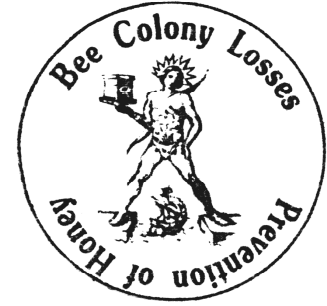




Action FA0803



**Proceedings**  
**COLOSS Working Group 3**  
**Workshop**  
*“In vitro larval rearing workshop”*

**18-19 November 2011**  
**La Rochelle, France**



Action FA0803



*Avec le concours financier de  
la Région Poitou-Charentes*



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## Agenda

### Wednesday 16<sup>th</sup> November 2011

- 6.30 pm: Reception  
8.00 pm: Dinner

### Thursday 17<sup>th</sup> November 2011

- 9.00 - 10.00 am: Karl Crailsheim: Outcomes from Graz workshop  
*10.00 - 10.30 am: Coffee break*  
10.30 - 11.00 am: Piotr Medrzicky  
11.00 – 11.30 am: Simone Tosi  
11.30 – 12.00 am: Gherardo Bogo  
*12.00 – 2.00 pm: Lunch*  
2.00 – 2.30 pm: Pierrick Aupinel: Effects of royal jelly storage on larval rearing and on hypopharygeal gland development  
2.30 – 3.00 pm: Coby van Dooremalen  
3.00 – 3.30 pm: Nicole Hanevald  
3.30 – 4.00 pm: Anna Gajda  
*4.00 – 4.30 pm: Coffee break*  
4.30 – 5.00 pm: Marianne Cousin  
5.00 – 5.30 pm: Mustapha N. Muz  
5.30 – 6.00 pm: Asli Oskirim  
*8.00 pm: Social dinner*

### Friday 18<sup>th</sup> November 2011

- 9.00 – 10.00 am: A. Decourtye: An example of the usage *in vitro* rearing method for research  
*10.00 – 10.30 am: Coffee break*  
10.30 – 12.00 am: Discussion and conclusion  
*12.00 Lunch*

**\*: about 20 mn for each talk, and 10 mn for discussion**

The objectives of this workshop are to share the knowledge about honeybee *in vitro* larvae rearing method, by comparing results of different experiences. To that purpose, each participant has made a rearing trial previously. The results were compared and discussed in the view of producing a method harmonized between the different laboratories.

This workshop also intended on working on the establishment and the validation of this method for risk assessment linked to pesticide usage and for the study of pathogens (virus and bacteria).

The global COLOSS network identifies the factors at the individual honeybee and colony levels causing severe colony losses and investigate synergistic effects between them. This enables the development and dissemination of emergency measures and sustainable management strategies to prevent large scale losses.

For this purpose, scientists, beekeepers and industry members from currently 55 countries collaborate with complementary approaches, thereby providing the crucial R&D link for the success of this Action. This worldwide integrated approach will mitigate the detrimental impact of honeybee colony losses for beekeepers, agriculture and natural biodiversity.

Pierrick AUPINEL

INRA Magneraud

Marie-Pierre CHAUZAT

ANSES Sophia Antipolis

## Outcomes of the workshop

**Initial objective:** each participant makes one non-treated plate and results are compared during the workshop.

After Graz workshop (2010), the *in vitro* larval rearing method has been followed. It was observed that experienced people had comparable results which makes true the remarks of Karl Crailsheim that the success is linked to the real experience (when the trained person actually sees the method).

Another observation for success is to acquire new (sterile) equipment dedicated to this method.

**Methodology:** This subject was studied by Marianne (picking tools), Stéphanie (acetone, MBC, grafting place), Pierrick (royal jelly conservation), Piotr & Simone & Mustafa (sanitary aspects), Gherardo & Anna & Coby (temperature rearing).

Optimal temperature should be constant within a range (i.e. amplitude and homogenization within the incubator). This point is essential to have comparable results.

The relative humidity and temperature should be monitored in the incubator and in the dessicator.

The capping is obligatory if the test requires producing reliable adults (better morphologic development of nymphs, possibility to test the adult rate of emergence, barrier against fungi infestation, better microclimatic conditions). If only larvae are studied, it is not necessary.

