

Improvement of artificial feeding in a standard *in vitro* method for rearing *Apis mellifera* larvae

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Abstract

An *in vitro* method for rearing bee larvae has been improved by studying three parameters of feeding: 1- the mode of feeding, 2- the amount of diet per larva, 3- the diet composition according to the age of larvae. The use of a uniform model of providing diet instead of adjusting the quantity according to the size of the larvae increased the homogeneity of larval weights. The total quantity of diet influenced the weight and the emergence rate. This last variable was also improved by increasing dry matter rate of the diet from the first to the last larval instar. This standardised method may be recommended to conduct various kinds of studies on brood, in particular regulatory trials for assessing pesticide toxicity to larvae.

Key words: honeybee, larval development, artificial food, *in vitro* method.

Introduction

In Europe the regulatory honey bee (*Apis mellifera* L.) risk assessment related to agrochemicals use follows the guidelines 91/414 EEC as well as the EPPO guidelines n°170 (OEPP/EPPO, 2001) and the attached decision making scheme (OEPP/EPPO, 2003). According to these guidelines a brood feeding test is requested to evaluate whether bee larvae may be at risk when exposed to a compound showing an IGR activity.

The recommended methods are those of Oomen *et al.* (1992) and Schur *et al.* (2003). Both are in-hive methods where experimental bees are free flying colonies. In the first method the artificial contamination is ensured by a syrup feeder fitted to the hive for 24 hours, in the second, the hive is exposed for one week to treated flowers grown in a tunnel, and then moved outside for the rest of the experimental period.

Due to environmental variations both methods may not be easily reproducible since the test product may be stored in the combs and not immediately dispensed to the brood by nurse bees. It may also be diluted by external nectar.

Referring to the related literature, other in-hive methods have been devised by several authors to assess pesticide toxicity to larvae. They imply either the use of feeders fitted to micro colonies in incubator (Barker and Taber (1977), or the injection of known amounts of product into brood cells of free flying colonies (Wittmann (1982), Atkins and Kellum (1986), Naumann and Isman (1996). Apparently none of these protocols inspired the authors of the official methods.

More research has been published on *in vitro* brood feeding test. Description of laboratory methods have been provided over almost half a century (Weaver (1955), Wittmann and Engels (1981), Rembold and Lackner (1981), Vandenberg and Shimanuki (1987), Davis *et al.* (1988), Czoppelt (1990), Engels (1990), Peng *et al.* (1992), Brodsgaard *et al.* (1998), Malone *et*

al. (2002). These methods generally provide a LD50 or a LC50 for the treated larval stage. In 1981, Wittmann and Engels suggested to use the *in vitro* brood feeding test as a routine method for screening insecticides and classifying chemicals according to their toxicity to larvae. Considering both the laboratory toxicity of a product to larvae and exposure data of brood to this product in natural conditions, the *in vitro* larval feeding test seems an appropriate starting point of the brood risk assessment in other terms a tier n°1 study.

However, objections have been raised against the *in vitro* method and its regulatory use, in particular doubts on the standardisation of a protocol, criticisms on the frequent high mortality and the presence of intercasts in the control samples. The difference of food quality and mode of dispensing between natural (Haydack, 1968) and artificial conditions described by authors may account for these weaknesses.

The objectives of the paper are to describe our *in vitro* method and with the aim of addressing the current criticisms, to present three experiments on artificial feeding:

- 1- on the mode of food dispensing;
- 2- on the amount of food per larva;
- 3- on the food composition according to the age of larvae.

Materials and methods

Collection of 1st instar larvae

We obtained our honey bee larvae from colonies of a *ligustica* strain reared in our experimental apiary at Le Magneraud. To obtain same aged eggs we adopted a method close to that from Peng *et al.* (1992). In a healthy colony the queen was confined for 36 hours on a comb containing emerging worker brood and empty cells and placed in an excluder cage (44.5 x 31.5 x 5.0 cm). The excluder box permitted worker bees to move freely from the encaged comb to other parts of the col-

ony for stimulating queen egg laying, and feeding larvae. In previous experiments, we generally noted that queens had to adapt themselves to their new confined conditions before laying. For this reason, to obtain a larger number of same aged eggs confining the queens about 30h was preferred instead of 24h as recommended by Peng *et al.* (1992).

After removing the queen from the excluder box, the frame with its excluder device was left in the middle of the brood chamber of the colony for three days for incubation. The comb was kept in the excluder box to prevent the queen from laying new eggs and to allow worker bees to feed new hatched larvae. On the third day, the frame was removed from the hive and brought into a climate laboratory (25 °C) for grafting first instar larvae into artificial rearing cells.

