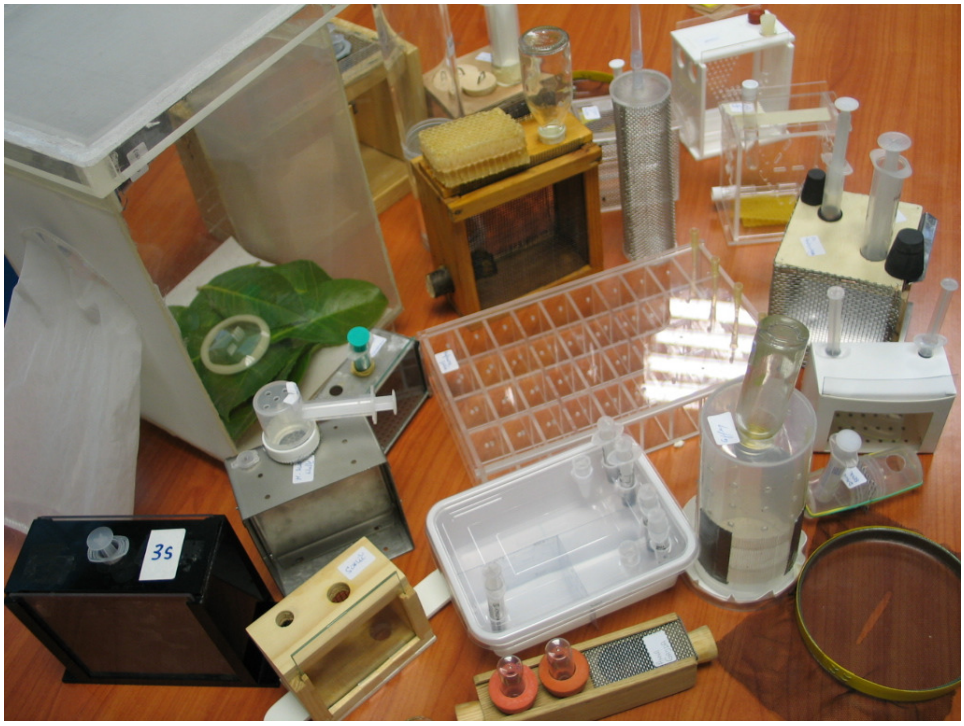
Action FA0803

Proceedings of the COLOSS Work Shop

Standardized methods for honey bee rearing in hoarding cages

25.-26.11.2010



Agricultural Research Council
Honey bee and Silkworm Research Unit (CRA-API)

Via di Saliceto, 80 – 40128 Bologna, Italy.

CRA
CONSIGLIO PER LA RICERCA
E LA SPERIMENTAZIONE
IN AGRICOLTURA
UNITÀ DI RICERCA DI APICOLTURA E BACHICOLTURA

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Agenda

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25.11.2010 (Thursday) CRA-API / CIN	
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	Session I: Laboratory cages in honey bee research
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11:00 – 11:30	Coffee break
11:30 – 13:00	Short presentations: - Use of cages in the study of effects of pesticides on food transfer between honeybee Workers. (Bevk & Kralj) - Imidacloprid effect on honey bees under laboratory conditions using hoarding cages (Hatjina & Dogaroglu) - Testing the effect of imidacloprid on honey bee mobility (Kence et al.) - Methods For Testing Pesticide Toxicity on Honey Bees in Cages (Laurino et al.) - BEE DOC: an EU project assessing the impact of pathogens and pesticides on honey bees. (Doublet et al.) - Neonicotinoid Insecticide Residues in Honey (Tanner & Czerwenka)
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16.30 – 17.00	Coffee break
17.00 -18.30	Short presentations: - Standardized methods for honey bee rearing in hoarding cages (Smith) - Understanding *Nosema* using caged honey bees: pathology, interactions between congeners, and the evolution of experimental conditions. (Williams et al.)

	- Laboratory infection of bees with viruses in Poland - some experimental conditions(Topolska & Gajda) - Evaluation of oral transmission of deformed wing virus variants between adult honey bees (*Apis mellifera*). (Yanez et al.) - Efficacy of a novel agent for the control of the Varroa mite *(Varroa destructor*). (Coffey & Kavanagh) - Survival and inclination to feeding of caged honey bees contaminated with oxalic acid. (Nanetti et al.)
18.30	Return to hotel
20:00 – open	Social dinner at "Trattoria dal Biassanot"
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10:00 – 10:30	Plenary talk "**Working with *Nosema ceranae* – laboratory and field results**" I. Fries
10:30 - 11.00	Coffee break
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13:00 – 14:00	Buffet lunch
14:00– 14:30	Practical demonstrations
14:30– 16:30	Discussion and conclusions
16:30 – 17:00	Goodbye coffee

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Title:

Impact of pesticides on the virulence of *Nosema ceranae*, an emerging parasite of the honeybee *Apis mellifera*

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Text of Abstract: (limit text to 250-400 words)

Massive colony losses of the European honeybee *Apis mellifera* have been observed worldwide during the last decade. As suggested by most scientists, such colony depopulation is multicausal (vanEngelsdorp *et al.*, 2010, *Inverteb. Pathol.* 103: S80) and may result from synergetic interactions between known stressors such as pesticides and pathogens. For example, many pesticides are known to alter the immune system of insects and to favor disease and pathogen development (Desneux *et al.*, 2007, *Annu. Rev. Entomol.* 52: 81).
Recently, Alaux *et al.* (2009, *Environ. Microbiol.* 12: 774) demonstrated that honeybees were significantly and synergistically weakened by exposure to the neonicotinoid imidacloprid and infestation with the microsporidian parasites of the *Nosema* genus. The proliferation of *N. ceranae* may thus be opportunistic and its virulence would depend on environmental conditions.
Our lab aims to investigate such combined effect of *N. ceranae*, a worldwide distributed pathogen, and other chemicals present in the environment assessing the virulence of the parasite on honeybees following a chronic exposure to sublethal doses of pesticides used in agricultural (Fipronil, Thiametoxam) or apiary (Amitraze) activities. The observation of changes in honeybee physiology (immune defences, energetic stress or detoxication activities) will give new insights in *N. ceranae* pathogeny and opportunism and on its impact on bee health.
Practically, new born encaged honeybees were exposed for seven days to sublethal doses of pesticide before being individually infected by *N. ceranae* (125000 spores per bee). Mortality and sucrose consumption were monitored every day and spore content was determined 5 days post-infection (dpi). Midguts were collected from sacrificed bees at 0 and 5 dpi in order to compare by RT-qPCR the expression of genes involved in the immune or detoxication systems. Moreover, proteomic analyses will be carried out to identify host's proteins that are specific to the cumulative parasite and pesticide treatment.

