FA 0803

Proceedings

of the COLOSS Work shop

"Method standardization for larval tests"

Graz, Austria, 7.-9.6.2010

Dear colleagues,

On behalf of the local organising team, it is my personal pleasure to welcome you to the Work shop “Method standardization for larval tests” in Graz, Austria.

I would like to thank all the people who have helped to organise and conduct this meeting and of course all contributors for submitting their abstracts, which I hope will stimulate rewarding discussions on the method standardization, trouble shooting and research conducted.

The method to rear larvae will further advance honeybee science and enable us to answer several questions that cannot be solved inside a colony due to the complex system of rearing and cannibalism inside a colony.

Financial support is granted by COST via the Action FA0803 COLOSS and the Karl-Franzens-University Graz.

I am looking forward meeting all of you, and hope you will enjoy this work shop in Graz.

Karl Crailsheim
Vice Chair of COLOSS

Graz, Austria, Tuesday, 25 May 2010

The local organising committee for the work shop and editors of this proceedings:

Department of Zoology, Karl-Franzens-University Graz
Robert Brodschneider, Karl Crailsheim, Ulrike Riessberger-Gallé, Jutta Vollmann

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Monday, 7.6.2010	
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10:30-10:50	Talk by Karl Crailsheim
10:50-12:00	Method standardization vs. method modification, open questions and troubleshooting. Talks by Hanewald, Peterek, Medrzycki, Nakrst (5-10 min each)
12:00-13:30	Lunch
13:30-15:00	**Open session** Method standardization vs. method modification, open questions and troubleshooting (see below).
15:00-15:30	Energy break
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9:00-10:00	Application of larval rearing methods I Talks by Charrière, Jensen, Forsgren (5-10 min each)
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10:00-12:00	Optional sightseeing walk

Registration on site is required. Registration fee: 30 € (incl. social dinner on 8.6.)

Open Session Method standardization vs. method modification, open questions and troubleshooting: gives every participant the opportunity to present specific questions or propose standardized procedures. To achieve conclusion and document this session, power point will be used. Open questions or (max. 4) slides necessary for this can be sent to us before the workshop until June 7th 14:00.

Open Session intended studies: gives newcomers to this method the opportunity to present and discuss their ideas and to find experienced partners if desired. Power point can be used.

A new larval *in vitro* rearing method to test effects of pesticides on honey bee brood.

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A new *in vitro* rearing method of honey bee larvae was devised to assess the effect on brood of any substance that can reach the hive with nectar and pollen. This method could be used in the risk assessment process since it meets with the following characteristics:

- low mortality rate when no treatment is applied,
- standardisation of the test,
- easiness of carrying out,
- sensitiveness to treatment,
- precise control of the ingested doses of diet and pesticide,
- control of larval mortality for each larval instar,
- control of pre pupae weight,
- measurement of adult emergence rate.

The method enables the study of 1/ lethal effects (calculation of LD50) 2/ sublethal effects (prepupal weight, duration of development, adult morphology and behaviour).The method can be used either to study acute effects by applying contaminated diet to one particular instar, or to investigate chronic effects by providing each day the larvae with the test substance.

The use of our rearing method is illustrated with thre insecticides:

- Dimethoate considered as a reference insecticide in toxicological tests on adults,
- Diflubenzuron which belongs to the IGR group.
- Fenoxicarb which belongs to the IGR group.

Aupinel, P.; Medrzycki, P.; Fortini, D.; Michaud, B.; Tasei, J.N.; Odoux, J.F. A new larval in vitro rearing method to test effects of pesticides on honey bee brood. Redia. 2007, 90: 91-94.

Aupinel, P.; Fortini, D.; Michaud, B.; Marolleau, F.; Tasei, J.N.; Odoux, J.F. Toxicity of dimethoate and fenoxycarb to honey bee brood (Apis mellifera), using a new in vitro standardized feeding method. Pest Management Science. 2007, 63 (11): 1090-1094.