Some aspects from the method should be changed:

- Nobody use MBC anymore (too expensive). It is replaced by the chloride product used for baby bottle sterilization.
- Feasibility criteria. There are some favorable seasons: spring and beginning of summer (Pierrick, Stephanie, Anna). It is related to the quality of the egg (linked to egg laying). Season also influences the quantity and quality of larvae to be grafted: it has been observed that the third plate could not be as good as the first one in terms of mortality rate. More statistically distributed design can overcome this difficulty (random distribution is important).
- The hive producing the larvae should be of a certain size (minimum number of frames) guarantying the production of sufficient quantity of larvae and insuring correct brood nest temperature. The colony should be healthy (no sign of any disease or intoxication, free of veterinary treatments of any kind) with a known background (the colony history should be known for one year prior the experiment). This information should be added in the report of the study.

The reproducibility of the method was checked through all these experiments: when sanitary and technical criteria are respected, mortality rate decreases to an acceptable level.

At Graz workshop, the acceptable mortality rates were 10% for day 7 and 25% for day 22. In the ring test (Aupinel et al.), the mortality threshold was 15%. The discussion is still on the acceptable mortality rate of 10 or 15% depending on the purpose of the tests (regulation or research). This question is linked to the seasonal effects on larvae survival.

The adaptation (flexibility) of the method is possible according to what is studied i.e. Piotr experiment with *M. pluton* and the feeding steps.

**Application:** the method can be applied to pesticides and pathogens studied singly or in synergy.

**Future:** the method should be applied on more studies and on more synergies. The study of the delayed effects should be a priority: expose larvae to a stress and subsequently test the adults for social & individual effects.

The effect of the food (royal jelly collected late or early in the season) on larvae and on subsequent adults should also be studied.

**Regulation:** the method was presented at the ICPBR meeting in 2005, subsequently ring tested. The ring test results were presented in Bucharest in 2007.

The SETAC (USA) adopted the method in 2011. Coloss will include it in the BEEBOOK. The method was sent to OECD in November 2011.

**Challenge:** the drone rearing until adult emergence remains to be completed. There are some difficulties in producing drones when it is not the correct season of drone laying.

## Effects of royal jelly storage on larval rearing and on hypopharyngeal gland development

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Royal jelly is one of the most important components of larval diet. Considering that royal jelly production is season dependant, it is necessary to store with no idea about storage effect on royal jelly properties. This issue was treated by testing the same royal jelly just after collecting (fresh royal jelly), and one year later in two temperatures of storage conditions: +5 °C and -18 °C.

In each experiment, larvae were reared until pupation. After emergence, adults were kept alive until the age of 8 days.

We noted for each trail:

- mortality at D8 (pre pupa stage), D15 (pupa stage) and D26 (8±1 days old adults)
- weight of pre pupa (D8)
- protein content of the head at D26 (correlated with hypopharyngeal gland size)

On most of the replicates (52 plates), mortality at D8 ranged from 0% to 15%.

We found a significant effect of storage time on mortality rates at D8 and D 15 with highest values for 3 month storage. This indicates that time effect was distorted by a seasonal effect. Temperature storage also influenced rate mortality.

We also found a significant effect of storage and temperature time on protein head content. This result comforts the idea to use fresh royal jelly if possible and in any case to have a control treatment in every experimental design.

## Effects of pesticides on adult bees obtained from *in vitro* rearing at two different temperatures

G. Bogo, S. Tosi, P. Medrzycki

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Previous studies indicate a greater susceptibility to intoxication by neonicotinoids of larvae reared at optimal temperature (34.5°C) than those reared at suboptimal temperature (33°C). With the present study we tested adult bee susceptibility to intoxication by Clothianidin. Honeybees were obtained by *in vitro* rearing at optimal and suboptimal temperature (34.5 and 33°C). Newly emerged bees were anesthetized using CO<sub>2</sub> and their mortality was tested by ingestion at 5 doses of the a.i. In order to calculate the LD<sub>50</sub> (24 and 48 hours). Unlike the larval LD<sub>50</sub> at two temperatures, the adults showed no significant differences between the response to intoxication at suboptimal and optimal temperature (LD<sub>50</sub> at 34.5°C = 3.35ng/bee; LD<sub>50</sub> at 33°C = 3.33 ng/bee), while, in a preliminary study carried out with Dimethoate, we saw a correlation between rearing temperature and susceptibility to intoxication.