Title:

Use of cages in the study of effects of pesticides on food transfer between honeybee workers

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Text of Abstract: (limit text to 250-400 words)

Trophallaxis is one of the main social behaviour in honeybees influencing food transfer. In this study we investigated whether acaricide coumaphos affects food transfer between donor and recipient bees using two experimental cage designs. The aim of our work was to determine if there are differences in the amount of food transferred by treated and untreated bees. Prior to the experiments donor bees of known age were fed individually with 10 µl of either coumaphos contaminated (1, 2, 5 µg) or control 30% sugar solution. Recipient bees were foragers of unknown age captured at the entrance of the same hive.

In the first experiment we measured the amount of transferred food between donors and recipients. Each of four single donor bees fed with contaminated or control solution was placed individually in its own plastic cage in the big plastic box (1000 ml). One hour later 30 recipient bees were added to the box. Donor cages were modified plastic 20 ml Rotilabo® containers (31 mm diameter x 43 mm long) with a feeder (a modified 5 ml syringe) containing a weighed amount of 20% sugar solution. A whole was made in the lid (25 mm diameter) and closed with the metal net that allows trophallaxis between donor and recipient bees. The boxes were placed into an incubator (34°C, darkness) and 24 h later the amounts of solution taken from the feeders were measured by weighing.

In the second experiment the number of dead recipient bees was recorded. A single donor bee was placed in the plastic cage made from two 20 ml Rotilabo® containers separated by the metal net. A side with the donor bee was provided with a feeder containing 2 ml of 20% sugar solution. On the other side of the cage 10 recipient bees were added one hour after donor bees had been treated with coumaphos contaminated or uncontaminated sugar solution. Cages were then placed in the incubator (34°C, darkness) and after 4 and 24 h the number of dead recipient bees was recorded.

Both experiments showed that coumaphos reduced food transfer between workers. This method could be used for testing the effects of pesticides on trophallaxis.

Title:

Experimental cages to study the effects of pesticides and diseases on adult honey bees

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Text of Abstract: (limit text to 250-400 words)

Pollinators are essential for pollinating 35% of the agricultural crops of global importance that serve as food for humans. They also play an important role in the pollination of natural areas. Among the pollinators, the social honey bees (*Apis mellifera*) have an economic value mainly due to their marketable products, as well as their activity of pollination in agricultural areas. However, the decrease in several species of bees from crop fields and other areas has been challenging many researchers worldwide.

Since the massive use of pesticides after the second world war, much concern has been raised on their interactions with honey bees. In order to better know the consequences of pesticide exposure, honey bees have been the subject of various tests. At the European level, guidelines have been edited in order to harmonize the tests for the pesticide homologation process. On the other hand, scientific publications report experimental conditions that are different to these standards. A brief review of these various conditions will be presented.

The effects of disease on honey bees have also been studied through cage tests. Parameters observed during tests are heterogeneous. It is worth noting that experimental conditions are also highly different from one laboratory to the other.

There is a need in the scientific community to compare data obtained on different subjects (pesticides or diseases) by different laboratories located all around the world. The variability in honey bee response to stressors is remarkably high. Therefore, it is important to apply the best experimental conditions in order to get reliable, repeatable and comparable data. More work should be done to meet harmonized standards. Coloss network is a great opportunity to gather practical knowledge on experimental conditions. However, ring tests are needed to compare conditions which would require some dedicated funds.

Title:

Efficacy of a novel agent for the control of the Varroa mite (*Varroa destructor*)

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Text of Abstract: (limit text to 250-400 words)

The aim of the study was to assess the efficacy of a novel agent for the control of Varroa mites in honeybee colonies. The product is known to be effective against mites in other domesticated animals, but its toxicity to honeybee colonies and its efficacy against the Varroa mite (*Varroa destructor*) had not been previously investigated. Since this product was not previously tested on honeybees, we initially carried out laboratory trials using hoarding cages. Newly emerged bees and developing larval were exposed to different concentrations of the product as a spray and in a syrup solution. The toxicity range of the product was known thus in this experiment we selected five different concentrations of the product plus a control, with three replicates per treatment group. Parameters assessed included: bee longevity, bee behaviour and larval development rate. The toxicity of the product to Varroa mites was also examined. To investigate if the product could permeate the cuticle of the adult mite and cause death, the product was dissolved in an aqueous solution and applied directly onto adult mites. The impact of the product on mites feeding on adult bees treated with the product was also examined. This was carried out by taking newly emerged bees, infesting them with a known number of mites and feeding the bees with similar concentrations of the product as described above. Mite mortality was assessed on a daily basis. Preliminary results indicate that the product had no negative effects on adult bee survival, but had negative effects of varroa mite survival. On completion of the laboratory trial, a small field trial was initiated to examine the effect of the product on mite population growth under field conditions. At present this trial is on-going, but preliminary results are positive and it is hoped to carry out a larger field trial 2011.

Title:

The risk of cross contamination by *Nosema* spp. in hoarding cage experiments

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Text of Abstract: (limit text to 250-400 words)

A trial was performed to asses whether it is possible to keep nosema-infected and non-infected bees in the same incubator when conducting experiments. Newly emerged bees (age < 24 h) were collected from combs kept in an incubator, and placed in small plastic cages (size 13 x 12 x 6 cm), which had previously held nosema-infected bees and had either been sterilised by soaking in sodium hypochlorite or only washed in hot water. Three experimental groups were set up: in the first group bees were placed in sterilised cages (30 bees x 3 cages) and were infected with 10 x 10^6 *Nosema* spp. spores by bulk feeding (0.1 ml of spore solution in 50% sucrose syrup); in the second and third group bees were not infected and were placed respectively in sterilised or washed cages (30 bees x 3 cages per group). All cages were placed in the same incubator on the same shelf, at a slight distance from each other (min. 5 cm). Five live bees per cage were collected on the 8^{th} and 16^{th} day post infection and analysed for presence and quantity of *Nosema* spp. spores. Dead bees were also checked for presence of *Nosema* spp. No spores were detected in any of the dead bees from the non-infected cages, however of the 60 not artificially infected live bees which were analysed, nosema spores were detected in one bee (75.000 spores), 16 days from beginning of experiment, from one of the sterilised cages.

