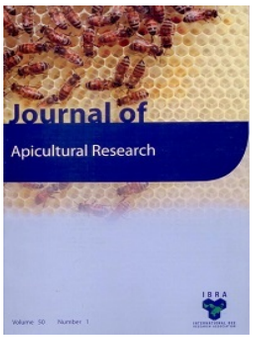

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ORIGINAL RESEARCH ARTICLE

Rearing honey bees (*Apis mellifera* L.) *in vitro*: effects of feeding intervals on survival and development

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Summary

A new and simple technique was developed to rear honey bees (*Apis mellifera* L.) *in vitro*. One day old larvae were grafted into Petri dishes and fed a basic diet at six different time intervals. There were no differences in the larval weights, survival rates or ovariole numbers of the bees among the groups that were fed at different intervals, but they were heavier and had larger ovaries than hive reared bees. It was shown that honey bees can be reared *in vitro* without replenishing their food daily. This simple mass provisioning technique reduces the labour involved, and enables the researcher to raise large number of bees *in vitro*.

Cría *in vitro* de abejas (*Apis mellifera* L.): efectos de los intervalos de alimentación en la supervivencia y el desarrollo

Resumen

Una nueva y sencilla técnica se ha desarrollado para criar abejas (*Apis mellifera* L) *in vitro*. Se dispusieron larvas de un día en placas Petri y se alimentaron con una dieta básica en seis intervalos de tiempo diferentes. No se hallaron diferencias en el peso de las larvas, tasas de supervivencia o número de ovariolas de las abejas entre los grupos que fueron alimentados en diferentes intervalos, pero mostraron ovaris más pesadas y grandes que las abejas criadas en las colmenas. Se demostró que las abejas pueden ser criadas *in vitro* sin el abastecimiento diario de comida. Esta sencilla técnica de aprovisionamiento reduce el trabajo implicado, y permite al investigador aumentar el número de abejas criadas *in vitro*.

Keywords: *Apis mellifera*, *in vitro* rearing, feeding interval

Introduction

The honey bee (*Apis mellifera* L.) has emerged as an important model organism for understanding the evolution of social behaviour, development, aging, and behavioural genetics and genomics (Page *et al.*, 2006; Robinson *et al.*, 2006). The social structure of the honey bee makes it attractive to study, but progressive brood rearing by nurse bees inhibits our ability to manipulate nutrition and study development and caste differentiation.

Honey bee larvae are fed mandibular and hypopharyngeal gland secretions produced by nurse bees. This food contains all the nutrients necessary for the development of queens, workers, and drones (Johansson, 1955; Rembold, 1965; Weaver, 1966; Haydak, 1970; Dietz and Haydak, 1971; Brouwers, 1984; Howe *et al.*, 1985). In this way, nurse bees control the composition and quantity of food given to larvae of the different castes, although larval signals (e.g., brood pheromone) may also influence nurse provisioning.

Under natural conditions, a queen larva is fed a total of 1600 times, and these feeding events last a total of 17 hours (Jung-Hoffmann, 1966). The number of feeding events increases with larval age from about 13 feedings / h at one day old, 16 feeding / h at three days, to 25 feedings / h at four days old (Jung-Hoffmann, 1966; Haydak, 1970). Larvae destined to be reared as queens receive abundant fresh royal jelly and grow to at least 1500-1700 times the weight of the egg, from 0.12-0.20 mg (Taber and Roberts, 1963; Roberts and Taber, 1965) to 250-346 mg (Haydak, 1970; Winston, 1987). Larvae destined to become workers receive the same brood food as queen

larvae up to 2.5 days old, and like queen larvae they float on the food. After the third day, however, they receive restricted food and finish all that is given to them. It is estimated that a worker larva is fed by the nurse bees 143 times, a total of about two hours during the whole larval stage (Lindauer, 1952). Although the genotype, cell size, nutrition and season influence the size and the weights of resulting adult worker bees, a worker larva grows about 900-1100 times of the weight of an egg or newly hatched larva and reaches approximately 175 mg (Wang,1965).