## Fungi reduction in honeybee larvae *in vitro* rearing

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During our experience in applying the *in vitro* rearing method, we sometimes found fungi on larvae and pupae. These propagated then from one to more cells, invalidating the test. To overcome this problem we decided to resort to the use of 7W UV sterilising lamps, switched on in the incubator for the entire duration of *in vitro* rearing. To evaluate their efficacy, we placed in the incubator, both outside and inside the desiccators used for *in vitro* rearing, Petri dishes and Eppendorf vials containing spore suspensions of two *Bacillus subtilis* strains and vegetative forms of *Enterococcus faecalis* in 0.9% NaCl solution. After about 20 hours in the incubator, the material was moved to a refrigerator. Then a series of dilutions of the different suspensions were prepared. These were then inoculated on plates containing agar medium appropriate for the bacterium and incubated at 36°C for 48 hours. The colony counts showed lower number of *E. faecalis* in material from the incubator with the UV lamps. No difference was recorded for the other bacterium.

Moreover specific agar contact plates were used in order to evaluate the microbial presence in the incubators and desiccators (both air and internal surfaces). The results showed the presence of resident saprophagous fungi, which seem to be genus *Paenicillium* and *Aspergillus*.

Thus we decided to sterilize incubators and desiccators with a 5% sodium hypochlorite solution.

We introduced also two modifications to the *in vitro* rearing protocol. The first one was to not use gloves, but a sanitizing hand gel, to prevent contamination. The second one was the plate sealing with a wax foil after D6, thus preventing the expansion of a possible fungi born to adjacent grafting cells.

## Artificial rearing of honeybee larvae to assess the toxicity of Paraquat

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Due to the lack of appropriate methods, little is known about the impact of pesticides on honeybee larvae. Now, the availability of *in vitro* larvae rearing methods promises to fill this gap. We studied the effects of sublethal doses of Paraquat, especially at the histological and cytological levels in larvae of different developmental stages.

In a first approach, larvae (from less than 24 h to 72 h old) were fed with a basic diet containing Paraquat at different concentrations (0, 0.001, 0.01, 0.1 and 1 µg/kg of food). Treatments were performed on larvae maintained in brood frames collected directly from the hives. Despite the fact that these conditions were close to natural conditions, the larvae survival was difficult to assess and the presence of pesticide residues in the wax could interfere with our treatments.

In a second approach, following the same experimental design, larvae were reared *in vitro* in artificial pesticide-free frames and under controlled laboratory conditions, as described by Aupinel *et al.* (2005).

Based on the estimation of mortality with the two methods, two conclusions can be drawn:

- the mortality is far to low when larvae are reared *in vitro* compared to "brood frame" *ex vivo* conditions (<10% and 29% respectively) ;
- the very low mortality level enables working in sublethal conditions, especially when mortality is considered as the end point.

## Current state in laboratory (*in-vitro*) rearing of honey bee larvae.

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Parasites, pathogens and pesticides are three of the major threads of honey bees that are, among others, also blamed to be responsible for colony losses. The effects of pesticides and pathogens, and the combination of two or more sublethal effects, are extensively investigated. An important tool for this research is the rearing of honey bee larvae *in vitro* because of much more controllable conditions compared to *in vivo*. Especially the testing of toxicity of plant protection products on brood can only be conducted in the laboratory in a reproducible and standardized way. In 2010, a work shop was held in Graz, Austria on "Method standardization for larval tests" to facilitate a common approach and discuss problems and different protocols. In particular, some open questions regarding study design, caging of queen and grafting of larvae, incubation conditions or quality of royal jelly were addressed. As an outcome of this workshop we prepared a draft article for the beebook. We will discuss basic findings on larval nourishment and how these are already adopted in larval rearing or may need more attention. We will summarize the crucial points for rearing honey bee larvae in the laboratory which are: 1. Study design, 2. Caging of queen and grafting of larvae, 3. Randomization, 4. Sterile environment, 5. Preparation of diets, 6. Storage of diets, 7. Feeding of diets, 8. Royal jelly, 9. Application of test substances, 10. Incubation conditions, 11. Assessing survival, 12. Data analysis, 13. Control mortality, 14. Capping, 15. Evaluation of rearing success, 16. Rearing reproductives in the laboratory.