It seems that the risk of contamination when using infected and non-infected bees in a same incubator does exist and standardisation of experimental techniques should specify whether infected and non-infected bees should be kept in separate incubators, or which procedures may be adopted when this is not possible. Also, protocols could foresee that non-infected cages should be kept together with infected ones as a negative control to verify the proportion of cross-contamination occurring in the incubator, and how the problem should be addressed if it occurs.

Of course care must be placed in keeping feeders, syrup containers, and other instruments used during the test well separated between infected and non-infected cages. When using plastic cages it also seems that a thorough washing in hot water may be sufficient to remove faecal residues containing spores, although standard protocols for experimental infection should specify the kinds of cleansing and sterilisation methods to be used for reliable comparable results.

Title:

The efficacy of Feedbee® pollen substitute on honeybee (*Apis mellifera*) in rearing cage by measuring total protein content

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Text of Abstract: (limit text to 250-400 words)

The use of pollen substitutes is becoming part of the routine beekeeping protocol more and more at present. Since digestion physiology of the bees is not yet fully understood, it is not an easy task to evaluate the conversion rate of a particular feed. The inclement weather conditions of recent years, monoculture farming and single crop pollination, bees are subjected to a single pollen source instead of the mixed pollen supply that provides bees with required profile of amino acids. This phenomena is considered to be an important contributing factor to the so-called CCD (Colony Collapse Disorder), causing rapid decline populations of the key pollinator in several parts of the world.

In our study we intended to evaluate the efficacy of a pollen supplement feed formula called Feedbee® (Saffari et al., 2004) in honeybees reared in roaring cages aiming to assess how it increases the total protein content of the bee's body.

For laboratory tests 4 groups of 10-15 newly hatched worker bees were confined in separate plastic cages, and all the cages were kept in an incubator at 25 ˚C with 70 % RH. Each group received only one of the experimental feeds of mixed pollen (beebread) candy, Feedbee® patty and the control group poor sugar candy only. Drinking water was readily available in the cages. The cages were checked daily to remove dead bees, and to refill feeders. On the 12^{th} day, the bees were anaesthetized with CO_2 and stored in deep-freezer at -70 °C until subsequent analysis for body protein content.

For the test homogenized total bee body was used preceeded by removing alimentary tracts. Protein analysis was carried out using the Bradford assay (Shu-Sheng and Lundahl, 2000), with the help of a Bio-Rad Microplate Manager and software.

The means of total protein of 5-5 experimental bees demonstrated the good conversion of Feedbee® (4,24 mg/bee), in comparison with the beebread containing candy (4,74 mg/bee). The protein level of the D0 control (5,69) and D14 control 2,55 clearly demonstrated the importance of protein diet in the development of young workerbees

Our protein assay seems to be reliable to analyze the total protein content of honeybees; it is suitable to measure storage protein in wintering bees, and to evaluate pollen substitute diets for honeybees.

1. Shu-Sheng Zuo1 and Per Lundahl (2000) A Micro-Bradford Membrane Protein Assay. Biochemistry Volume 16.162-164.
2. Saffari, A M, Kevan, P G, Atkinson, J L (2004) A promising pollen substitute for honey bee. American Bee Journal, 3. 230-231.

Title:

BEE DOC: an EU project assessing the impact of pathogens and pesticides on honey bees

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Text of Abstract: (limit text to 250-400 words)

BEE DOC comprises of a network of eleven partners from honey bee pathology, chemistry, genetics and apicultural extension aiming to improve colony health of honey bees. BEE DOC will empirically and experimentally fill knowledge gaps in honey bee pests and diseases, including the 'colony collapse disorder' and quantify the impact of interactions between parasites, pathogens and pesticides on honey bee mortality. Specifically our research on adult honey bees within BEE DOC aims to test how interactions among pathogens and pesticides affect individual bees. We shall use one model parasite (*Nosema*), two model viruses (Black Queen Cell Virus, Israel Acute Paralysis Virus) and two model pesticides (thiacloprid, τ-fluvalinate). Preliminary analyses by BEE DOC colleagues have developed methods for administration of pathogens and evaluated threshold levels of viruses in individual honey bees held in cages. They have also determined interactions between *Nosema* species, allowing administration of a single dose of fixed proportion of *N. ceranae/apis*. We now aim to extend these analyses by examining interactions between BQCV, IAPV, *Nosema* spp. and the two target pesticides in individual adult honey bees held in cages. We aim to implement standardized methods for rearing honey bees in hoarding cages, as recommended by the current workshop.

Title:

A review on role of haemocytes in honeybee immunity

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Text of Abstract: (limit text to 250-400 words)

Haemocytes classification:

- Prohaemocytes
- Plasmatocytes
- Granular Cells
- Spherule Cells
- Cystocytes
- Oenocytoids

Haemocytes in immune defence:

- Phagocytosis and encapsulation are the most common mechanisms in bees against pathogens.
- Wound repair
- Regeneration of tissues
  - Phenoloxidase pathway
- Encapsulation
  - Components of the proPo system could function as signaling molecules to promote encapsulation.
  - The invader is enclosed in several layers of cells and the capsule-like so formed melanizes and strictly isolates the parasite from circulation.
- Melanization
  - Nodule formation is a phenomenon in response to both animate and inanimate substances that cannot be removed from circulation by phagocytosis.
  - In this cellular reaction, the haemocytes loaded with bacteria are entrapped by a coagulum that is produced by the degranulating granular cells and then centrally melanized.

Humoral reactions

- Immune proteins such as:
- Anti-bacterial and anti-fungal proteins.

