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Original Article

REARING DRONES IN QUEEN CELLS OF *APIS MELLIFERA* HONEY BEES

Georgios Goras[1]
Chrysoula Tananaki[1]
Sofia Gounari[2]
Elissavet Lazaridou[1]
Dimitrios Kanelis[1]
Vasileios Liolios[1]
Emmanouel Karazafiris[1]
Andreas Thrasyvoulou[1]*

[1]Aristotle University of Thessaloniki, Greece
[2]Institute of Mediterranean Forest Ecosystems, NAGREF, Greece

*corresponding author: thrasia@agro.auth.gr
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Abstract

We investigated the rearing of drone larvae grafted in queen cells. From the 1200 drone larvae that were grafted during spring and autumn, 875 were accepted (72.9%) and reared as queens. Drone larvae in false queen cells received royal jelly of the same composition and of the same amounts as queen larvae. Workers capped the queen cells as if they were drones, 9-10 days after the egg laying. Out of 60 accepted false queen cells, 21 (35%) were capped. The shape of false queen cells with drone larvae is unusually long with a characteristically elongate tip which is probably due to the falling of larvae. Bees start the destruction of the cells when the larvae were 3 days old and maximised it before and after capping. Protecting false queen cells in the colony by wrapping, reversing them upside down, or placing in a horizontal position, did not help. The only adult drones that emerged from the false queen cells were those protected in an incubator and in push-in cages. Adult drones from false queen cells had smaller wings, legs, and proboscis than regular drones. The results of this study verify previous reports that the bees do not recognise the different sex of the larvae at least at the early stage of larval development. The late destruction of false queen cells, the similarity in quality and quantity of the produced royal jelly, and the bigger drone cells, allow for the use of drone larvae in cups for the production of royal jelly.

Keywords: drones, false queen cells, royal jelly, sex recognition

INTRODUCTION

Drone larvae feed on royal jelly during the first three days of their development while the rest of the days they feed on what is called a modified diet. However, in nature some queen cells are started over drone larvae (thus, producing false queen cells) instead of worker larvae and occasionally drones may complete their development in queen cells (Manley, 1936; Wolsterncroft, 1936; Woyke, 1956; Smith, 1961). Fell and Morse (1984) studying the production of emergency queen cells in the honeybee colony, found that 25 out of 268 queen cells (9.3%) were started over drone larvae. This percentage increased to 40.6% (26 drones out of 64 queen cells) in colonies which were overturned to depress swarming (Thrasyvoulou et al., 1992). It seems that the sex of the larvae in queen cells does not appear to be a significant factor. Drone larvae grafted into queen cells are accepted by the bees for further rearing (Woyke, 1956, 1965). This led Obata and Nonogaki (1965) to transfer drone larvae instead of worker larvae into queen cells to produce royal jelly. The acceptance (94%) and the production of royal jelly (average 244.8 g) from queen cells with drone larvae, was slightly higher than those with worker larvae (92% and 222.2 g, respectively). This result does not support Fell and Morse's (1984) statement that the level of acceptance in queen cells with drone larvae is generally lower than for worker larvae.

The fate of drone larvae in queen cells is not

clearly understood. According to Smith (1961) drone larvae die before reaching pupation. Naulleau (1962) stated that drone larvae died soon after the cells were sealed probably because of the abnormal conditions, especially the vertical position. Fell and Morse (1984) found that most of the false queen cells with drone larvae were torn down before sealing. Von Rhein, cited by Smith (1961) and Woyke (1971), suggested that normal drones can develop on royal jelly. Woyke (1971a) examining reasons why the drones do not develop in queen cells, concluded that drone larvae become detached from the royal jelly and slide fast to the base of queen cells. Some larvae dropped either out of the queen cells or at the edge of the queen cells, where they were eaten by worker bees. Another reason for the destruction of false queen cells may be the inability of drone larvae to spin cocoons (Woyke, 1965).

According to Woyke (1956), rearing drones in queen cells by bees is suggesting that nurse bees do not recognise the sex of bee larvae. This opinion is in contrast to earlier reports (Haydak, 1958; Naulleau, 1962) and recent beliefs (Sasaki, Kitamura, & Obara, 2004) that honeybee workers can discriminate the sex of the brood. Woyke (1956) proved his suggestion by introducing worker combs with fertilised and unfertilised eggs into a queenless colony and found 6 out of 11 emergency queen cells (54.5%) over drone larvae.