## An ecotoxicological application of standard *in vitro* method for rearing *Apis mellifera* L. larvae: impact of a juvenoid on larval development and behaviour of adult

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The insect growth regulators (IGRs) are hormones mimics, which disrupt moulting process (juvenile hormone or ecdysone mimics) or cuticle formation (chitin inhibitors). According to typical actions of these compounds, IGRs cause potentially damage to parameters necessary to successfully development of bees (Tasei, 2001; Desneux et al., 2007). Most observations, which were reported on the IGRs effects, relay on measurements of brood quantity. The official recommended method is to consider the number of cells containing different bee brood stages as a parameter to detect possible adverse effects on the growth larvae (Oomen et al., 1992). But such methods in field conditions did not allow for precise control of larvae exposure to the insecticides. According to difficulties to rear larvae in reliable conditions, there have been few quantitative studies to assess the impact of IGRs on physiology or development during this stage. The individual exposure of larvae in the *in vitro* brood test described in Aupinel et al. (2005; 2009) is one way to ensure a more precise assessment of the effects of insecticides on honeybee larvae. Using this advantage, our study at individual level of the effects of IGRs on the growth is suitable. We found that pyriproxyfen (juvenile hormone mimic) chronically applied on larvae of *A. mellifera* L. (18 or 54 ng per larvae) resulted in an early emergence time and an increase of morphologic malformations in the emerging adults. When the morphologically normal adults were introduced inside a colony, their brood-rearing capabilities (nurses) were reduced. Finally, we have found an increased ejection rate of exposed workers outside the hive which was a possible consequence of the behavioural disruption. The goal of this presentation is to discuss the strengths and weaknesses of the *in vitro* larval-rearing test, by accounting for the context of our ecotoxicological approaches.

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## First try to introduce the *in vitro* larval rearing method by P. Aupinel to WULS Laboratory of Bee Diseases.

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An attempt to introduce the Aupinel method to our laboratory was conducted in the middle of August 2011. It was performed without using any pathogen/toxic exposure. We wanted to see if the method itself is applicable in WULS laboratory. Since all our colonies are infected with *Nosema ceranae* and infested with *Varroa destructor* but do not show the symptoms of disease we used one of them as egg donors and we also collected royal jelly and pollen from our colonies. During the experiment the mortality rates were very high on the first few days: up to 8 dead larvae per day (probably due to manual errors during grafting). It was increasing up to day 5 and decreasing afterwards. At day 22 only 2 not emerged bees were found, however, all in all, only 8 bees emerged from the 48-well plate. They were fed sugar syrup from a bird feeder and pollen patty from Petri dish, both *ad libitum*. We encountered following problems with adjusting the method to our conditions. The push-in cage for confining the queen had to be replaced with an empty comb, because the workers were removing the eggs from plastic cups. The size of boxes (chambers) used in the method was too big to fit more than 3 of them in an incubator, because the ones in our laboratory are rather small. The rest of the method setup, generally, was very easily applicable to our laboratory, the equipment is easily accessible and the feeding only once per day (with the diet prepared earlier and stored in refrigerator) allows to minimize the time of maintenance to an hour per day.

## Abstract for the workshop “Method standardization for larval test” in La Rochelle

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BASF SE

The new regulation 1107/2009 for placing plant protection products on the market requires data on honeybee brood for every active substance. Therefore, the standardization of the method proposed by AUPINEL et al. (2005/2007) will provide a valuable contribution to address effects of plant protection products on honeybee larvae already in the laboratory.

To develop a method that produces reliable and robust for regulatory purposes BASF is conducting tests with either active substances or formulations since 2007.