  Cationic peptides have been found to have activity against:
  - Gram negative and Gram positive bacteria
  - Fungi
  - Eukaryotic parasites
  - Viruses
  - Most importantly, cationic peptides are effective against strains of antibiotic resistant bacteria.
- Lysozymes
- Lectins

Title:

Working with *Nosema ceranae* – laboratory and field results

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Text of Abstract: (limit text to 250-400 words)

The influence of *Nosema ceranae* on honey bee colonies (*Apis mellifera*) appears to be very different in different parts of the world. The impact from this parasite, based on laboratory results as well as on field results, suggests that the parasite may have severe implications in some regions, whereas the damaging effects from this parasite appears to be negligible in other regions. Possibly, this reflects that the severeness of this infection may be influenced by factors not present in the entire distribution area of the parasite, or the existence of strains of the parasite with different levels of virulence.
Studies of virulence of *N. ceranae*, both at the individual larval level and at the colony level, will be discussed.

Title:

Oral inoculation of individual bees with the pathogen *Nosema ceranae*

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Text of Abstract: (limit text to 250-400 words)

In the honeybee *Apis mellifera* infection with pathogens is a standard procedure. Nevertheless the methods used to provoke an infection vary in many ways for example in the infection mode. Here we present a method to achieve an oral infection of individual workers with the microsporidian *Nosema ceranae*. For the inoculation workers (24 hours old) were placed in 1.5 ml tubes and to limit bee movement another smaller tube [1.6mm length] was placed behind and the 1.5 ml tubes were looked. After the bees starved for two hours each bee was fed with 5µl of freshly prepared *N. ceranae* spore solution (10^5 spores/workers in 50 % sugar water) through an opening at the end of the tube. The controls were fed with 5µl of pure 50% sugar water. The infection with *N. ceranae* was achieved consistently. The data show that controlled infection of individual bees in short time can be obtained and this method could be used for individual inoculation of big sample sizes. Moreover, the used materials are cheap and can even be recycled given suitable sterilization. Older workers can also be infected after immobilizing at 4 °C for 30 min. Finally, we suggest to take advantage of this method for other honey bee pathogens by adjusting the infectious dose and post-infection separation time or other fields like neurobiology and ecotoxicology as well.

Title:

Imidacloprid effect on honey bees under laboratory conditions using hoarding cages

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Text of Abstract: (limit text to 250-400 words)

A current project in Hellenic Institute of Apiculture concerns the evaluation of the effects of the neonicotinoid imidacloprid on honey bees using *in-vivo* and *in-vitro* methodology. One of the *in-vitro* methods already used in our Institute is the use of hoarding cages where the effects of imidacloprid was evaluated on adult honey bee longevity, food consumption, and development of hypopharygeal glands (HPGs).

Imidacloprid was administered through sugar solution and pollen patty given to the bees in a concentration of 2ppb and 3ppb respectively (concentrations match those found on plants developed from seeds dressed with imidacloprid). No effects of the imidacloprid were detected on bee longevity or food consumption. However, a very high effect was found on the size of the HPGs, with 9 days old treated bees having much shorter diameter of acinus compared to control bees.

Our aim is to define and standardize the methodology in accordance with other laboratories in order to have undisputable results on risk assessment of plant protection products on honey bees and to discuss other possible applications of the methodology.

Title:

Testing the effect of imidacloprid on honey bee mobility

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Text of Abstract: Please fill in the table with the data relevant to the trials described in your abstract.

What kind of tests do you use hoarding cages for ? (eg toxicity, bee vitality, longevity, development of disease, efficacy of medicaments, synergic effects of several factors, etc)	Toxicity of pesticides, Disease resistance,
What kind of bees do you use (subspecies, age) ?	Apis mellifera anatoliaca, A.m. carnica, A.m. caucasica, A. m. syriaca
What kind of cage do you use (size, material, etc) ?	Wooden cages with wire screen sides to hold one stansart bee frame.
Technical / practical problems encountered (eg reduced bee vitality, death caused by handling, palatability of administered food)	

Short description and results of your trials using hoarding cages (limit text to 250-400 words):
We have used wooden cages with wire screens sides to hold one standart bee frame. Cages were kept in an incubator at 34° C where combs in the frame were filled contaminated and uncontaminated honey. Contaminated honey was obtained from dead colonies near the sunflower fields. The seeds of the sunflowers were treated with a systemic insecticide containing imidacloprid. Honey bees were monitored for a week in two types of cages by taking their videos. The effect of contaminated honey was the reduced mobility of honey bees. Honey bees that were fed on contaminated honey were grouped at one corner of the frame and they stood still whereas honeybees feeding on uncontaminated honey were running all over the frame.

We plan to compare the reactions of different races in Turkey to pathogens and chemicals. To use a standardized method for doing such experiments will be very valuable for us in our research.

Title:

Methods for testing pesticide toxicity on honey bees in cages

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Text of Abstract: (limit text to 250-400 words)

Honey bees were kept in plexiglas and nylon net cages (20x20x30 cm, 300 μm mesh); all activities were performed through a circular opening (10 cm diameter) in a net wall, connected to a 20 cm long sleeve.

Compounds were tested at the highest concentration among those recommended for crop treatment on the label (field concentration) and were gradually diluted down to the concentration that caused mortality not significantly different from that of the untreated controls.

Ten foragers, taken from the flight board of a single hive - which was periodically checked to exclude the presence of the most common honey bee diseases - were placed in each cage and introduced not more than 15 minutes from capturing. In some instances honey bees were starved for 2 hours in order to overcome pesticide repellence.

Tests were performed in a dark room at 28-30 °C and 70% relative humidity.

For oral toxicity tests honey bees were administered a 25% sucrose solution, pure for untreated controls or with known amounts of the compounds to be tested. Solutions were administered through a dish feeder (7 mm high and 28.2 mm internal diameter) in which a test tube (25.9 mm x 70 mm) was inserted. The resulting 1.15 mm annular space allowed the foragers to suck the liquid, but prevented contact with the legs. Solutions were made available to the honey bees for one hour; sugar candy was administered throughout the remaining part of the trial.

For indirect contact tests leaves were collected in areas far from possible pollution sources and soak-sprayed with pure water, for untreated controls, or with water suspensions of the products to be tested. They were left to dry in the shade for at least three hours and then introduced into the cages so as to cover completely the floor. Honey bees could walk freely on them for three hours, then the leaves were removed.

During the trial, honey bees were fed with sugar candy from a feeder obtained by opposing two watch glasses so as to obtain a 1 mm slot; so honey bees did not touch the sugar candy, except with their proboscis.

Honey bees were considered "dead" when they remained absolutely still when touched with a brush or during a 10 second observation period.