Many attempts have been made to rear honey bees in the laboratory (Rhein, 1933; Michael and Abromovitz, 1955; Weaver, 1955, 1962, 1970, 1974; Smith, 1959; Hoffmann, 1960; Mitsui *et al.*, 1964; Rembold, 1965; Jay, 1965; Dietz, 1973; Rembold *et al.*, 1974; Asencot and Lensky, 1976; Schuel *et al.*, 1978; Schuel and Dixon, 1986) but the methods were labour intensive, yielded only small numbers of viable individuals, and were not sufficient to consistently produce individuals of specific castes.

Rembold and Lackner (1981) developed a larval diet for rearing honey bee queens *in vitro*, using royal jelly (RJ) , D-glucose, D-fructose, and distilled water. They obtained 75% adult survival, and most adults were workers. Adding Difco bacto-yeast extract (YE) to this basic food increased the survival of the larvae to 80% and 30% of the individuals developed into queens. Hanser (1983) used royal jelly diluted with nutrient solution which consisted of 35 g of honey instead of glucose and fructose, 10 g of Torula yeast, 0.3 g Nipagin and 100 ml double-distilled water to rear queen bees. Royal jelly was diluted at a 2:1 ratio for feeding younger larvae and a 1:1 ratio for older larvae. One to two days old larvae weighing 1-2 mg were grafted onto 0.25 ml of royal jelly solution in plastic cups (thimbles). They were kept at 35^{0}C and 85-90% RH in an incubator and fed twice a day with 0.1 ml royal jelly solution. Unconsumed diet was removed from the cups and fresh food was supplied. Just before the larvae started spinning, they were transferred to new cups and pupated in the incubator. Vandenberg and Shimanuki (1987) compared plastic and beeswax queen cell cups to rear worker honey bee *in vitro* and fed them with the mixture of 50% RJ, 6% D-glucose, 6% D-fructose, 1% YE and 37% distilled water in an incubator which was kept at 34^{0}C and 96% RH during the larval stage and 70% RH during the pupal stage. They obtained up to 90% larval survival in beeswax and 57% survival in plastic cell cups. Peng *et al.*, (1992) modified Vandenberg and Shimanuki's method and raised honey bee larvae in 24 well plates to study the effects of chlortetracycline on the development of worker honey bee larvae reared *in vitro*. The larval mortality and post-defecation mortality rates were 6.3% and 18.1% respectively. Aupinel *et al.*, (2005) also used Vandenberg and Shimanuki's diet and improved the technique by altering the quantity (130 µl *vs*. 160 µl), sugar content (6%, 7.5% and 9%), and yeast extract content (1%, 1.5% and 2%) of the diet for different instars. They used plastic queen cell cups and placed them in 48 well plates. The larvae fed with 160 µl of diet were heavier than larvae fed with 130 µl. The survival rate also increased from 33.64% to 69.7% when the amount of food increased. Brodshnider *et al.* (2009) reared bees *in vitro*, observed the flight behaviour and compared it to natural workers. They did not find morphological differences between hive reared bees and *in vitro* reared bees.

With all the above techniques, honey bee larvae were fed once or twice daily, effectively mimicking progressive provisioning. These techniques are labour intensive and time consuming. We tested the efficacy of reducing the number of times *in vitro* reared larvae are fed in order to reduce the effort needed and to enhance mass rearing of worker bees. Here we report the effects of different feeding intervals on survival, adult weight, and ovariole numbers of honey bees reared *in vitro*.

Materials and methods

Collection of honey bee larvae

A healthy honey bee colony (*Apis mellifera ligustica*) headed by an open mated queen was used as the larval source at the Bee Research Facility of the Arizona State University, Mesa, AZ. 85212, USA. To obtain one day old larvae, the queen was confined to a fully drawn comb in an excluder cage for one day, and the comb with the larvae was removed from the hive on the fourth day (Peng *et al.*, 1992).

Composition of diets and feeding intervals

The basic larval diet consisting of 53% (w/w) commercial frozen royal jelly (RJ), 6% glucose, 6% fructose, 1% Becton Dickinson (BD) Bacto^{TM} yeast extract (YE) and 34% distilled water was used throughout the experiment. There were six treatment groups, three replicates per treatment, and ten larvae in each replicate. The 1st group was fed every day of larval development, the 2nd group every other day, the 3rd group on the first and third days, the 4th group on the first and fourth days, the 5th group on the first and fifth days, and the 6th group was fed on the first day only. The feeding intervals and the amounts of foods given to different interval groups each day are given in Table 1.