Aupinel, P.; Fortini, D.; Dufour, H.; Tasei, J.N.; Michaud, B.; Odoux, J.F.; Pham-Delègue, M.H. Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae. Bulletin of Insectology. 2005, 58 (2): 107-111.

How stress during larval development affects immature and adult honey bees?

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Bees are exposed to various stress factors during development such as diseases, pesticides and afflicted weather conditions. Development of honey bees could be thus affected directly by pathogens and other factors or indirectly by food. Impact of different stress factors in the period of bee development can be successfully tested by an *in vivo* method of rearing bee larvae. Bees are reared artificially in controlled environment by feeding larvae with known composition and amount of food. The method enables to test effects of stress factors on weight, larval development, survival and performance of adult bees. The method of artificial rearing of larvae demonstrated in workshop in Graz will be applied at the National institute of biology in Slovenia to test influence of pathogens and sub-lethal doses of pesticides applied during larval development on longevity, flight activity, orientation and learning of adult bees.

Effect of insect resistant (Bt) GM corn pollen on the development of honeybee (*Apis mellifera*) larvae *in vitro.*

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There is no officially adopted method for rearing honeybee larvae in vitro. The method recommended by the 91/EEC directive for testing bee larvae for toxic compounds, hides several errors (Aupinel et al., 2005). Publications of Rembold and Lackner (1981); Brodsgaard et al. (1998); Malone et al. (2002) have been criticized recently, because of high mortality rate along rearing.
In our experiments larvae of 3 days were transferred into U-bottom plastic cell culture plates. Our basic larval diet (BLD) contained d-glucose, d-fructose, yeast extract and gentamycin. It was mixed with 1:1 fresh royal jelly. This feed was given 20 % pollen (1:1 beebread and Bt (MON810)- or isogenic corn pollen. The plates were incubated at 35 ˚C with a relative moisture of 90 %. Beebread with royal jelly and pure royal jelly served as control feeds. The larvae were fed and weighed one by one two times a day.
According to the results of our experiment the difference in weight between Bt- and iso groups was 18 % which may demonstrate the development breaking effect of Bt-pollen. After 72 hours larval mortality suddenly increased and only the 5-10 % of the larvae survived longer.
In histological preparations of the midgut, the peritrophic membrane of larvae fed with Bt-pollen, seemed to be discontinuous and incomplete that might explain deficient digestion and lower weight gain.

Quality of artificially reared honey bees.

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To evaluate the quality of semi-defined diets for honey bee larvae we compared physiological parameters of adult bees reared according to the protocol of Aupinel et al. (2005) to sister bees reared under natural conditions in the colony. Artificially reared honey bee larvae gain sufficient nutrients to develop into adults capable of long, persisting flights. We demonstrated differences between artficially and naturally reared honey bees in top performance flight, wing size and dry weight of thorax in one experiment (Brodschneider et al., 2009a) and in 16 out of 18 or 17 out of 17, respectively investigated parameters of body weight or size in another experiment including two colonies (Brodschneider et al., 2009b). Artificially reared larvae were always slightly inferiour compared to their naturally reared sisters, suggesting deficiencies in the larval diet or the way it is applied. The method for artificial larval rearing, though not yet being a totally chemically defined synthetic diet, enables research on nutritional requirements and malnourishment of developing larvae to be conducted *in vitro*. This method could be used in experiments helping to understand the effects of sublethal protein malnutrition during larval development as it may occur during shortage of pollen.

Aupinel P., Fortini D., Dufour H., Tasei J.N., Michaud B., Odoux J.F., Pham-Delègue M.H. (2005) Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae, Bull. Insect 58, 107–111.

Brodschneider R., Riessberger-Gallé U., Crailsheim K. (2009a) Flight performance of artificially reared honeybees (*Apis mellifera*), Apidologie 40, 441–449.

Brodschneider R., Steiner D., Moder A., Vollmann J., Riessberger-Gallé U., Crailsheim K. (2009b) Synthetic larval diet produces lighter and smaller honeybees (Apis mellifera), Apidologie 40, 663–664.