Title:

Observations on pathogens

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Text of Abstract: (limit text to 250-400 words)

The study of pathogens under laboratory conditions requires the use of cages to confine the bees during the study. During those test, honey bees are kept inside the cages, under different conditions and for a time variable depending on the pathogen to study.

Up to now, no detailed standard regulations have been described. As a general rule, cages are recommended to be easy to clean and well-ventilated (OECD213, OECD214, but they are for toxicity tests). Stainless steel, wire mesh, plastic, cartoon or wood are the most commonly materials used. The size of test cages has been established either and for example OECD guidelines (for toxicity test) only mention they should be appropriate to the number of bees, providing adequate space.
However, in the specific case of pathogens, some factors are very important since cages should guaranty they are not a source of pathogens (from previous assays, for example) and they should satisfy right conditions for the development of bees under study.

Under the absence of standards, it seems every research team has developed cages of different size, form and material (sometimes even more than one) that they have been adapting to any research they performed.

The necessity of standardization of cages for the pathogen studies in honey bees should be discussed, since this could be a first step when trying to standardize the pathogens research in honey bees.

Title:

A review on different laboratory rearing conditions and materials

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Text of Abstract: (limit text to 250-400 words)

Researchers studying the effects of different stressors (intoxication, pests, diseases) on honey bees apply either official protocols or local methods. There are several guidelines that outline trial conditions. Nevertheless in most cases the material used for bee incubation is not precisely characterised. The present contribution doesn't give indications regarding optimal trial conditions and best material to be used for all trials but is aimed at rising some technical issues for discussion.
The following trial parameters will be considered: bee collection for the trial (age, collection method); introduction into hoarding cages (eg. anaesthesia with CO2, chilling, manually with no aid); stressor administration (contact, ingestion, feeding method); incubation (incubation conditions, duration).
The following material topics are discussed: incubator (precision, settled parameters); hoarding cage (material, dimension, structure); feeders.
A wide range of potentially encountered problems will also be presented for discussion on standardisation.

Title:

Size does matter: a new depth adjustable metal cage adapts to its task

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Text of Abstract: (limit text to 250-400 words)

Hoarding cages are used for a huge variety of experiments in honeybee science. Depending on the nature of the test and the number of bees, different sized cages are in use. Here we present a novel stainless steel cage that can be adjusted in depth. Whereas the largest volume (600cm³) can contain a high number of bees even with a piece of comb; the smallest setting (100cm³) allows bees just to move in two dimensions. The latter is fundamental for behavioural studies in which automated camera and analyses systems monitor tagged bees from the top. Since the cage is made out of stainless steel it is easy to autoclave which is an essential feature in cage tests with pathogens and pesticides. So this cage provides multifunctional applications in a wide range of tests.

Title:

Survival and inclination to feeding of caged honey bees contaminated with oxalic acid

Authors and Affiliations:

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Text of Abstract: (limit text to 250-400 words)

Mortality and food consumption were observed in caged honey bees that had been contaminated with oxalic acid (OA) in the field. A preliminary stage was performed to determine the time when the highest OA amount was present in the intestines. Two administration methods were considered: trickling (1.7g OA in sucrose solution) and sublimation (1.4g OA). They were applied to two different colonies of similar size from which samples of 50 adults were taken at 0, 24, 48, 72, 96 and 168h. After gentle washing, the bee intestines (except honey-sacs) were slipped off and macerated. Analyses were made on both intestine pools and washing waters to measure intestinal and external OA, that were summed up to calculate the overall OA contamination. At 24h, the bees treated by trickling and sublimation had similar total OA load (about 8.5 microg per bee). Lower amounts were detected later, but a distinctly sharper decrease occurred for sublimation, which also implied a lower proportion of the total OA being in the intestines. A further step was made on bees collected from a colony 24h after an OA treatment by trickling that previously showed the highest intestinal OA amount. 30 bees were introduced into each of 4 laboratory cages and *ad libitum* fed with 1:1 honey solution. Pre-treatment bees from the same colony served as a control. Food consumption and bee mortality were registered for 16d. At 24 and 48h, the food uptake was significantly lower in treated, but the average individual consumption in the whole period (1022.5mg per bee) was not significantly different. The trial showed that the OA contamination due to the treatment may play a temporary influence on the bee attitude to feeding, that is likely to be associated to the intestinal aliquot.

At 16d, the cumulative mortality was 45.8 and 42.8% in treated and controls respectively. The difference was not significant, which contrasts with the hypothesis of an acute OA toxicity at the usual treatment dosage.

When the honey bee mortality along the timeline is evaluated always on the same groups, like in this case, a time sequence of non-independent observations is generated. This casts the problem of a correct statistical approach. (Throne *et al*., 1995).

Title:

The use of hoarding cages for the evaluation of single and synergistic effects on individual bees after artificial infestation with Nosema spores

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Text of Abstract: (limit text to 250-400 words)

We use hoarding cages in order to evaluate the longevity of bees and the course of infestation after individual feeding of young bees with spores of *Nosema spec*. and/ or with pesticides. A large number of tests with Nosema infested bees in hoarding cages have been published, however, the details of these test systems are often not described sufficiently and a comparison of the methods reveal an alarming amount of variations.

In preliminary tests we therefore compared crucial details of the test performance, i.e. the exact age of the bees at the time of infection, the number of spores for the start infestation, the way and quality of feeding, the condition within the incubator and the number of bees per cage. On the basis of these experiences we will describe and discuss our current test system and present preliminary results from the recent season which are performed in the framework of the EU project BEEDOC. These results indicate that (i) *Nosema ceranae* is the predominant Nosema species in South Germany and (ii) the effect of this new parasite on the longevity of bees in hoarding cages is lower than expected. An open question is to what extent additional parameters like social behaviour (trophallaxis) and/ or components of the immune system can be included in the evaluation of hoarding cages.

