate, 2-methyl-2-butenyl caffeate and 3-methyl-2-butenyl caffeate. The concentrations of these substances were higher in propolis of resistant colonies and the differences observed were statistically significant. Higher concentrations of two further caffeates, CAPE and cinnamyl caffeate were present in the samples from resistant colonies but these differences were not statistically significant.

Propolis from resistant colonies from Gotland contained higher concentrations of the black polar markers pinocembrin, galangin, pinobanksin acetate, pentenyl caffeates. It is important to note that caffeic acid and caffeates (all three prenylcaffeates, CAPE and cinnamyl caffeate) were present in statistically significant higher concentration in propolis from Varroa-resistant colonies, just like in the resistant colonies from Avignon.

Task 7.4 Treatment with honeybee peptides

Royalysin is an authentic antimicrobial peptide of royal jelly and honey. It is recognised as exogenous antifoulbrood factor of honeybee nutrition. The amount of royalysin in larval diet is relative low and therefore the aim of our task is production of recombinant royalysin and testing of its effect in prophylaxis of honeybee against microbial and fungal pathogens.

The biotechnological preparation of royalysin by PCR amplification of cDNA-royalysin from universal honeybee cDNA library and by cloning into several expression systems. Prepared constructs were transformed into expression E. coli strains BL21(DE3) and AD494, respectively. The expression of recombinant peptide was achieved in all prepared cloning systems and was confirmed immunochemically using specific polyclonal antibodies.

Large Scale Production of Royalysin.

This was performed at the University of Ghent by Partner P6. The expression conditions determined by P11, were used to set up 50 L expression cultures. Royalysin was produced as GST tagged protein and also possess a His-tag which corresponds with a molecular weight from 37 kDA. Bradford analysis of the purified Royalysin resulted in a final yield of 800 mg Royalysin. The protein was finally dialyzed against PBS overnight and the protein was solubilized at concentrations of 0.5mg/ml.

Task 7.5 Develop interactive web based treatment tool 'The Bee Doc'

P3 integrated the Varroa mite model published by Calis et al. (1999) into a new version of the modeling program 'Stella'. The integration of different compiled brood data sets into the model and M7.1 has been completed. The model was adapted to brood rearing scenarios representing northern Europe (original data), central Europe (data provided by P9) and southern Europe (data provided by Dr. Marco Lodesani, Bologna, Italy). The simulation has a link from the BEE DOC web site and can be reached at:
<http://forio.com/simulate/ingemar.fries/varroa-dynamics/simulation/> (D7.2)

As an interactive web based treatment tool is also included (D7.2) the wikiCOLOSS BEEBOOK which is a 28 chapter manual nearly complete thanks to the efforts of over 170 honey bee experts, including numerous BEE DOC investigators that are either lead (six chapters) or contributing (12 chapters) authors.

WORK PACKAGE 8

TRANSFER OF RESULTS

WP 8 was fully dedicated to dissemination, which turned out to be most important for the BEE DOC particularly in light of its integration into global activities to fight the honeybee decline

Specific objectives of WP8 had been to:

- transfer the results to all stakeholders responsible for bee health
- train extension specialists and enhance the public awareness for honeybee health

Task 8.1 Direct Contacts and Participation

8.1.1 Societies

i) The European Association for Bee Research (EURBEE)

In September 2010 and 2012, a special symposium was organized by BEE DOC at EurBee 4 and 5, respectively, thus ensuring direct contact with all stakeholders. Additionally, BEE DOC partners 1 and 2 are executives of this Association, and hosted EurBee 5, and are therefore fully aware of all on-going activities.

ii) APIMONDIA

In September 2011, BEE DOC hosted a symposium at Apimondia 2011 in Buenos Aires, Argentina to further ensure direct contact with both beekeepers, researchers, and national authorities. This allowed Work Package leaders to each highlight their project accomplishments. Additionally, Partner 4 was name Vice President of the Apimondia Scientific Commission on Biology, thus ensuring a close link between BEE DOC and Apimondia.

iii) The International Bee Research Association (IBRA)

BEE DOC investigators were either lead (six chapters) or contributing (12 chapters) authors of the COLOSS BEEBOOK: Standard Methods for *Apis mellifera* research (32 chapters in total). This peer-reviewed manual appears as a special issue in the Journal of Apicultural Research, a scientific publication of the IBRA.