Virulence of different *Melissococcus plutonius* strains tested by an in vitro larval test.

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In Switzerland, European foulbrood (EFB) is a honeybee disease which requires control in accordance with the Swiss Animal Epidemic Regulation. After having been under control for the last 30 years, cases have recently been reported with increased frequency. Between 1970 and 1998, approximately 20 to 50 diseased apiaries per year were sanitized by the veterinary authorities. However, since 1999 there has been a significant increase of reported cases, with more than 790 apiaries affected in 2009 alone. This represents a prevalence of 4.2 %.

At the moment, we have no explanation for the dramatic expansion of this highly infectious brood disease. Significantly, we also observe that currently several regions in Switzerland are free of EFB as in most of Europe where EFB reports are anecdotal and present with low morbidity. An exception being the UK where the prevalence of EFB is also rather high.

One hypothesis to explain the resurgence of EFB in Switzerland and the other isolated regions where EFB is problematic could be the emergence of a more virulent *Melissococcus plutonius* strain. To test this hypothesis, we used the larval in vitro rearing method developed by Aupinel et al. (Pest. Manag. Sci., 2007) with minor modifications. Here, we compare different Swiss, French and Italian EFB strains and present the first results of these tests

Evaluation of the effects from probiotic bacteria on *Paenibacillus larvae* using *in vitro* rearing of honeybee larvae.

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Exposure bioassays (*in vitro* rearing of honeybee larvae) were used to evaluate the antagonistic effects of newly identified lactic acid bacteria (LAB) originating from the honey stomach on the honeybee pathogen, *Paenibacillus larvae*. To rear larvae *in vitro* a protocol by Aupinel *et al*. (2005) was followed with minor modifications. Worker honey bee larvae were reared in 48-well tissue culture plates on a diet consisting of 50% royal jelly (v/v), and 50% of an aqueous solution of D-glucose (12%) and D-fructose (12%). Before any experiment, the required amount of diet was prepared and then stored at +4°for the duration of the experimental feeding. Before grafting, each plastic well was supplied with the pre-warmed diet. The control group was provided with uninfected diet while the experiment groups were either provided larval diet spiked with known amounts of *P. larvae* spores or *P. larvae* spores mixed with the LAB mixture. First instar worker larvae (less than 24 hours) were grafted from larval combs and transferred to the surface of the larval diet of the different treatments. The larvae were kept in an incubator at 35℃ until the experiment was finished 14 days post-infection. Throughout the experiment dead larvae were removed daily and cultured on agar plates to verify presence or absence of *P. larvae*.
Adding the LAB mixture to the larval food significantly reduced the number of AFB infected larvae in the exposure bioassays. The results demonstrate that honey bee specific LAB possess beneficial properties for honeybee health. The use of exposure bioassays as well as possible benefits from enhancing growth of LAB or from applying LAB to honeybee colonies will be discussed.

Experiences with artificial feeding in a standardized *in vitro* method for rearing larvae of the honeybee *Apis mellifera.*

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BASF works on the standardization of the method proposed by Aupinel since 2007 (member of the ring test group). After establishing the method and reaching acceptable control mortalities we started to work on simplifications and improvements to the method of Aupinel. To give a few examples: we used one instead of three diets, reduced the caging time of the queens, reared in different climatic chambers and incubators, etc. LR_{50} and NOECs were determined for Dimethoate and Fenoxycarb as proposed by the ring test group led by P. Aupinel.
As observations during the last years showed further research should be done concerning:

- healthiness of the colonies during the course of a year (increasing control mortalities starting end of July), stage of the larvae and diseases
- quality of the Royal Jelly
- the need of three different kind of diet
- caging time of the queens
- climatic conditions during the conduct of the study
- comparison acute and chronic feeding

BASF would be very interested in a comparison of results derived from studies done in the laboratory and results from semi-field and field trails to have a kind of validation and an evidence of the sensitivity of the method.