Title:

Methods for observation of honey bees in hoarding cages for different aims

Authors and Affiliations: Please list all authors and their affiliations. For the contact author only (typically the senior author), include email address, mailing address, and phone number. Denote contact author with an asterisk = *. (font size 12, Times new Roman)

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Text of Abstract: (limit text to 250-400 words)

Especially for toxicity tests, the observation of adult bees is very useful by using hoarding cages. For this aim, 10x10x10 cm cages in cubic shape are used . They are covered by wire-mesh among wooden parts. If they are used for disinfectan products activities, the product should be applied before putting adult honey bees inside it. In case they are used for development of infections it could be filled by honeybees directly. Anyway, they should be clean before use. 50 newly emerged honey bees are collected from combs and put inside the cage. Small plastic injectors 5 ml and 10 ml are used for feeding them by syrup (1:1) from two small holes top of the cages. Avarage 5 ml syrup and av. 1.5 gr pollen cake are consumed by bees per day .If the experiment is about toxicity of some products, two kinds of syrup must be prepared; one of them contaminated with testing chemical. All groups contain control and 3 replicated cages are proceeded maximum for 20 days. Every two days dead bees are counted and recorded the numbers for statistical analysis.

Title:

Toxicity tests on *Apis mellifera* and *Varroa destructor* in hoarding cages – oxalic acid toxicology

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Text of Abstract: Please fill in the table with the data relevant to the trials described in your abstract.

What kind of tests do you use hoarding cages for? (eg toxicity, bee vitality, longevity, development of disease, efficacy of medicaments, synergic effects of several factors, etc)	Toxicity/mortality tests, screenings and development of medicaments
What kind of bees do you use (subspecies, age)?	*Apis mellifera carnica*, worker bees about 5-10 days old brushed from brood or food combs
What kind of cage do you use (size, material, etc) ?	Wood cage with glass plate amd wire mesh either side (100x65x110mm)
Technical / practical problems encountered (eg reduced bee vitality, death caused by handling, palatability of administered food)	Lab conditions/methods: **bees** Temperature: 20-22°C; R.H. 55-62%; no wind draft Food ad libitum: 50% sugar-syrup or food dough Individual administration: oral 10µl in sugar-syrup 50%; dermal 5µl solution in water 10 bees per cage, 3 cages per test + replicate, n minimum = 60 bees Control group – same handling but administration of placebo Lab conditions/methods: **mites** Mites foraging on bees are recruited from colonies and these bees get treated as described above (oral/dermal application), to every bee with a mite another uninfested bee gets placed – this way mites can change host from infested to healthy bees and mortality as a result of handling can be avoided 10 bees with mites + 10 bees without mites per cage, 3 cages per test + replicate n minimum = 60 mites

	Control group – same handling but administration of placebo Cages are placed in shallow dishes to collect fallen mites, no Vaseline to exclude combinatoty effects on mortality data collection

Short description and results of your trials using hoarding cages (limit text to 250-400 words):

The toxicity of a substance depends to a great extent on the route of administration. So far toxicology data of oxalic acid treatment on individual bees derived in the laboratory were not available without combinatory effects allowing to establish a dose-response-relationship for oxalic acid.
We investigated toxic effects of oxalic acid dehydrate (OA) on *Apis mellifera* after dermal respectively oral application. 5 to 10 day old worker bees were kept in small groups (3x10 bees per dosage, one replicate) in cages in the laboratory. After individual application of OA sugar-syrup solution in different dosages the mortality was determined after 24/48/72 hours to establish a dose-response-relationship, threshold value, LD_{10} and LD_{50}. The control groups were treated with a placebo (50% sugar-syrup) and kept under the same laboratory conditions. Statistical analysis was conducted using the Mann-Whitney *U*-Test ($p \leq 0.05$) on the total bee mortality after 72 hours and Probit analysis to derive LD_{10} and LD_{50} values. Dermal application of OA was well tolerated by the bees. The dosage of 175µg/bee, corresponding to the 3.5% solution used in beekeeping practise, did not cause mortality different from controls. Application of 250µg increased the mortality, 375µg OA per bee caused mortality significantly higher than in the control group (MWU, $p \leq 0.05$). Generally, bees reacted much more sensitive to the oral application of OA: 10 and 50µg did not cause mortality different from the control group, while 75µg resulted in significantly higher bee mortality (MWU, $p \leq 0.05$). 100µg killed 55% of treated animals. The threshold values are: 285µg/bee (dermal) resp. 75µg/bee (oral), the LD_{10}: 151µg/bee (dermal) resp. 62µg/bee (oral) and the LD_{50}: 582µg/bee (dermal) resp. 98µg/bee (oral).

Title:

Evaluation of pesticide toxicity to honeybees by indirect contact

Authors and Affiliations:

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Text of Abstract: (limit text to 250-400 words)

At field conditions, the main ways of exposure of honeybees to pesticides are ingestion, direct (topical) and indirect contact. To assess the environmental risks of plant protection products to honeybees, it is important to study these ways of exposure in laboratory, semi-field and fields tests. While EPPO guidelines provide standardized methods for oral and direct contact toxicity tests, almost no protocols are available for indirect contact toxicity tests.

From 2003 to 2008 the Bee Protection Group (researchers of the University of Bologna and CRA-API), conducted acute toxicity tests with 63 pesticides on adult honeybees in laboratory conditions. With the aim of obtaining *fast* and *applied* data, useful to growers and Regional Extension Services, we tested the toxicity by ingestion and indirect contact of the commercial products applied at recommended field rates. For the indirect contact tests, a standardized protocol of Arzone and Vidano (1980) modified according to EPPO guidelines was used. Ten adult honeybees housed in small cages were fed *ad libitum* with a 50% sugar solution until the end of the trial and maintained in darkness at 25 °C. For each tested agrochemical, three replicates were used. Apple leaves were sprayed with the test product at the recommended field rate (spray volume: 200 µl/leaf of solution) and left to dry completely. The bottom of each cage was then covered with two apple leaves treated with the tested product, and the honeybees were introduced into the cage. After 3 hours, the treated leaves were removed from the cage. For each agrochemical, corrected mortality after 12 h from the beginning of the trial was calculated by using Schneider-Orelli's formula and its toxicity class was established. In 2009 and 2010, an appropriately modified version of this method was used to study the toxicity of dusts derived from corn seed dressed with three neonicotinoids (imidacloprid, clothianidin and thiametoxam) and one phenylpyrazole (fipronil) in laboratory conditions.