iv) The World Organisation for Animal Health (OIE)

BEEDOC experts were invited to contribute to the new edition of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Seventh Edition): Chapter 2.2.2. American Foulbrood (Partner 6), Chapter 2.2.3. European foulbrood (Partner 6), Chapter 2.2.4. Nosemosis (Partner 3), and Chapter 2.2.5. Small Hive Beetle Infestation (Partner 4). Additionally, four BEE DOC investigators presented research results at the OIE symposium 'Diagnosis and Control of Bee Diseases' that was held in September 2011 in Buenos Aires, Argentina, thus maintaining close contact with this inter-governmental organization.

Task 8.1.2 Symposia at Apimondia, COLOSS, and EURBEE conferences

BEEDOC symposia were organized at five major apidological events.

- 1)EurBee 4 and 6th COLOSS Conference, September 2010 Ankara, Turkey
- 2)Apimondia 2011, September 2011 Buenos Aires, Argentina
- 3)EurBee 5 and 8th COLOSS Conference September 2012 Halle, Germany

At each event, updates on the BEE DOC progress were provided by each work package leader as oral presentations.

Task 8.1.3 Annual European Parliament Bee Breakfast

The envisaged annual bee breakfast did not occur because of unforeseen MEP reasons. However, various BEE DOC partners interacted with MEPs and other EU authorities on a regular basis.

Specifically, BEE DOC members attended five official events hosted by the EU in Brussels, Belgium.

Date Location

- 1)EU Parliament: 'Bee declines in Europe: the evidence Symposium March 2011 Brussels, Belgium
- 2)EU Reference Laboratory of Bee Health kick-off meeting June 2011 Brussels, Belgium
- 3)EU COST Networks of Science and Technology' exhibition; October 2011 Brussels, Belgium
- 4)EU Parliament Inter-group meeting on Climate Change, Biodiversity, and Sustainable Level: Tackling the bee health problem December 2011 Brussels, Belgium
- 5)EU Parliament 'Bee Health in Europe' symposium February 2013 Brussels, Belgium

Significant results

Overall, BEE DOC met the objectives outlined. By participating in learned societies and events relevant to apiculture, members transferred consortium results to stakeholders responsible for bee health and the general public. Additionally, by co-authoring several chapters in the COLOSS BEEBOOK, and by hosting four training schools, BEE DOC educated and informed researchers studying honey bees and extension specialists.

Task 8.2 Publications

Overall, BEE DOC members authored or co-authored 38 peer-reviewed articles on honey bee health relating to BEE DOC projects in 17 different scientific journals. Additionally, BEE DOC members participated in 265 dissemination events or activities.

Significant results

BEE DOC publication and other dissemination efforts occurred in 19 countries specifically in their native language at local or regional events, as well as international events (e.g. central Europe, Europe, North America, global) in English. This enabled BEE DOC to disseminate consortium results, as well as general knowledge of honey bee health, to a broad audience.

Task 8.3 BEE DOC and WikiCOLOSS

The WikiCOLOSS concept evolved from a storage place for general BEE DOC information and data collection to one that maintains relevance of the COLOSS BEEBOOK. This is because the WikiCOLOSS concept could be more effectively used as a tool to allow readers to dynamically comment on current versions of the COLOSS BEEBOOK (see <http://www.coloss.org/beebook> online). Thirteen articles of the COLOSS BEEBOOK have been published online with open access; the remaining 20 will follow suit in spring 2013. BEE

DOC members developed the wikiCOLOSS BEEBOOK concept to provide dynamic updates to the manual. It allows the scientific community to directly comment on text electronically on the COLOSS website (coloss.org/beebook). Comments will be considered during updates of the manual, and therefore promote both the COLOSS BEEBOOK's relevance and novelty to the apiculture community.

The 'BEE DOC Interactive Bee Health Tool' became active on the consortium's website (see http://www.bee-doc.eu/info_beekeepers_sim.php online) during the reporting period. It specifically informs and educates beekeepers about timing of Varroa destructor management methods.