Having a high variability of control mortalities since working with this method, in 2011 low control mortalities (< 25%) were achieved. One important factor contributing to these results could be the use of honeybee colonies free of the following diseases: *Nosema cerana*, *Nosema apis*, AFB; DWV, SBV, ABPV, KBV and CBPV.

Other critical factors which turned out to improve control mortalities are carrying out all steps (grafting, feeding, assessments) under controlled conditions of a climatic chamber during the larval phase as well as performing the test in early season. During a time period of 5 months control mortalities increased with progressing season: control mortalities (based on the emergence of adult honeybees) were in a range of 12.2% to 24.4% during the months April, May and June and in a range of 31.1% to 68.9% while July and August.

For testing active substances it is usually necessary to solve them in organic solvents. Therefore, the tolerance of honeybee larvae to organic solvents (e.g. acetone) is an important factor to avoid high solvent control mortalities. To know the tolerance of honeybees to certain organic solvents could help to explain why in some cases higher doses of an active substance could not be tested.

Additional to the development of a reliable test design to test honeybee brood in the laboratory it is essential to develop a common understanding on how the results are used in the context of regulatory risk assessments. For this purpose more detailed discussions are needed.

## **Assessment of the effect of pesticides on the physiological parameters of honeybees, such as HPGs development and their protein activity, when exposure starts at larva stage**

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Our current research using laboratory cages has shown that imidacloprid administered to honey bees in sub-lethal doses can affect the development of their hypopharyngeal glands as well as their respiratory rhythm. However, in the field, the bees are encountered with the substance even since there are larvae. Although the contamination in larval stage is lower than in adult stage, nevertheless it is the crucial time of development. In fact the bees are in contact with the substance for longer periods. The total chronic exposure then measures from the day they are encountered with the substance as larvae and continues till they are adults. In our experiments the contamination will start at Day 3 of larval stage and continue as adults from Day 1 in controlled conditions till the bees are 14 days old. The reason for this is that at the age of 10-11 days old we measure the full development of the HPGs in order to show the full development of the HPGs, size and protein activity, and at the age of 14 day we measure the reduced size of the glands a phenomenon in parallel with the age dependant role of the honey bees.

## **Affect of Antioch propolis as food additive on viability and viral load of artificially reared and natural drone’s semen**

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Colonies of *Apis mellifera syriaca* in the apiary of the Mustafa Kemal University in Antioch were used for experiments. Colonies (adult and larvae) checked for viruses, bacteria, microsporidias and protozoans. We have no any DWV free colonies in Antioch. Drone larvae were collected from asymptomatic colonies which were treated against to varroasis organically before 1 months ago. A semi-artificially prepared diet was used which include extract of Antioch propolis. Procedures of Aupinel’s was used according to manuel of “Method for rearing honeybee larvae in laboratory conditions”. Adult and larvae of drones were compared. qRT-PCR was used for determination of virus levels. SYBR-14 and PI were used for sperm viability test. Sperm viability test was done according to Colins A.M. Second repeat of the full experiment will be done at next spring. Preliminary results are hopeful.

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## Observation of entomopathogenic nematodes growing in *Galleria mellonella* and honey bee larvae by using larval rearing method in vitro

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The aim of the study was to use entomopathogenic nematodes as biopesticides on the Greater wax moth *Galleria mellonella* larvae causes big damages in hives. Besides, it was tried to test their activities on honeybee larvae because honeybees are also insects and entomopathogenic nematodes would have been pathogenic on honeybee larvae too. Steinernematid and Heterorhabditid genus were tested in the experiments. *Galleria mellonella* larvae were removed from their artificial nutrient medium (dried milk, honey, flour, bee wax, bran) and transferred in to a 24-well tissue culture plate. Honey bee larvae (preferred 3 days old) were collected from uncapped brood cells by using grafting tool and placed in to small cells (9mm). Each cell was placed in to a well of a 24-well tissue culture plate. Honeybee larvae were reared by 50% royal jelly, 50% mixture of 4% yeast extract, and 35% honey and distilled water. Although two genus of nematodes were observed in high pathogenity on *Galleria mellonella* larvae, Mortality of honey bee larvae exposed to any of the nematode species was less than 10%, and there was no evidence of nematode infection when dead larvae were dissected. Nonsusceptibility of the Honey Bee, *Apis mellifera* (Hymenoptera: Apidae), to Steinernematid and Heterorhabditid Nematodes was detected in these experiments.