Artificial rearing cells

Traditional plastic queen starters made of crystal polystyrene (Nicoplast[®]) were disinfected for 30 minutes in 0.4% methyl benzethonium chloride (MBC) in water, before grafting the new larvae. The cells were drained and dried in a laminar-flow hood, then individually weighed (+/-0.1 mg), and placed into a 48-well tissue culture plate. Each well was previously half filled with a piece of dental roll wetted with 15.5% glycerol in 0.4% MBC (Vandenberg and Shimanuki, 1987).

Diet preparation

The larvae were fed with a basic diet (diet A) containing 50% of royal jelly (RJ) and 50% of an aqueous solution of yeast extract (2%), D-glucose (12%) and D-fructose (12%) (Vandenberg and Shimanuki, 1987). Osmosed and inorganic water was used for the solution. The sugar solution was filtered at 0.2 µm.

We used fresh RJ from a French beekeeper. This RJ provision was split into 5g doses which were frozen at -18 °C. Chemical analyses and residues research were also performed on samples by the "Laboratoire de repression des fraudes, Marseille, France" and the "Groupement Interrégional de Recherche sur les Produits Agropharmaceutiques, Beaucauzé, France" respectively. Our RJ contained 33.10% of dry matter (DM), 44.40% of proteins/DM, 10.60% of fructose/DM, 12.10% of glucose/DM, 2.70% of saccharose/DM, 2.57% of 10HDA (10-hydroxydecanoic acid). The multi residues analysis did not reveal concentrations beyond the detection limit for the 28 insecticides, and 9 fungicides tested.

Before any experiment the required amount of diet was prepared and stored at +5 °C during the 6 days of feeding. The diet was provided to the larvae with a micro pipette once a day.

Grafting and incubation of larvae

Before grafting, each plastic cell was filled with 10 or 20 µl of the diet (according to the experiment). The diet was warmed at 34 °C prior to each trial. The worker larvae were grafted from the comb to the plastic cells at the rate of one larva per cell, using a thin paintbrush (double-zero size). Viable larvae of the first instar were not collected if they did not stick to the paintbrush. The

plates containing the cells were transferred into a hermetic Plexiglas desiccator (30 x 30 x 30 cm) containing a dish filled with K₂SO₄ saturated solution on its bottom in order to keep 96% RH. The desiccator was placed into an incubator at 34 °C (Vandenberg and Shimanuki, 1987) for 6 days.

Transfer of larvae for pupation and emergence

On the 7th day, the cells containing the larvae were individually weighed (+/-0.1 mg). Generally at this stage, larvae begin to defecate and spin their cocoon. Then the 48 well plates were transferred for one week into a new desiccator at 34 °C, where the relative humidity was kept at 80% with a dish filled with a saturated solution of NaCl. After this period, the plates were finally transferred into alimentary crystal polypropylene pupation boxes (11 x 15 x 12 cm). Their cover were aerated with wire gauze, and they were equipped with external bird feeders filled with 50% sugar solution in water (25% fructose, 33.5% glucose, 20.5% maltose, 21% maltotriose and polysaccharides), and fixed to the boxes; the pupation boxes were placed into an incubator at 34 °C and 70% RH. until adult emergence.

Experimental layout

After some preliminary trials we adopted the rearing conditions described above, for conducting our main experiments concerning larval feeding. Our sample sizes varied from 96 to 107 larvae per treatment.

Experiment 1: Mode of feeding

The mortality and the weight of larvae fed on diet amounts adjusted to their size (individual mode as described by Vandenberg and Shimanuki, 1987) were compared to those of larvae fed each day uniformly, according to the following model (uniform mode) (table 1).

In this experiment, we used only diet A, the basic composition. The daily food amounts of the model U1 were based on the daily mean consumption computed after preliminary experiments, where food supply was adapted to the size of the larvae.

Experiment 2: Amount of diet per larva

We compared the U1 model to U2 model (table 1) for which the total amount of food per larva reached 160 µl instead of 130.

Experiment 3: Diet composition according to the age of the larvae

We compared the U2 model (unique and uniform diet) to the sequence of three A, B, C diet compositions. Diet A was fed on days D1 and D2, diet B was fed on D3 and diet C on D4, D5 and D6 (table 2).

Data and observations - Statistical interpretation

Every day the dead larvae were counted. At the age of 7 days (pre pupa stage), the larvae were individually weighed. Except for the first experiment, emerged adults were counted and observed to check the presence

Table 1. Daily food volumes provided to the larvae with the U1 and the U2 models.