Significant results

Overall, the wikiCOLOSS BEEBOOK concept proved to be very useful to further develop the research field. It will become the definitive, but evolving, honey bee research manual, composed of 33 peer-reviewed chapters authored by more than 200 of the world's leading honey bee experts. Additionally, the 'BEE DOC Interactive Bee Health Tool' will be used by beekeepers to properly manage the arguably single greatest threat to honey bees, the mite Varroa destructor. By enabling beekeepers to make more informed choices, this will promote bee health, and therefore reduce colony losses.

All information concerning BEE DOC project background information, objectives, and references to important BEE DOC publications can be found on the consortium website (see <http://www.bee-doc.eu> online).

Task 8.4 Implementation of GAP (Good Apicultural Practices)

Training schools and dedicated publications were the main tools to implement this goal. Four joint BEE DOC / COLOSS training schools were organized

1)Diagnostics in honeybees, Ghent - <http://coloss.org/publications/ghentproceedings>

2)Varroa control, Hohenheim

<http://coloss.org/publications/hohenheim-training-school>

3)Honeybee genetics, Murcia

<http://coloss.org/documents/proceedings-of-the-pop-workshop-murcia-2012>

4)Extension: connecting stakeholders, Bern

<http://coloss.org/publications/bee-doc-bern-training-school>

with approximately twenty participants attending each event.

Teaching material in the form of Proceedings is available for professional apiculturists in a pdf printer friendly format on the COLOSS website.

Additionally, three GAP manuals were produced in several BEE DOC languages (French, German, and Italian). They are available for download in a printer friendly pdf format on the COLOSS website:

-French (see http://www.coloss.org/publications/zbf_gap_f online)

-German (see http://www.coloss.org/publications/zbf_gap_d online)

-Italian (see http://www.coloss.org/publications/zbf_gap_i online)

Significant results

Overall, the BEE DOC consortium significantly contributed to further improvement of apicultural practice through events that trained and engaged researchers and students, and GAP manuals and the 'BEE DOC Interactive Bee Health Tool' that allow beekeepers to make more informed management decisions that will help promote bee health, and ultimately help to reduce colony losses.

Potential Impact:

Impact

Addressing the call text

'The European added value lies in the pooling of interdisciplinary research expertise, thus creating economies of scale to address a cross-border issue and provide support to agricultural policies' (call text)

In spite of the substantial economic and enormous ecological significance of honeybees and other pollinators for our society and environment, the research field of apiculture was small and scattered in Western Europe. In Central and Eastern Europe, in contrast, the significance of bees was politically much better perceived. This caused a very unbalanced situation for the expanded EU. There were (and still are) many extension institutions of mixed quality in the Eastern Europe, which have not attained top international scientific level. In contrast, scattered across the old member states, there are a few excellent groups with internationally highly competitive research programmes.

These high level research groups, however, had been only rarely in contact with applied research institutions or the industry. Their main impact was in fundamental research and through publications in top scientific research journals including Nature, Science, Cell and PNAS. Their impact on applied research was consequently considerably less, resulting in an urgent need for knowledge transfer in both directions between pure and applied research. Excellent applied research institutes and beekeeping enterprises often had insufficient impact in international research simply because they reported in more specialized, low impact, national journals. As a result they did not draw attention at an international level. This was very unfortunate, because the cutting edge research groups (although superbly staffed and equipped) often had insufficient beekeeping capacity for experiments requiring large numbers of bee colonies.

Within pure or fundamental biology, bee research disciplines are far apart and many honeybee researchers (e.g. in genetics, in physiology, in ecology) have never dealt with a honeybee colony themselves or only know the product of honey from their daily breakfast. They are usually completely unaware of and uninterested in the important role their study animals have for agriculture. Researchers are usually focused on a small facet of the bee's biology, ignoring any impact it may have on the final product: the honey on the consumer's daily bread. Similarly many researchers working with honeybee colonies have very little knowledge about the individual capacity of a single bee. As a result, much of what we know

from honeybee biology is not being used to enhance colony health in modern bee management. It is here where the BEE DOC broke this barrier and developed its major synergy leap. Because of the intimate connection of the extension partners with the basic research teams, the research quality exceeded by far previous efforts to improve colony health.