Artificial larval rearing as a tool for evaluating the effects of imidacloprid on honey bees under laboratory conditions.

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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 effect of imidacloprid was evaluated on adult honey bee longevity and development of HPGs. However, artificial larval rearing is increasingly being used as an *in-vitro* method for assessing the toxicity of different substances to developing stages of honey bees. Imidacloprid may be very dangerous to the larvae, and cause damages at the colony level later on, even if apparently not harmful to individual adult bees. The first test on artificial larval rearing will be run in our laboratory during April-May 2010 using 96-well tissue culture plates according to Aupinel *et al*. 2005 and Aupinel *et al*. 2007.
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.

Artificial rearing of honey bee larvae to investigative economically important diseases in South Africa.

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There are a number of reasons for rearing worker bee larvae (*Apis mellifera*) under laboratory conditions, such as the effects of antibiotics and toxicity of insecticides and the impact of transgenic products. The recent outbreak of the American foulbrood disease in South Africa calls for applied research under controlled conditions to avoid the further spread of this disease. It therefore is our objective to learn a new technique that will enable us to investigate this disease in *Apis mellifera scutellata* and *Apis mellifera capensis.*
Since AFB has been around for >100 years it is surprising that there was no earlier outbreak of this disease in Southern Africa. South African honeybees may be more resistant to this disease than workers of other subspecies and therefore may need a much higher infection dose than has been previously reported for other subspecies. By using the rearing of honeybees under laboratory conditions we can control important parameters, like nutritional status (protein:carbohydrate ratio), amount of jelly, and additional substances (like pesticides) which might reduce the immune capability of the workers. Honeybee research in South Africa has the world wide unique situation of having a natural population and all the diseases present, but no obvious decline.

Variation in chalkbrood susceptibility of in vitro reared honey bee larvae.

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Several microorganisms (virus, bacteria and fungi) are able to infect, kill and exploit honeybee larvae for their proliferation. These brood pathogens can cause recognized diseases like foulbrood, sacbrood and chalkbrood. In the combat of the brood disease honeybees have evolved several defence mechanisms, which can be divided into individual (e.g. antimicrobial peptides) and social defences (e.g. hygienic behaviour). Hygienic behaviour is strongly induced by the presence of chalkbrood mummies. This behaviour is an excellent weapon against chalkbrood, but makes studies of individual defence mechanisms in the hive context more difficult. Yet studies conducted on larvae reared under laboratory conditions allow for investigations without behavioural interference.
Thus by use of *in vitro* rearing and chalkbrood, *Ascophaera apis,* spores exposure we investigate individual larval chalkbrood resistance. Larvae were grafted from *A. m. carnica*, *A. m. ligustica* and *A. m. mellifera* colonies, respectively. Three day old larvae were fed with different dosages of *A. apis* spores. The response differed significantly between the colonies with LD_{50} estimates of 55 to 905 spores. Even within colonies of the same subspecies high variation was shown (up to a factor ten). Another issue we have addressed using *in vitro* reared larvae is the variation of virulence between different pathogen isolates.
We would like to discuss how such variation could be taken into consideration when standardizing bioassay protocols of *in vitro* reared larvae and brood pathogens.

Determination of effects of pests, parasites, and pesticides on the artificially reared honey bee (*Apis mellifera*) larvae populations of different strains of Turkey.

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We have a project supported by the Turkish Scientific Research and Technology council (TUBITAK) for determination of the effects of pests, parasites, and pesticides on honey bee colonies of different races in Turkey. Specifically, the effects of Varroa and Nosema, and DWV infections, the effects of Perizin, Imidacloprid, Terramycin, and also mismanagement. The artificially reared larvae are ideal to study the effects of pesticides, pathogens on genetically different races on the same environmental conditions. For these purposes we need to breed homogenous populations of larvae (the same age and size) from different strains, and nobody in our research group have experience in this technique. The Workshop on artificial rearing “Method standardization for larval tests”, to be held in Graz, Austria from 7th to 9th June 2010 will enable me to learn artificial larvae rearing technique and to show to our students and design experiments for this goal in the project.
Thus, artificially reared larvae to study the effects of pesticides, pathogens, growth and metabolism, genetics, nutrition or rearing environment, in other words, the application aspect will be a great use for our research purposes.
My contribution migth involve the analysis of pesticide applications since I have worked on genetics and evolution of insecticide resistance, and the effect of the resistance on life table parameters in the presence and absence of the stressors in another insect species, *Musca domestica* populations previously.