## Interactive effects of *Melissococcus plutonius* and fipronil on honey bee larvae

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The Aupinel’s method for rearing honeybee larvae in laboratory conditions allows to test the effect of pesticides on bee brood. In fact, toxicity tests carried out in field are influenced by a lot of factors that are difficult to control, as weather, alimentation, pathologies, etc. But how strongly these other factors can influence the effect of an intoxication by a pesticide?

European foulbrood is a bacterial brood disease caused by the Gram-positive bacterium *Melissococcus plutonius*. The disease has a worldwide distribution and it is an increasing problem in some areas. A slight presence of the bacterium within the beehive do not always cause the pathology, but how much could a sub-clinical dose of *M. plutonius* administered to the bee brood influence the bee susceptibility to an intoxication by fipronil?

To investigate the effects on honey bees of both a sub-clinical contamination by *M. plutonius* and an intoxication by fipronil, a laboratory study was carried out.

In order to administer *M. plutonius* and fipronil to the larvae the Aupinel’s method was modified. Four preliminary tests and the rearing of more than 1000 larvae were necessary before obtaining the definitive test protocol.

The bees were reared *in vitro* from the first hours of larval life until few days after the emergence. The larvae were contaminated by *M. plutonius* and then intoxicated by fipronil. Larval mortality and adult emergence were recorded.

The results show that the emergence rate of honey bees inoculated with the bacterium or intoxicated by the pesticide is lower than the emergence rate of control bees. Besides, it seems that the inoculation with a sub-clinical dose of *M. plutonius* does not have any significant influence on the emergence. On the other hand, the administration of 1 or 3 ng/larvae of fipronil significantly influences the emergence rate, and it seems that this influence is greater when the bees are not infected with the bacterium. In addition, emergence mortalities corrected with the Schneider-Orelli’s formula in relation to both the pesticide or the bacterial factor were calculated. The results of this analysis show that the effects of both the inoculation with *M. plutonius* and the intoxication by fipronil seem to be mitigated by the association of these two factors.

For these reasons it seems that the administration of the two stressors on honey bee larvae cause anti-synergic effects.

The definitive protocol we defined was tested only once, thus in order to confirm the results obtained it is necessary to carry out other experiments.

## **In vitro rearing of honey bees at Bees@wur**

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The rearing of larvae in a laboratory (*in vitro*) is highly attractive because of controlled laboratory conditions and the reproducibility. Biologically relevant factors such as weight, development (e.g. hypopharyngeal gland) and the survival of the larvae, or longevity and behaviour (e.g. flight performance) of artificially reared adult workers can be easily monitored under laboratory conditions. Hence, a large advantage of *in vitro* rearing of larvae is that feedback mechanisms on the colony level can be excluded to study the pure effects of the factors of interest. Many *in vitro* tests are hampered by high mortality of the test subjects, lack of standardization and repeatability. In our lab, we are able to rear larvae up to day 6, but after that, they die and turn black. Out of the >2000 larvae we started to rear between April and October 2011, we only managed to rear one sad little bee, which died a few days after it was born. We used the protocol of Aupinel (2005), occasionally using the nicotplast egg laying method from Hendriksma (2011). During the season, we observed differences in laying behaviour between colonies. Some queens refused to lay eggs in the nicotplast, even when the nicotplast were waxcoated. Towards the end of the bee season, survivability of the larvae decreased, which could be a natural phenomenon. Within batches, we observed that that units (wells plates) that were filled with crafted larvae first and were subsequently fed first, showed higher larval survival than units crafted and fed last. We assume that larvae crafted last, were longer exposed to the cold and therefore less vital. Additionally, due to the cooling down of feed during feeding, larvae fed last suffer from cold food. Conclusion, craft fast and rewarm food between units.