Rearing day	D1	D2	D3	D4	D5	D6	TOTAL
Model U1	10 µl	10 µl	20 µl	20 µl	30 µl	40 µl	130 µl
Model U2	10 µl	10 µl	20 µl	30 µl	40 µl	50 µl	160 µl

Table 2. Daily food volumes provided to the larvae and variation of the composition of the diet according to the age of the larvae.

Rearing day	D1	D2	D3	D4	D5	D6
Diet	A	A	B	C	C	C
Volume (µl)	10	10	20	30	40	50
% royal jelly	50	50	50	50	50	50
% yeast extract	1	1	1.5	2	2	2
% D glucose	6	6	7.5	9	9	9
% D fructose	6	6	7.5	9	9	9
% Dry matter	29.55	29.55	33.05	36.55	36.55	36.55

Table 3. Comparison of different treatments relative to feeding larvae in the *in vitro* rearing process. Comparisons are realised between treatments of the same experiments with: t test for weight comparisons, χ^2 for rate comparisons. Data bearing identical letters are not significantly different (P = 0.05).

Experiment	Mode of feeding		Amount of diet per larva		Diet composition according to the age of the larvae	
	May 2004		September 2004		October 2004	
Date of experiment	May 2004		September 2004		October 2004	
Daily treatment	individual, according to larval size	uniform	uniform	uniform	uniform, unique diet	uniform, three diets
Diet composition	A	A	A	A	A	A/B/C
Amount of diet/larva (µl)	$\bar{X} = 132.94$ ($\sigma = 17.72$)	130	130	160	160	160
Number of larvae	96	96	107	99	106	105
Mean weight (mg) of pre pupae (σ)	96.9 (17.2) a	96.5 (9.5) a	98.0 (7.6) a	120.3 (10.1) b	121.7 (8.3) a	123.8 (10.0) a
Larval mortality rate (%)	11.46 a	8.33 a	12.15 a	7.07 a	21.70 a	24.76 a
Number of pre pupae	85	88	94	92	83	79
Number of adults	-	-	36	69	54	69
Emergence rate/larvae (%)	-	-	33.64 a	69.70 b	50.94 a	65.71 b
Emergence rate/pupae (%)	-	-	38.30 a	75.00 b	65.06 a	87.34 b

of corbiculae and tongue length which enables to estimate the percentage of workers, queens and intercaste individuals. T tests were used for weight comparisons, and χ^2 tests for rate comparisons.

Results

In experiment 2, larvae fed with 160 µl of diet were significantly heavier than larvae fed with 130 µl of diet (120.3 mg and 98.0 mg respectively, $t = 17.01$, $P < 0.0001$) (table 3). In a previous experiment, the mean weights of 7 day old larvae reared in two different colonies (*in vivo* conditions) and calculated on 10 individual samples lead to the following results: 128.6 mg

($\sigma = 16.2$) and 126.8 mg ($\sigma = 26.47$) (unpublished results). These weights were close to the mean values observed in larvae fed with 160 µl of diet.

We did not observe any effect of the treatments on larval mortality. In the third experiment conducted in October, we noticed a higher larval mortality rate (21.7% and 24.76%) than in the two previous experiments in May and September (from 7.07 to 12.15%).

Emergence rates relative to the number of larvae and pupae were twice higher when larvae were fed with 160 µl than when larvae were fed with 130 µl of a uniform diet.

The use of sequenced diet according to the age of the larvae also increased significantly the level of adult emergence, and the homogeneity of larval weights. The

coefficient of variation of weight was 17.8% when diet was provided to larvae according to their size, and from 6.8% to 9.6% when larvae were uniformly fed (table 3).

The comparison of the two similar treatments (160 μ l uniform and unique diet) in experiments 2 and 3 revealed significant difference between larval mortalities ($\chi^2 = 8.767$, $p = 0.003$), and no significant difference between emergence rates relative to pupae ($\chi^2 = 2.064$, $p = 0.151$) and larvae weights ($t = 1.03$, $p = 0.307$).

The comparisons of the two similar treatments (130 μ l uniform and unique diet) in experiments 1 and 2 did not show any significant difference between mortalities ($\chi^2 = 0.795$, $p = 0.373$) and weights of the larvae ($t = 1.18$, $p = 0.239$).

In all experiments, 100% of adults had corbiculae and were typical workers.

Discussion

The generally low larval mortality rates observed are acceptable for a test procedure, except for the third experiment, and particularly if we compare larval mortalities between the two similar treatments (160 μ l of diet A per larva) in experiments 2 and 3. This last trial was realised in October, at a period where the decrease of egg laying activity of the queen did not offer a large number of larvae for grafting. This lower availability resulted in longer period in laboratory conditions due to the time consumed to collect the larvae, and might have reduced larval viability. Therefore the feasibility of our test method is bound to the egg laying activity and then to the season. We also noted similar results between the two similar treatments realised in May and September. Therefore, experiments will be more easily comparable if conducted in similar seasonal conditions. In the previous descriptions of *in vitro* methods, no author mentioned seasonal variations.