Deformed wing virus infection of honeybee *Apis mellifera* larvae with different degrees of Varroa infestation.

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We are a research group focused on honeybee viruses, their diagnostic and distribution. We have already several publications about these topics (see references) and the next step in our research is to perform ‘*in vivo’* assays based on controlled honeybee viral infections to define the real effect of the virus in the honeybee health. Specifically, we established a research plan for ‘*in vivo’* inoculation of DWV in honeybee larvae and adult bees that have different degrees of *Varroa* infestation.
To be able to develop this kind of assays, we need some information about how to manage with the honeybees in an ‘*in vitro’* environment as we are aware that there are no available stable cell lines derived from honeybee. Because of this, the complete study of honeybee viruses relies on ‘*in vivo’* experiments. This kind of methods are not yet well developed and standardized but have a huge potential to keep going further in the honeybee viruses research. They will be helpful to answer questions such as the real pathogenicity and virulence of honeybee viruses and their relation with other pest and with different environmental conditions.
As the development of these techniques is still starting, standardized protocols are needed to validate future assays as well as to compare inter-experiments results. Topics like good management practices, ideal laboratory conditions, troubleshooting and meaning of ‘in vitro’ rearing results are questions that we would like to know before establishing our ‘*in vivo’* study and therefore, we think this workshop would be really helpful for us.

Kukielka D., Esperón, F. and Sánchez-Vizcaíno, JM., 2007. A sensitive one-step real-time RTPCR method for detection of deformed wing virus and black queen cell virus in honeybee *Apis mellifera*, *J. Virol. Methods* 147, 275–281.

Kukielka D., Pérez, A., Higes, M., Bulboa. M. And Sánchez-Vizcaíno, JM., 2008. Analytical sensitivity and specificity of a RT-PCR for the diagnosis and characterization of the spatial distribution of three *Apis mellifera* viral diseases in Spain. *Apidologie* 39: 607–617

Kukielka D., Sánchez-Vizcaíno JM. Development and adaptation of one-step real-time quantitative PCR assays based on SYBR Green for the detection and field study of Sacbrood honeybee virus and Acute bee paralysis virus. J. of Virol. Methods 2009 Nov;161(2):240-6.

Kukielka D., Sánchez-Vizcaíno JM. First detection of Israeli Acute Paralysis Virus (IAPV) in Spanish honeybees.In press, Spanish Journal Agricultural Research.

Technical problems encountered during the 5-years experience in the in vitro brood rearing.

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Following the numerous cases of bee malformation in areas where IGRs were used in agriculture, our group carried out experiments in order to evaluate the influence of sublethal intoxication by most common IGRs at the larval stage on the morphology and functionality of the emerged adults. Very quickly it appeared that the *in vivo* trials are biased by many uncontrolled factors. Thus we began to apply the Aupinel’s method in order to run the tests in controlled conditions but at the beginning we encountered many technical difficulties. The first problem was related to insufficient hermeticity of the desiccator with the consequent lower RH level. Notwithstanding the correct saturated solution, 24 hours after grafting the diet jellified preventing larvae from aliment themselves. The problem was solved by using more hermetic desiccators. Moreover, since June 2009 we found almost impossible to produce adult bees from the *in vitro* reared brood and every cycle was interrupted between 7th and 8th day by the death of larvae. Till now we didn’t understand the mechanism of this phenomenon but in the same period this unexplainable phenomenon was observed for the first time also at the laboratory where the method was elaborated (INRA Le Magneraud, France).
The high potentiality of the Aupinel’s method was exploited during the years 2005-2009 when we carried out several experiments, including the international ring-test of the method. We studied also the influence of the slight variations of the rearing temperature on larvae and on emerged adult bees. We found that slight decrease of brood temperature, even if not influencing development mortality and emergence rate, had strong negative effects on the emerged adults, reducing their longevity and resistance to intoxication by pesticides. On the contrary, the same factor increased significantly the larval resistance to pesticides. These data point out the extreme importance of precise control of rearing temperature in order to make the results comparative between different trials and labs. Moreover adult bee longevity seemed influenced according to the trial season. Thus also this factor should be taken into account.