Based on the experimental design, aliquots of 200-4200 mg food were placed in the centre of polystyrene petri dishes (60 x 15 mm) and 10 one day old larvae were grafted on each aliquot (Fig. 1A). The larvae were re-grafted on fresh food in new petri dishes in groups 1-5 each time they were fed and the unused food was discarded (Fig. 1B-E). All groups received a total of 4200 mg of food throughout the larval stages (Table 1). The petri dishes were kept in an incubator at 34^{0}C and 90% RH during larval stages, and 34^{0}C and 70% RH during the pupal stages. Pre-defecation stage larvae were removed from the feeding dishes, weighed and transferred to 100 x 15 mm petri dishes lined with Kimwipes® tissue paper. Dirty tissue papers were removed on the next day and the larvae were transferred to 24 well plates for pupation and emergence (Fig. 1G, H).

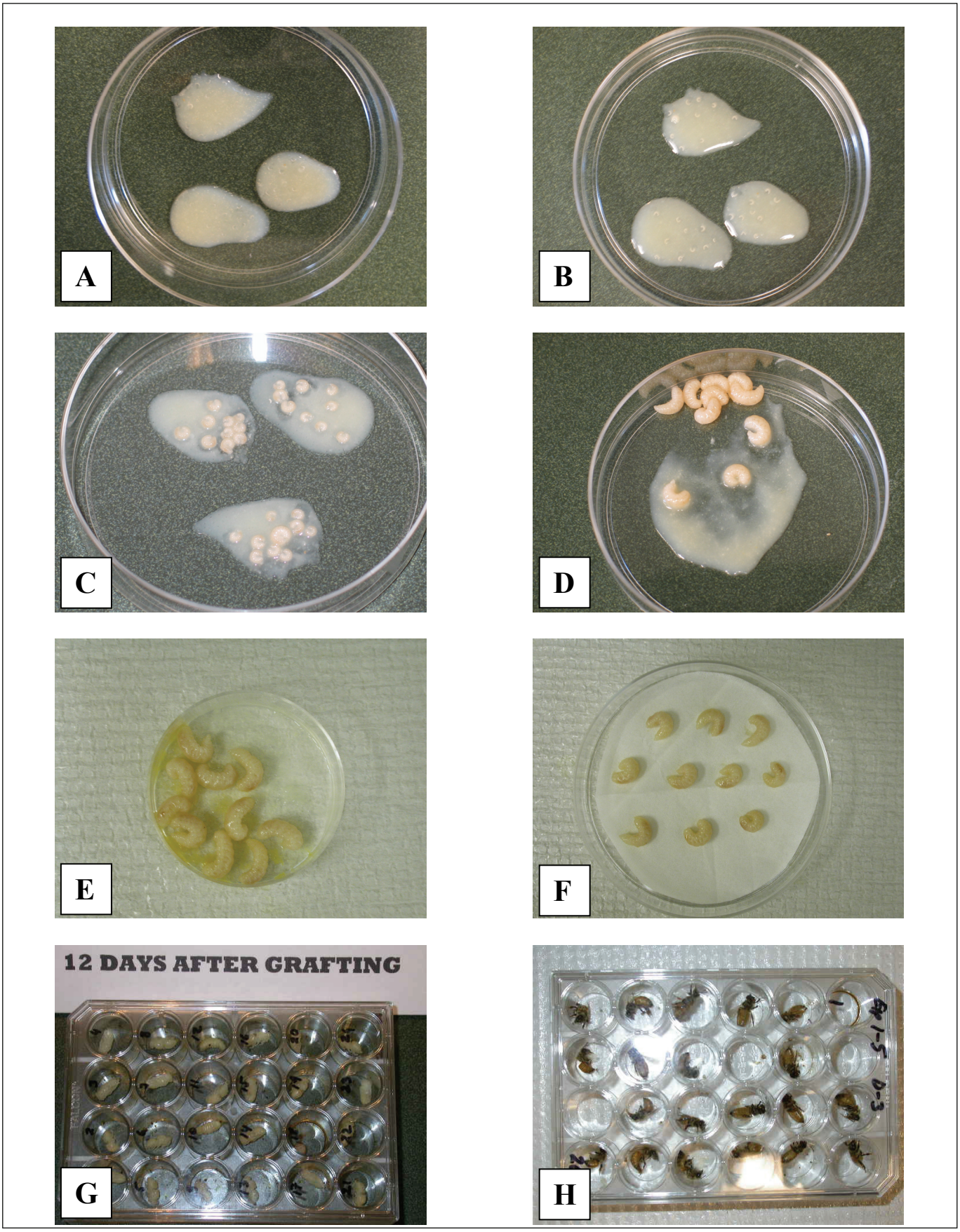

Fig.1. Rearing honey bee larvae *in vitro*: A: grafting 1-1.5 day old larvae into the aliquots of larval diets (Day 1); B: Day 2; C: Day 3. After Day 3, each group of larvae except groups 4-6 were transferred to new dishes with diets; D: Day 4 and 5; E: pre-defecation stage larvae; F: larvae placed on filter paper; G: pupae in 24 well plates; H: adult bees reared *in vitro.*

Table 1. Amounts of food given to each group of larvae in each day (mg).

	Amounts of food given to per larvae in each day (mg)						
Group	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Total**
1	20	40	60	80	100	120	420
2	80	-	100	-	240	-	420
3	100	-	-	320	-	-	420
4	150	-	-	-	270	-	420
5	300	-	-	-	-	120	420
6	420	-	-	-	-	-	420

Adult survival rate (SR) was the survival from grafting larvae to emergence of adults and calculated as: SR = (number of adults / number of larvae grafted) x 100.

Ovariole numbers

Newly emerged adult bees were pinned by the thorax and the last segment of the abdomen in a dissection plate (a petri dish half filled melted paraffin wax). Distilled water was added into the plate to cover the whole abdomen of the bee. Abdominal tergites were cut laterally by using a pair of McPherson-Vannas® scissors and gently removed from the abdomen. The ventriculus was lifted upwards very gently with a pair of fine forceps to free the ovarioles (Fig. 2A). Ovaries were removed by pulling and cutting them from the lateral oviducts under an Olympus stereo-zoom (SZ-60) microscope. The ovaries were placed on a microscope slide with a drop of distilled water, and a cover slip was placed on them. Ovarioles were then counted using a Leica® DM 400B dark field (10X) objective (Fig. 2B, C).

Results

Effects of feeding intervals on larval survival and larval weights

Larval survival rates are shown in Table 2. There were no significant differences among groups for larval survival (ANOVA $F_{5,173} = 1.203$, $P > 0.05$) or for pre-defecation larval weights (ANOVA: $F_{5,168} = 1.314$, $P > 0.05$). Kaftanoglu *et al.* (2010) compared seven different diets to study the effects of carbohydrates on the development of honey bee larvae reared *in vitro*. Their Diet 3 was the same diet as used in this experiment. Their larval weights using Diet 3 (203.1±32.69 mg) were also similar to the average larval weights in the present experiment.

Effects of feeding intervals on survival and adult weights

A total of 125 adult bees were reared from 180 larvae (Table 3). Even though the survival rate was higher in Group 5 which were fed twice during the larval stages, there was no significant difference among the groups (ANOVA $F_{5,178} = 0.796$, $P > 0.05$). The survival rates in all the groups were satisfactory compared to previous studies where larvae were fed every day or twice a day (Vandenberg and Shimanuki, 1987; Peng *et al.*, 1992; Aupinel *et al.*, 2005).

Feeding interval affected the adult weights of bees (Table 2). The highest adult weights were observed in the 2nd group which was fed every other day (ANOVA: $F_{5,124} = 2.694$, $P < 0.05$). Most of the *in vitro* reared bees appeared morphologically indistinguishable from normal hive reared bees, but the weights of the *in vitro* reared bees (140.09±21.52 mg) were greater than the hive reared control bees (114.2±7.11 mg) as previously reported (Kaftanoglu *et al.*, 2010). The average ovariole number was 23.3±1.74 among all the groups, and there was no significant difference in the ovariole number of bees fed

Fig. 2. Ovariole counts: A: intact ovaries stained with methylene blue; B: small ovary; C: large ovary.