The BEE DOC pooled the expertise of leading European laboratories on honeybee pathology, genetics, food chemistry, and apicultural extension to concert a broad methodological approach that could only be conducted in a pan European research effort. The variety of lab facilities needed, the large beekeeping operations required, and the European scope of the problem itself went far beyond national resources. The BEE DOC was only successful because it could synergise not only the activities within Europe but also those at a global level. The problem of colony losses was and still is a global and not just a European one, and it was therefore essential to integrate and coordinate European activities with those in other continents with similar problems. With the participation of European SMEs, the BEE DOC not only contributed towards a halt in honeybee decline, it also put European Enterprises into a globally leading position for treating honeybee diseases and providing beekeepers with easy-to-use quantitative, diagnostic tools.

'Indeed, the massive loss of honeybee colonies may impact on agriculture via the pollination network. ' (call text)

The BEE DOC aimed at understanding the role of interactions between pathogen and pesticides in multiply infected honeybees using a series of integrated experiments and observations. From the fundamental results and knowledge arising from its research programme, the BEE DOC was able to identify the main culprits of colony death (Varroa and its associated virus infections) which now allow for an even more focused therapy of honeybee colonies. The novel therapies and selection tools for resistance towards diseases will evolve into appropriate strategies preventing large scale colony losses. A suite of novel diagnostic tools allow for timely and routine identification of outbreaks of diseases well before clinical symptoms can be seen at the colony level. Moreover, the BEEDOC was not just be a doctor for diseased bees. It reached deep into society through its broad transfer concept and dissemination activities through all possible media for the general public (including TV, Radio, Newspapers).

'The role of pollinators is important both for our food supply and for the preservation of natural ecosystems' (call text)

The success of the BEE DOC did not only enhance colony health, it also had a considerable impact on societal perceptions of bees and their economic and ecological value. Many of the participating groups had recurring contacts with the public media and political decision

makers. The BEE DOCs dissemination plan ensured that the media and the public were reached to raise awareness of the value of honeybee pollination services for our societies.

The BEE DOC further rose the appreciation at the political level of the services honeybees and other pollinators provided for our society and environment. As a most remarkable result are numerous novel calls for national research initiatives in several member states addressing the honeybee decline. Also EU policy makers realized the needs for sustained research efforts, resulting in a second call on honeybee health within the 7th frame work. It was a major achievement of the BEE DOC to develop compelling arguments to convince political decision makers to reroute subsidies in agriculture more strongly into topics supporting honeybee colony health. In light of the ecological and economic significance of honeybees, BEE DOC envisaged the clear need to set new incentives for beekeeping, considering the pollinator decline in Europe. Most important in this context is to make beekeeping again attractive as a hobby, which requires healthy colonies. If we are to succeed in making beekeeping an attractive pastime activity and a profitable business again, we first need to deal with colony health and allow the beekeeper to assess and treat diseased colonies before they die.

Contributions to standards and policy development

The results of BEE DOC allowed to implement novel diagnostic tools for pathogens that may find broad use in the on-going national and EU wide concerted monitoring of colony health. The BEEDOC developed novel treatments for honeybee diseases which may have considerable impact on EU and national support policies for apiculture. BEE DOC's research aims at sustainable beekeeping techniques that allow for bee management without chemical treatments. This BEE DOC philosophy has apparently convinced many policy makers and became part and parcel of the currently open call on colony health in the 7th framework. In addition, the BEE DOC co-ordinator was chosen as member of the permanent expert group on organic farming (EGTOP), which gives advice to the commission

Dissemination activities of BEE DOC

The main goal of was the dissemination of project results as well as the transfer of knowledge to various stakeholders. A wide range of dissemination paths and methods were employed for a successful realization. Target stakeholders included researchers, but also government authorities, the beekeeping industry as well as the general public and were approached by direct contact in form of symposia at major international congresses (3) and active BEE DOC participation at conferences (57), workshops (22) and different events at the EU parliament (5), as well as by publications of relevant results in scientific (38 articles) and/or popular (17 articles) journals in various languages. Special emphasis was given to the COLOSS BEEBOOK: standard methods for *Apis mellifera* research, which is co-authored by several BEE DOC members in cooperation with nearly 200 other global scientists. It aims to