Artificial larval rearing of honey-bees (*Apis mellifera carnica*) in Slovenia.

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To determine the effect of each factor on the development of worker bees’ larvae and worker, we started in 2009 with (artificial) breeding of worker-bees from larvae in the lab conditions. At the beginning no one at the institute had any experiences working with this method. In the autumn 2009 we have reached good results and have successfully breed approximately 80 % of adult worker-bees. The most successful results were reached with the delineate procedure; grafting of up to 24 hours old larvae in the plastic disinfected artificial cups (30 min in 0,4 % methyl benzethonium chloride (MBC) in water). Cups were placed on wetted dental rolls which were positioned in 48-well tissue culture plate. The larvae were daily fed with a basic diet containing 50 % of royal jelly and 50 % of an aqueous solution which consist of the following ingredients: yeast extract (2 %), D-glucose (12 %) and D-fructose (12 %). Each larva was daily fed with warmed prepared diet, lasted for 6 days. The plates with the larvae were placed in the incubator for the first 6 days at 34 ℃ and a relative humidity of at least 96 %. The seventh day the humidity was reduced below 80 %. Eleventh day larvae were placed in a horizontal position, we have removed dental roll and coved larvae with a thin plate prepared wax. After successful optimization of this method and the treatment with form of development with different acaricides (amitraz and coumaphos), infected with *Nosema* spp. spores, we will continue monitoring the development of larvae and observing the mortality of brood, included histological researches at the level of cells.

Eurofins-GAB takes the challenge of establishing honey bee larval tests.

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In 2009 we carried out some practical honey bee larval studies according to the protocols of Aupinel et al. and Peng et al. All studies were conducted with untreated food. In none of those test runs did we gain satisfactory results and were faced with different problems in each test design. The main problem in the test runs according to Aupinel et al. was the high larval mortality in the first days. But the few larvae which were able to develop to pupae survived until hatch. Nevertheless we had high mortalities in most runs. One problem of this test design could be the risk of drowning or injuring the larva when feeding them.
In the tests conducted according to the test design of Peng et al. more larvae survived the larval stage and were able to develop to pupae compared to the Aupinel et al. test design. But most pupae died due to fungal infections and only a few individuals were able to reach adult stage (hatch).
We are convinced that a routine handling, strong and healthy bee hives, and a sanitary work environment are the main factors for the success of the honey bee larval studies. Even if a standard protocol would be developed it will stay a very difficult and challenging test system.
We are eagerly looking forward to continuing our research to find a suitable test design for our laboratory this year.

Comparison of protein content of artificially and naturally reared bee brood.

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In the scientific landscape it is quite popular nowadays to use artificially reared honeybee larvae for different experiments, it became an important tool for various investigations. Inside the brood nest of a regular honeybee colony a larva gets the best care we can think of, this has to be compensated in the lab. All the ambitious efforts of in vitro rearing deal with one big question: How fit are these artificially reared larvae? Can they withstand against different treatments with pesticides or pathogens in the same way as naturally reared larvae?
To verify the quality of artificially reared bee brood we compared the protein content of artificially and naturally reared larvae. For our investigations we grafted larvae into plastic queen cups at the age of 5-10 h, fed them 6 days once a day with a solution consisting of royal jelly and sugar-yeast dilution (slightly modified after Aupinel et al, 2005). The protein content of the artificially and naturally reared brood was analysed.
On average artificially reared larvae showed a lower protein content than naturally sisters until they reached an age of 3.5 days. After their 4th day an equal or higher protein content was detected in artificially reared larvae than in naturally reared ones.
These results indicate that a proper supply of artificially reared bee brood is ensured by using the method after Aupinel et al. (2005).