Academic purposes aside, we believe that this aspect of honeybee biology has not been studied adequately. We still do not have answers about the morphological characteristics of those drones that they reared on a great amount of royal jelly. It is known that royal jelly has the physiological activity and capacity of changing the process of organogenesis in developing individuals (Melnichenko, Kapralova, & Shmelena, 1983). Furthermore, we do not have a clear view about the developmental period of the larvae in these false queen cells, or about the practical application of this phenomenon in producing royal jelly.

We performed experiments so as to give answers to several questions about the described phenomenon. We grafted queen cells with drone larvae (false queen cells) and we compared their acceptance to queen cells with worker larvae (real queen cells). We gave the bees the option of choosing between queen cells with drone larvae and queen cells with worker larvae and we followed the capping and the destruction of the false queen cells. To be certain of the type of food that drone larvae received in the false queen cells we compared the composition of the food with royal jelly collected from adjacent queen cells. Finally, we used different techniques to protect the false queen cells. Through these actions we were able to describe the characteristics of the imago that developed in queen cells with drone larvae.

MATERIAL AND METHODS

Differences in Acceptance of Queen Cells Containing Either Worker or Drone Larvae

This investigation was conducted at the Apiary of the Aristotle University of Thessaloniki, Greece, with bee colonies of *Apis mellifera macedonica*. Three queenless colonies received forty artificial plastic queen cells grafted with larvae which were less than 24 h old. In the first colony, 20 cells containing drone larvae and 20 cells containing worker larvae were put in, one after the other. The artificial cells of the second colony had worker larvae and of the third colony had drone larvae. Every third day, the grafted cells were removed and replaced by another 40 cells, to complete 10 consecutive graftings. The experiment took place in May of 2012 and was repeated in November of 2012. The procedure meant that 1200 drone larvae and 1200 worker larvae were grafted and their acceptance as well as the amount of royal jelly in cells was compared. The three queenless colonies received one comb of sealed brood every six days and half a liter of syrup (1:1) every three days.

Destruction of False Queen Cells

In each of three queenless colonies 20 accepted queen cells with drone larvae were left and their development was monitored every day.

The destroyed cells were removed.

Efforts to Protect False Queen Cells

In another trial, we protected the false queens from destruction by wrapping them in tinfoil and leaving only the tip uncovered, putting them in a horizontal position, turning them upside down, transferring them into an incubator, and finally protecting them in a colony with a push-in grid cage. Twenty sealed cells for each case were used with the exception of the push-in grid cages where twelve were used. All false cells were protected or removed immediately after capping.

Morphological Characteristics

The morphological characteristics of drones from false queen cells were compared to drones of the same age that had emerged from ordinary drone combs of the same colony. These drone combs had been constructed in the year of the experiment. Measurements were also made on worker bees of the same colony. We chose morphological characteristics that are mainly genetically determined, highly constant, but are nevertheless subject to certain influences; like the quantity and the quality of brood food as proposed by Ruttner (1988). Those morphological characteristics chosen were the length, the width, and the marginal cell of the fore-wing, the hooks of the hind-wing, the length of the femur, the tibia, and the length and width of the metatarsus of the hind leg. The measurements were performed using the Image Pro Plus 6 software on ten drones one day after their emergence from the protected queen cells.

The Composition of the Diet

Pool samples consisted of the content of 5 accepted false/real queen cells that were collected three days after grafting with 12h old larvae. From five consecutive graftings, we collected 5 pool samples that were analysed for moisture, crude protein, sugars, and 10-hydroxy-decenoic acid (10-HDA) content.

Moisture Analysis

A quantity of 0.5 g of royal jelly was weighed in a porcelain evaporating dish, placed at 105°C in a drying oven for 3 hours (until its weight was stabilised) and the water content was estimated using the formula of Sesta and Lusco (2008):

Water %= $M-M_1/M-M_2 \times 100$

Where M is the sum of the dish and sample weight (g), M_1 is the mass weight of the sample after drying (g), and M_2 is the weight before adding the analysed sample (g).

Crude Protein Analysis

A quantity of 0.2 g of royal jelly was placed into the tubes of a digestion unit (K-435, Buchi), with 20 ml sulfuric acid and one tablet of catalyst (K_2SO_4,- Na_2SO_4, - $CuSO_4$, - TiO_2). The tubes were placed in the digestion unit and heated. When the digestion was completed the samples were removed and left to cool at room temperature. After cooling, the solutions were distilled in the presence of a sodium hydroxide (NaOH) solution (KjelFlex, 360 Buchi) and the nitrogen content was estimated by titration with 1 M HCl (Mettler Toledo, T-50). Crude protein was calculated by multiplying the content of nitrogen by 6.25 (Garcia-Amoedo & Almeida-Muradian, 2007).