promote standardization of honeybee research methods globally. The manual is encouraged to remain relevant to the research community as new techniques are developed via the wikiCOLOSS BEEBOOK, an online portal (see <http://www.coloss.org/beebook> online) that allows direct commenting to the BEEBOOK text. Specific knowledge about honeybee health research in general and BEE DOC result in particular was provided by four training schools that were primarily attended by extension specialists and researchers. Additionally, BEE DOC also sought to promote education, cooperation, and standardization among beekeepers, including usage of the BEE DOC's Bee Doctor tool. To reach that goal, good apicultural practice manuals were produced to transfer know-how to the apiculturists. The 'BEE DOC Interactive Bee Health Tool' on the consortium's website provides advice across Europe for honeybee management (see http://www.bee-doc.eu/info_beekeepers.php online). Further dissemination activities included the distribution of newsletters to government authorities (5), interviews (5) and press releases. Additionally, the project homepage enabled all interested people to access information about BEE DOC throughout the whole duration of the project. The number of the reported dissemination efforts via numerous channels as outlined in BEE DOC Annex I alludes to the successful transfer of results and knowledge by the BEE DOC consortium to as many stakeholders interested in, or responsible for, honeybee health as possible. It is these international dissemination efforts listed below, which will promote a sustainable apicultural industry.

Direct contacts and participation in conferences

i) The European Association for Bee Research (EURBEE)

In September 2010 and 2012, a special symposium was organized by BEE DOC at EurBee 4 and 5, respectively, thus ensuring direct contact with all stakeholders. Additionally, BEE DOC partners 1 and 2 are executives (president and secretary) of this Association, and hosted EurBee 5, and are therefore fully aware of all on-going activities. The conferences were attended by over 400 participants from over 50 countries.

<http://www.eurbee2012.uni-halle.de/stats/>. At each event, updates on the BEE DOC progress were provided by each work package leader as oral presentations.

ii) APIMONDIA

In September 2011, BEE DOC hosted a symposium at Apimondia 2011 in Buenos Aires, Argentina to further ensure direct contact with all three beekeepers, researchers, and national authorities. This allowed Work Package leaders to each highlight their project accomplishments. Additionally, Partner 4 was elected as Vice President of the Apimondia Scientific Commission on Biology, thus ensuring a close link between BEE DOC and

Apimondia. Updates on the BEE DOC progress were provided by each work package leader as oral presentations. The conference was attended by over 2000 participants.

iii) The International Bee Research Association (IBRA)

BEE DOC investigators were either lead (six chapters) or contributing (12 chapters) authors of the COLOSS BEEBOOK: Standard Methods for *Apis mellifera* research (32 chapters in total). This peer-reviewed manual appears as a special issue in the Journal of Apicultural Research, a scientific publication of the IBRA.

iv) The World Organisation for Animal Health (OIE)

BEEDOC experts were invited to contribute to the new edition of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Seventh Edition): Chapter 2.2.2. American Foulbrood (Partner 6), Chapter 2.2.3. European foulbrood (Partner 6), Chapter 2.2.4. Nosemosis (Partner 3), and Chapter 2.2.5. Small Hive Beetle Infestation (Partner 4). Additionally, four BEE DOC investigators presented research results at the OIE symposium 'Diagnosis and Control of Bee Diseases' that was held in September 2011 in Buenos Aires, Argentina, thus maintaining close contact with this inter-governmental organization.

Contacts with Members of Parliament (MEP)

The envisaged annual bee breakfast did not occur because of unforeseen MEP's declines. Nevertheless, various BEE DOC partners repeatedly interacted with MEPs and other EU authorities on a regular basis.

Specifically, BEE DOC members attended five official events hosted by the EU in Brussels, Belgium

1)EU Parliament: 'Bee declines in Europe: the evidence' symposium (March 2011, Brussels, Belgium)

2)EU Reference Laboratory of Bee Health: kick-off meeting (June 2011, Brussels, Belgium)

3)EU COST: 'Networks of Science and Technology' exhibition (October 2011, Brussels, Belgium)

4)EU Parliament: Inter-group meeting on Climate Change, Biodiversity, and Sustainable Level: Tackling the bee health problem. (December 2011, Brussels, Belgium)

5)EU Parliament: 'Bee Health in Europe' symposium (February 2013, Brussels, Belgium)

Overall, BEE DOC exceeded the objectives outlined in its dissemination plan. By participating in learned societies and events relevant to apiculture, members transferred consortium results to stakeholders responsible for bee health and the general public. Additionally, by co-authoring several chapters in the COLOSS BEEBOOK, and by hosting two training schools, BEE DOC educated and informed researchers studying honeybees and extension specialists.