Study of DWV effects in larvae and emergent honeybees development (*Apis mellifera iberiensis*).

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Through these tests we want research DWV effects in larvae and emergent honeybees development (*Apis mellifera iberiensis*). Fifth instar larvae will inoculate with different dose DWV viral particles (1, 10, 100, 1000). These larvae will be sealed in the hive. Sealed larvae will keep at 35 ºC in a drying hot chamber. Every 24 hours we will slaughter larvae for analyzing viral load and morphological changes. In emergent honeybees we will proceed same form.

Standardization of protocol for rearing Africanized honeybee larvae and its application in bee biology studies and risk assessment of pesticides.

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The present abstract intend to show a compilation of the results obtained by our Brazilian research group which works with honeybee larvae reared in laboratory conditions in order to identify toxicological effects of pesticides and other chemical compounds in the mortality/survival tax, besides the histophatological analysis of larval organs. Two different protocols (Vandenberg and Shimanuki, 1987; Aupinel, 2005) described for European honeybees were tested in Africanized Apis mellifera whose obtained data showed different efficacy to increasing survival tax in these artificial conditions. Additionally, we noted that some modifications in these protocols are necessary to adapt them to Africanized honeybee larvae. There are difficulties to obtain a homogeneous population of larvae of defined age and, while this difficulty remains, our group suggests some standardization during the choice of larvae to morphological, imunohistochemical and/or biochemical analysis. In relation to research applications, our group could present data about the evaluation of different pesticides in artificially reared larvae in order to discuss the use of this method for the risk assessment of plant protection products, including cautions in relation to bee biology that could affect the quality of produced workers.

Application of artificial larvae rearing method in Croatia.

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The larval test will be included into a PhD thesis of a young researcher, as a part of her experimental design. The Faculty of agriculture in Zagreb currently has 100 hives situated on an experimental apiary, which are used for the purpose of teaching and training of undergraduate, graduate and postgraduate students. The artificial rearing of honey bee larvae has not yet been applied in our studies, but we plan to include it in our research and teaching. The fields of our interests on that subject are related to the effects of nutrition and rearing environment, as well as the influence of pests and pathogens. Our intention is to provide the students an opportunity to explore this novel and advanced method and to answer certain practical questions, which will contribute to the improvement of our beekeeping extension program. To be able to carry out the above mentioned activities, there is a need to acquire basic knowledge of the artificial larvae rearing method.

First, second, third larval instar, who knows? An abstract of the relevant scientific literature.

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For *in vitro* rearing of honeybees standardized methods are necessary. For standardization of grafting the age of the larvae has to be determined. Two possibilities to define the age are known, the biological age (larval stage) and the chronological age, respectively. The chronological age can be determined by caging of the queen, whereas the biological age can differ from this and therefore morphological parameters, have to be investigated. During their development honeybee larvae undergo various larval instars, whereas each larval instar lasts different time (Bertholf 1925, Rembold et al. 1980). To determine the chronological age and the biological age you have to be aware that a larva which had reached the age of 1 day has reached the second instar, respectively (Bertholf 1925, Rembold et al. 1980). Each larval instar or each larval age shows typical characteristics. According to Rembold et al. (1980), head diameter is a proper parameter to characterise each of the five larval instars. According to Myser (1954) the developmental stages of the mouthpart, wing buds, leg buds and the gonapophyses are useful parameters to determine the chronological age of honeybee larvae too.

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Myser W. C. (1954): The larval and the pupal development of the honey bee *Apis mellifera* Linnaeus. Ann. Ent. Soc. Am. 47, 683-711.