The mean larval mortality rate observed in our 2 experiments of May and September was 9.7%, which is generally equal to that of Peng *et al.* (1992), and lower than those of Weaver (1974), Rembold and Lackner (1981), Brodsgaard *et al.* (2000), and Brodsgaard *et al.* (2003). No comparison with Vandenberg and Shimanuki (1987) and Brodsgaard *et al.* (1998) is possible since the first authors did not take into account the mortality during the first 24 hours, and the second did not indicate precisely the mortality of larvae grafted at the first instar.

The use of a uniform model to provide food instead of adjusting feeding according to the larval growth did not induce mortality, and reduced the variability of weight. Haydak (1968) noted that all the larvae of the same age and in the same area on a comb received the same amount of food at the same time. This explains that uniform models are adapted to the production of homogeneous larvae, on condition that the grafted larvae are collected from a compact new brood area of a comb. In our experiments conducted in May and September the egg laying intensity was favourable to the uniform feeding model whereas in October, larvae collected from sparse and less abundant brood might have been

less adapted to the uniform method. Furthermore, this original process avoids subjective observations for adjusting the amount of food, susceptible to vary with the operator, and not compatible with a standardised method. The individual rearing and the standardised daily volume of food supplied also allow knowing the exact amount ingested by each larva.

The mean weights of the larvae fed *in vitro* with 160 μ l of diet in our experiments were close to those observed *in vivo* in our colonies (120-124 mg and 126-128 mg respectively), and also on larvae reared in other *in vitro* conditions by Vandenberg and Shimanuki, (1987) who found mean weights from 98 to 116 mg.

Ability to metamorphose was also influenced by the quantity and the metamorphose of food ingested by the larvae. The low rates of emergence observed in the 130 μ l treatment in experiment 2 may be the consequence of the lower level of feeding. On the contrary, larvae fed with 160 μ l of diet were more able to metamorphose. The lower rate observed for the 160 μ l constant diet in experiment 3 was linked to the higher larval mortality rate in this experiment. Therefore, even with a high larval mortality rate in the A, B, C treatment in experiment 3, the adult emergence rate relative to larvae was close to that observed in the 160 μ l treatment in experiment 2, and higher than the values measured by Weaver (1974), Malone *et al.* (2002) and Brodsgaard *et al.* (2003). The emergence rate relative to pupae was maximum in the third experiment with the A, B, C treatment, and also higher than that observed by Weaver (1974) and Malone *et al.* (2002) who fed the larvae with unique diet. The increase of dry matter in the diet provided by workers to last instar larvae was described by Haydak (1968). This author mentioned that before the age of 2 days, the worker larvae were fed with a diet containing 26.49% of dry matter, and after 2 days, 35.10%. These values were close to those of diets A and C used in our method.

Our method offers many advantages in term of time saving compared to those already described which recommend to feed at least twice a day and to move more or less frequently the larvae from their cell to a new one. In our method we used the same cell from the first instar to the emergence, and we fed the larvae only once a day. Furthermore, the use of plastic queen starters as rearing cells avoided any additional handling of larvae after their grafting. The use of a unique plastic cell also permits to weigh individually the larvae without direct contact with a metallic tool as described by some authors, with the risk to injure the larva.

The uses of this method are multiple. Ecotoxicology researches as well as lethal or sublethal routine evaluation trials on worker or queen larvae may require such process, when interaction with nurses is not desirable. Inherent toxicity of products to larvae will be estimated by their oral LD50 obtained by using a range of concentrations of the substance mixed with the food and provided to individuals on the 4th day of their larval development. Sublethal effects may be assessed by measuring weight and larval development variations, and the morphology changes of adults. Such adults could also be tested with behavioural tests such as the conditioned proboscis extension response (Pham-Delègue *et al.*, 2002).

Brood risk assessment could be performed thanks to the *in vitro* method by the comparison of the lethal concentration revealed by the test and the predictable exposure concentration measured in agronomical conditions.

Other type of studies may require this kind of method. It can be used for alimentary test (nutritive value of R.J., honeys, syrups, pollens etc.). It also can be used for studies in pathology by controlled inoculation of pathogens to study their effect on the development, or the effect of a treatment, or synergy of a pathogen with an insecticide.

In conclusion this method is fully adapted to routine tests, irrespective of the subject studied, because it is relatively simple to use and easy to standardise. The next step will be to validate the method by ring testing.

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