Publications

Overall, BEE DOC members authored or co-authored 38 peer-reviewed articles on honeybee health relating to BEE DOC projects in 17 different scientific journals.

Apidologie: 7 publications;

Applied and Environmental Microbiology: 1 publication;

BMC Genomics: 1 publication;

Brain, Behavior, and Immunity: 1 publication;

Environmental Microbiology Reports 1 publication;

Food Chemistry: 1 publication;

Food Control: 1 publication;

Insect Molecular Biology: 1 publication;

Journal of Agricultural and Food Chemistry: 2 publications;

Journal of Apicultural Research: 9 publications;

Journal of Apicultural Science: 1 publication;

Journal of Chromatography A: 1 publication;

Journal of Invertebrate Pathology: 5 publications;

NeuroToxicology: 1 publication;

PLoS One: 3 publications;

Trends in Parasitology: 1 publication;

Veterinary Microbiology: 1 publication;

BEE DOC members participated in 265 dissemination events or activities summarized as follows:

Articles published in the popular press: 17;

Conference: 57;

Flyers: 5;

Interviews: 5;

Media briefings: 6;

Posters: 16;

Presentations: 69;

Press releases: 3;

Publication: 39;

Theses: 17;

TV clips: 7;

Websites/Applications: 2;

Workshops: 22;

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specifically informs and educates beekeepers about timing of Varroa destructor management methods.

Overall, the wikiCOLOSS BEEBOOK concept proved to be a major step forward to unify the fields of fundamental and applied apidology. It became the definitive, but constantly evolving, honeybee research manual, currently composed of 33 peer-reviewed chapters authored by more than 200 of the world's leading honeybee experts. Additionally, the 'BEE DOC Interactive Bee Health Tool' will be used by beekeepers to properly manage the arguably single greatest threat to honeybees, the mite Varroa destructor. By enabling beekeepers to make more informed choices, this will promote bee health, and therefore reduce colony losses.

Implementation of Good Apicultural Practices

Training schools and dedicated publications were the main tools to implement this goal. Four BEE DOC training schools were organized for extension specialists, students and COLOSS members. Approximately twenty participants attended each event.

BEE DOC training schools:

1)Diagnostics in honeybees: August 2010 in Ghent, Belgium:
<http://coloss.org/publications/ghentproceedings>

2)Varroa control: August 2010 in Hohenheim,Germany
<http://coloss.org/publications/hohenheim-training-school>

3)Honeybee genetics:1/5/10:February 2012:Murcia, Spain
<http://coloss.org/documents/proceedings-of-the-pop-workshop-murcia-2012>

4)Extension: connecting stakeholders: February 2013 in Bern, Switzerland
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Teaching materials in the form of Proceedings are available for professional apiculturists in a pdf printer friendly format on the COLOSS website.

Additionally, three GAP manuals were produced in several BEE DOC languages (French, German, and Italian). They are available for download in a printer friendly pdf format on the COLOSS website:

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-German (see http://www.coloss.org/publications/zbf_gap_d online)

-Italian (see http://www.coloss.org/publications/zbf_gap_i online)

BEE DOC web site

The BEE DOC web site served as a multifunctional platform allowing for communication within the project and to the outside. The web site had global visibility with visitors from over 62 countries and an average of 20 visits per day. Even towards the end of the project the web site still has an average of 13 new visitors every day resulting in a total over 14000 unique visitors searched the web site throughout the funding period. The web site will be maintained and updated also after the end of the project.

Overall, the BEE DOC consortium significantly contributed to further improvement of apicultural practice through events that trained and engaged researchers and students, and GAP manuals and the 'BEE DOC Interactive Bee Health Tool' that allow beekeepers to make more informed management decisions that will help promote bee health, and ultimately help to reduce colony losses.

List of Websites:

<http://www.bee-doc.eu/>