Indirect contact toxicity tests most closely resemble exposure of honeybees in the field, but the following methodological problems may arise:

- it is not possible to determine the exact quantity of active substance uptaken by each single bee;
- the exposure time of the bee to the tested product may not be realistic;
- the residual toxicity of the active substance may depend on the time delay between the contact of the honeybees with the treated surface and the application of the treatment;
- the toxicity of the pesticide may vary depending on the type of substrate used in the trial (leaf, polystyrene, filter paper);
- the results obtained in indirect contact tests are hardly comparable with those observed in topical (direct) contact trials.

The impact of each of these factors was evaluated in specific experiments.

Title:

Standardized methods for honey bee rearing in hoarding cages

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Text of Abstract: (limit text to 250-400 words)

Rearing newborn honey bees in cages for use in *Nosema ceranae* infection experiments, we require them to be:

1. Free from infection
2. As similar to their natural counterparts as possible

Newborn bees are necessary, because they are free from infection, unexposed to spores within the hive. Previously, newborn bees have been fed a diet of 50/50 sucrose solution, sometimes supplemented with "pollen patty," a paste containing sucrose and naturally collected pollen (sterilized for spores in a -80°C freezer). While this is an improvement over a purely liquid diet, we have incorporated nurse bees into our rearing technique. Using two compartments, separated by a screen, bees collected from the hive are placed in one compartment, newborns in the other. Both have access to sucrose and pollen *ad libitum*. The elder bees take the role of nurses, able to feed via the screen. In our experiments, qualitative measurements show bees raised by nurses to be more active, with less mortality. They also begin to build comb. Furthermore, none of the newborns developed *N. ceranae* infection during their raising, confirming them as uninfected for future trials.

Title:

Pathogen and pesticide experiments in bees using laboratory hoarding cages

Authors and Affiliations: Please list all authors and their affiliations. For the contact author only (typically the senior author), include email address, mailing address, and phone number. Denote contact author with an asterisk = *. (font size 12, Times new Roman)

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Text of Abstract: (limit text to 250-400 words)

Toxicity, bee longevity tests and experimental infections were conducted on caged workers in our laboratory. For the purpose wooden cages (7'5 x 4'0 x 5'0 cm) with one wired and one glass wall on each side were used. Small hole is drilled in wooden top bar of the cage for insertion of the standard micro test tubes (1'5 ml) used as gravity feeders. In our experiments caged bees were normally provisioned with tap water, sugar candy and sugar syrup (1:1). Caged bees are kept in an incubator at 30±1°C in 24-hour dark period with a pan of water placed at the bottom of the incubator to ensure 60 – 70 % RH. We used pincers to collect bees from the cages through the bottom board opening and anaesthetize them with CO_2 before further procedures. Dead bees were removed from the cages regularly. The problem we encountered during rearing honeybee in hoarding cages was the blockage of the small holes made in standard micro test tubes used for provisioning honeybees with sugar water due to crystallization. One day old caged workers were used for *Nosema spp.* spore multiplication. In this case worker bees were individually infected by feeding spores in sugar solution. Inoculated workers were then introduced into hoarding cages for defined period of time and checked regularly for spore loads. We have performed similar experiments in order to monitor the effects of pesticides or pathogens on individual worker bees. Caged workers were therefore inoculated with *Nosema spp.* spores or treated with pesticides for longevity test and sampled for dissection and further histological, molecular or cell biology analyses. We have also used caged workers in experiments to test potential infection transmission between workers. In these experiments we caged pathogen inoculated workers together with untreated control workers marked with pin paint on the thorax. Combination of two or more cages joint together was used to study trophallaxis activities among workers. Workers were separated in different cages, but could communicate through wired wall and thus transfer food or other fluids among them. Appropriate number of test and control cages was included in all experiments in order to perform statistical analyses. Further experiments will be conducted regarding studies of longevity, effects of different factors on alimentary tract, hypopharyngeal glands and immune system in pesticide treated, *Nosema spp.* or virus infected workers.

Title:

Neonicotinoid insecticide residues in honey

Authors and Affiliations:

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Text of Abstract: (limit text to 250-400 words)

Neonicotinoid insecticides are of growing importance in today's agriculture and are widely used as a measure of pest control for a broad range of pest insects. These highly potent insecticides work in a very specific way as agonists on the postsynaptic nicotinic acetylcholine receptors of the insect's central nervous system causing the blockage of signal transmission. However, non-target effects on beneficial arthropods cannot be excluded *per se*. Indeed, different exposure pathways may result in the contamination of beehive products with neonicotinoids. Here we developed and validated an analytical LC-MS/MS approach to detect and quantify neonicotinoid residues in honey. All commercially available neonicotinoids as well as their residues (see EU residue definition) were included. The method proved to be rapid, sensitive and reliable and was used for the analysis of 28 Austrian flower and forest honey samples collected in the framework of the Austrian residue control program in 2009. Two out of eight neonicotinoids could be detected in the samples. Thiacloprid residues were found in both flower and forest honey samples and acetamiprid residues in flower honey. Details of the method and the obtained will be displayed and the relevance of residues will be discussed.

Title:

Factors which influence the variability of honeybee cage experiments

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Text of Abstract: (limit text to 250-400 words)

Caged experiments with honey bee *Apis mellifera* revealed high variability of results, although same modes of experiments were done. This variance is influenced by the development of hypopharyngeal glands in individual bees. Homogeneous nutrition after hatching is necessary for low variability of physiological condition (mainly in individual resistance to pathogens, filling of rectum and longevity of bees).

The best and comparable results are obtained if bees are from one brood frame. This frame with sealed brood is removed from the hive, all stored pollen are cut off or dropped with wax and the frame is put in an incubator (34 ± 1°C). Newly hatched bees are moved to cages. Suitable number of bees is 10 – 20 for Petri dishes and 50 – 250 for cage experiments. Bees should be in cages with a part of comb (thermal treated). Bees are fed with standardized glycide and protein food since first hour after hatching. Dead bees cause stress of living bees, thus dying bees are removed from cages at least once per day.

In special experiments when feeding of royal jelly is excluded, bees are fed with sugar solution only. For this purpose, keeping of drones is possible without workers. Drones are hatched from drone frame in an incubator as described above and are fed with sugar food injected to combs.

Oxygen consumption of bees depends on experiments burden. Then fresh air supply is necessary for cage experiments.

Last but not least source of variability is, if the cages are queenless or queenright.

The community without the queen is completely different to normal honeybee biological unit.