Table 2. The effects of feeding intervals on larval survival and larval weights.

	Larvae			Pre-defecation larval weights (mg)			
Group	**Grafted**	**Survived**	**% Survival**	**X±SD**	**Min**	**Max**	**% CV (Coefficient of Variation)**
1	30	26	86.7	197.6±30.68	135	257	15.53
2	30	29	96.7	200.6±25.50	152	278	12.71
3	30	29	96.7	184.1±24.00	134	222	13.04
4	30	30	100.0	191.6±22.36	145	232	11.67
5	30	28	93.3	191.4±31.85	133	256	16.64
6	30	27	90	198.6±35.94	139	278	18.10
Total/Average	180	169	93.9	193.6±28.73	133	278	14.84

Table 3. Effects of feeding intervals on survival and adult weights of bees reared *in vitro.* * Different letters indicate significant differences ($P < 0.05$).

Group	**N**	**% Survival**	**Adult Weights (mg)**			**Ovariole Numbers**		
			X±SD	**Min**	**Max**	**X±SD**	**Min**	**Max**
1	22	73.3	143.95±21.61ab*	103	187	24.8±23.3	4	119
2	18	60.0	151.44±20.30b	111	183	16.1±10.5	3	43
3	19	63.3	131.42±15.62a	103	162	30.3±27.7	6	101
4	22	73.3	142.86±19.47ab	116	203	19.2±8.3	8	33
5	24	80.0	131.96±20.90a	93	170	28.3±22.6	10	125
6	20	66.6	140.60±25.51ab	93	203	19.6±10.3	4	46

at different intervals (ANOVA $F_{5, 122} = 1.704$, $P > 0.05$). All the *in vitro* reared bees had more ovarioles (overall average 19.6±10.3) than the hive reared bees (9.1±3.52). Even though the average ovariole numbers varied between 16 and 30, the difference was not statistically significant due to high variation within the treatment groups ($P > 0.05$).

Discussion

Honey bees require proteins, carbohydrates, lipids, sterols, vitamins, minerals and trace elements for development and metamorphosis. Brood food has all these requirements in balance, and royal jelly has all the proteins, essential amino acids, fats, sterols, vitamins and minerals. Nurse bees add sugars (nectar) to the brood food for energy and possibly for metabolism or the synthesis of hormones. Young larvae (0-3 days old) receive abundant brood food and float on it. As they grow, they receive restricted food and finish all the food given to them by the nurse bees. There is obesity in honey bee larvae and they can consume more food and grow bigger when fed *ad libitum*. Nurse bees regulate the amount of food, however, stop feeding when they reach 6th instar larvae and cap the cells. Hive reared bees are therefore more uniform in size than *ad libitum* fed *in vitro* reared bees. Larval weights and adult weights can be regulated by the amount of food given to larvae. We have successfully reared smaller and larger bees by differential feeding of the larvae *in vitro* (Kaftanoglu *et al.*, unpublished data).

Ovariole numbers of the *in vitro* reared bees depend on the quality and the quantity of the food. The sugar content of the larval food significantly affects the ovariole numbers and formation of queens and intercastes (Asencot and Lensky, 1975). Ovariole numbers of hive reared bees also vary from colony to colony and from season to season due to genetics, colony strength, nutrition, availability of nectar and pollen and age distribution of the nurse bees, but because worker bees regulate the amount of food given to the larvae, the variation is much smaller than *in vitro* reared bees. We have successfully reared worker bees with high and low ovariole numbers by regulating the quality and quantity of the diet given to the developing larvae *in vivo* and *in vitro* (Kaftanoglu *et al.*, unpublished data).

This experiment shows that honey bees can be successfully reared *in vitro* when only once, i.e. mass provisioning instead of progressive provisioning. This has not previously been reported. This new technique greatly reduces the labour associated with *in vitro* honey

bee rearing, and the survival rate is higher than techniques requiring multiple feeding events due to less handling and a reduced chance of damaging larvae, especially during moulting. We have found in subsequent experiments that the quantity of food given to each larva can be strictly regulated by rearing larvae in individual cups (Kaftanoglu *et al*., unpublished data) and feeding them only once.

Acknowledgements

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