Rembold H., Kremer J.-P., Ulrich G. M. (1980): Characterization of postembryonic developmental stages of the female castes of the honey bee, *Apis mellifera* L. Apidologie 11 (1), 29-38.

Establishing the honeybee brood laboratory test according to P. Aupinel at BioChem agrar

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Following the INRA workshop in La Rochelle, France in 2008, where first insights into theory and practical accomplishments of the honeybee brood laboratory test according to Pierrick Aupinel had been gained, BioChem agrar, in particular Mr. Markus Barth joint the ring test and started to set up the test.
Initially problems occurred concerning the mortality rate of the untreated larvae due to bacterial infection or mycosis. During last season the control mortality has been reduced significantly and is now ranging below 10 % on day 7 (beginning of pupation). The number of hatching adult bees is still very volatile and is frequently exceeding 15 %. In the course of the meeting in Graz an exchange of experiences might improve our success in breeding honeybees until hatching of adults.
Some of the following subjects are not in complete accordance with the first INRA method description but showed good results and therefore need to be discussed in Graz:

- Either by applying an excluder cage or by daily observation and marking of the brood it was secured that freshly hatched larvae of approximately the same age were used in the test.
- For grafting a "chinese grafting tool" was used rather than a paint brush.
- No adverse effects had been noticed by placing the larvae during test in a standard climatic chamber instead of a hermetic Plexiglas desiccator.
- During the 22 days of the test the larvae were not removed from their grafting cells to avoid any mechanical damage. It should be clarified if this could be the reason for a higher mortality until adulthood.
- An uncertainty occurred concerning the statistical evaluation based on the study layout (minimal requirements for the number of larvae per test concentration and number of replicates).
- Another question of interest is the issue of an increased larvae sensitivity over the season, which was not noted in our trials.

Scientific report of COLOSS Work shop “Method standardization for larval tests” Graz, Austria, 7.-9.6.2010

A tool for assessing the effects of different factors on bee brood is necessary. Such a test is the artificial larval rearing (in vitro in the laboratory). This method is very important and useful to investigate the effects of plant protection products, IGR pesticides, brood pathogens or other stressors of honey bees. The in-vivo testing is easier and requires less technical preparation but other test conditions, especially the uptake and transfer of tested compounds to larvae in the colony, are far much less controllable. Thus a standardized laboratory protocol is needed. This work shop was the first activity bringing together experienced scientists and representatives from the industry as well as interested honey bee experts without previous experience regarding this method. Altogether, 24 scientists coming from 15 different countries participated in this work shop. Actually, several protocols or modifications are used to rear larvae in vitro, and we seek consensus on which sub-steps of laboratory rearing of larvae need standardization and where modifications are suggestive.

A crucial question is the design of the study, and this is where we recommend consulting a statistician. It is controversial if different replicates of an experiment are made up of testing several colonies within a short or longer period, or one colony should repeatedly be tested within a short period of time. Decision about the above subject should also consider the aim of investigation, i.e. if genetic or seasonal diversity is desired or unwanted.

Consensus was found and suggestions will be proposed regarding the standardized caging of the queen, the grafting of larvae, the importance of incubation temperature and humidity, how to assess the survival of larvae and the accepted maximum control mortality. When experiments addressing the quality of honey bees produced in the laboratory are conducted, capping of the rearing plates and turning them into a horizontal position is suggested as an additional feature. This activity was also demonstrated. The importance of the quality of the royal jelly used to prepare the larval diet and the storing conditions of prepared diets were discussed. Another crucial point for successful larval tests is to provide a sterile environment and thus the sterilization of the used materials and chemicals. A regular survey of sterility is recommended, otherwise infections from fungi or bacteria will make experiments impossible. Decision was made that every participating laboratory will try to achieve the optimum level of sterility, using their preferred method of choice for further methodological approaches.

A suggestion for further cooperation among laboratories in standardization, future workshops or ring tests for the efficiency of each laboratory was welcomed by the audience. As a possible date for the next work shop on the same subject autumn 2011 was suggested to be proposed by work group 3 to the next MC meeting of this action.

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