Title:

Laboratory infection of bees with viruses in Poland - some experimental conditions

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Text of Abstract: (limit text to 250-400 words)

In the Laboratory of Bee Diseases at Warsaw University of Life Sciences the method learned at the Rothamsted Experimental station is used to perform experimental infection of bees with viruses. Bees, together with the comb to which they adhere (from a hive super), are taken to the laboratory, anaesthetised with CO_2 and placed in experimental cages (30 bees per cage). They are supplied with distilled water and 60% sugar syrup (w/v) and kept until the next day in the incubator at 30°C and relative humidity about 26%. Then they are anaesthetised (in groups) and injected with the different dilutions of virus suspension or control fluid. Three cages of bees for each suspension and for control bees are used. For injection a Burkard microapplicator is used. Each bee receives 1µl of fluid under the 3rd abdominal tergite. During injection the bee is held by its wing. After injection, bees are placed in cages kept in room temperature until they start moving around and next are placed in an incubator in appropriate temperatures (depending on the virus). During the two following days dead bees are removed from each cage. From this time on, bees are observed carefully each day (for the presence of disease signs) and dead bees are collected from each cage every day and placed in a deep freeze (temp -18). The water and sugar syrup are replenished when needed. The time of the observation (in days) depends on the virus.

Last time we used the method to work with chronic bee paralysis virus. However, then we used foragers (collected into a black veil placed at the entrance to the hive) and bees at the age of 11-12 days (marked on the combs on the first day after emerging in an incubator, placed in colonies and picked from combs – by catching by wings – on the appropriate day). When injected bees became less vigorous (after a few days) the cages were opened for a while under a transparent glass shade for easier observation of bee movements.

Under the described conditions very few control bees (injected with the control fluid) died during two days after injection (probably because of different manipulation injuries), and next no worrying symptoms were observed until the tenth day of the experiment. This was sufficient time to observe the development.

Title:

Understanding *Nosema* using caged honey bees: pathology, interactions between congeners, and the evolution of experimental conditions

Authors and Affiliations:

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Text of Abstract: (limit text to 250-400 words)

Two microsporidians, *Nosema apis* and *Nosema ceranae*, parasitize western honey bee populations worldwide. Symptoms of parasitism by *N. apis*, the historical microsporidian species of western honey bees, are relatively well understood compared to those of its recently detected congener, *N. ceranae*. Curiously, it is not known why *N. ceranae* appears to have displaced *N. apis* in many regions of the world, or why *N. ceranae* is highly virulent and associated with increased colony mortality in some locations, but not in others. Because of recent large-scale honey bee die-offs in parts of North America, Europe, and Asia, data on pathology associated with *N. ceranae* infection and interactions between *Nosema* congeneric parasites are of significant interest. Here, we describe a cage study performed in Atlantic Canada that used regional *Nosema* parasites and western honey bees to investigate these questions. We also discuss how experimental design and methods, such as growth chamber conditions, and sources of *Nosema* spores and bees, may affect comparisons among published cage studies.

Title:

Evaluation of oral transmission of deformed wing virus variants between adult honey bees (*Apis mellifera*)

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The social behaviour of honey bees (*Apis mellifera*) represents an ideal model to study the interaction between mode of transmission and virulence of pathogens. Deformed wing virus (DWV), the most studied virus disease of honey bees, has been frequently associated with honey bee colony losses when vectored by the ectoparasite *Varroa destructor*. DWV is a single strand RNA virus, and therefore naturally prone to producing a variable quasi-species of related variants, due to the high replication rates and the lack of proofreading enzymes associated with RNA replication. This cloud of mutants is maintained and their internal relationships represent the genetic variability of the virus population. Two major, co-existing DWV variants have been recently identified in natural Swedish bee populations, comprising a multi-modal quasi-species. These are used to look at the epidemiological interaction between virus genetics, virulence and bee-to-bee transmission.

Trophallaxis is used by honey bees for several activities, such as feeding, communication inside the colony and the transfer of nectar. However, this social behaviour can also potentially transmit pathogens between adult bees. To investigate the effects of trophallaxis between adults on the dynamics of DWV variant transmission, DWV-negative and DWV-positive adult bees were placed in contiguous hoarding cages. The groups of bees were physically separated by a metal screen. Nectar and pollen feeders were provided to the cage with the DWV-infected worker bees, which then fed the uninfected bees from the contiguous cage by trophallaxis, thus potentially facilitating virus transfer. The oral transmission of these variants between different honey bee development stages is evaluated quantitatively to understand what selective processes act in maintaining these DWV variants in the honey bee population and if there are differences in relative virulence between the variants.

Conclusions:

The final discussion focused on the different parameters involved in pathogen and pesticide tests, ranging from those related to:

- Bee
- Cage
- Tested stressor
- Feeder
- Nutrition
- Incubator

It therefore emerged that there is an extreme diversity of laboratory conditions and materials. Of this multitude of factors we decided that some of them influence experimental results more than others, and standardisation for these is more important. The following factors:

- Protein nutrition
- Kind of sugar
- Bee number/density in cage
- Age of bee
- Season collection
- Temperature
- Acceptable control mortality

were selected as those with a priority in standardisation needs.

It emerged quite clearly that the amount of data and knowledge available is not sufficient to decide which conditions are better than others, apart from the following:

- Reusable cages should be sterilised and cleaned, for all experiments;
- 10-50 bees are recommended for average cage size (10-15 x 10-15 x 5-10 cm);
- It is recommended that in experiments involving pathogens control and treated cages should be kept in separate incubators;
- Minimum level RH should be 60%.

All participants agreed that best conditions are those that ensure the highest possible survival of control bees, indeed, their survival is object of standardisation as no threshold currently exists.

To achieve standards for testing bees in hoarding cages specific studies and experiments must be performed in ring test mode, in order to yield results that can be used to establish reliable and repeatable conditions. To do this, specific funding is needed, as this work would require at least a 3 year full time position to deal with coordination of a group and collection and processing of the resulting data.

The participants thereby agreed to ask the Action Chair to forward such a request to European and international agencies, such as EMEA, EFSA, OIE, FAO, and private companies involved in honey bee research.

Furthermore the participants agreed that aside from the establishment of standards, many conditions (detailed in a specific file) should be specified and reported in materials and methods of publications, and that this list should thereafter be forwarded to researchers involved in honey bee research and to editors of scientific journals. The file will be published on the Coloss website.