Sugar Analysis

The chromatographic method suggested by Sesta (2006) with some modifications, was used to determine the sugars in royal jelly. A quantity of 0.5 g of royal jelly was placed in a volumetric flask, diluted with a methanol/water solution (25:75) and 0.1 ml of Carrez I, and Carrez II reagents were added. Finally, the flask was filled to 5 ml with the methanol/water solution, the content was homogenised, and the mixture was filtered through a 0.22 μm disposable syringe filter. Sugars were determined by liquid chromatography (HPLC) with refractive index detection (RID) (Agilent, 1200), using a Zorbax Carbohydrate analysis column (4,6mmID x 150mm x 5μm) at 30°C.

10-Hydroxy-2-decenoic acid (10-HAD) Analysis

For the 10-HAD determination, the method described by Zhou et al. (2007) with some modifications was used. A quantity of 0.2 g of

royal jelly was placed in a 10 ml volumetric flask with 0.6 ml HCl, 40µl internal standard (methyl-4-hydroxybenzoate solution of 1mg/ml) and 1 ml purified water. The rest of the volume was filled with absolute ethanol. The solution was mixed, placed into an ultrasound bath for ten minutes and filtered through a 0.22 µm disposable syringe filter. The concentration of 10-HDA was determined by a liquid chromatography instrument equipped with a DAD detector (Agilent, 1200) using a Nucleosil® 100-5 C_{18} (4,6mmID x 150mm x 5µm) at 35°C.

Statistical analysis

The one-way Analysis of Variance (ANOVA) and Duncan's multiple range test were used to compare: the number of accepted queen cells, the produced quantity of royal jelly, and the seasonal differentiation between grafted drone and worker larvae, the morphological characteristics among drones that emerged from queen cells, regular cells, and workers that emerged from regular cells, and the differences regarding the chemical composition of royal jelly produced from queen cells with drone larvae and those with worker larvae.

The analyses were carried out using SPSS v.17.0 (SPSS, Inc., Chicago, IL) for Windows (Microsoft Corporation, Redmond, WA). The significance level of all the statistical tests was set at α=0.05.

RESULTS

Acceptance

It can be seen in Table 1 that bees equally accepted rearing drone larvae and worker larvae in queen cells (one sex grafted per colony) (spring F=0.101, p=0.754; autumn F=0.043, p=0.838). Where they had to choose between worker and drone larvae (two sexes grafted per colony), bees still failed to recognise the difference (spring F=0.071, p=0.793; autumn F=0.241, p=0.629). Even in autumn, when bees are not so prompt to rear drone brood, the acceptance of the queen cells with drone larvae was high (78.5%). Actually, the acceptance of queen cells in November was higher than in May for both false and real queen cells. No statistically significant differences were found regarding the acceptance of worker and drone larvae grafted during the same period (May or November). From the 1200 drone larvae that were grafted during spring and autumn, 875 were accepted (72.9%) and reared as queens.

The amount of royal jelly per cell that was harvested from the queen cells ranged from 0.07 g to 0.68 g for worker larvae (the average 0.28±0.09 and 0.31±0.08) and from 0.06 g to 0.63 g for drone larvae (the average 0.28±0.12 and 0.31±0.05) in both seasons (Tab. 1). The pool average of the amount of royal jelly for spring and autumn was 0.28 and 0.31 g per cell,

Table 1

Total of the accepted queen cells with drone and worker larvae, and the produced quantity of royal jelly

	Drone larvae in queen cells (false queen cells)		Worker larvae in queen cells (real queen cells)	
	May	November	May	November
Larvae grafted using one sex per colony (n=400)	290[a] (72.5%)	307[a] (76.7%)	298[a] (74.5%)	302[a] (75.5%)
Larvae grafted using two sexes per colony (n=200)	114[a] (57%)	164[a] (82%)	120[a] (60%)	159[a] (79.5%)
Quantity R.J. (g/cell)	0.28[a] ±0.12	0.31[a] ±0.05	0.28[a] ±0.09	0.31[a] ±0.08

The same alphabetical letter indicates no significant differences (α=0.05, t-test). The comparison was done horizontally for each season separately, and horizontally for each larva sex separately

respectively, both for false and real queen cells. No statistically significant differences were found regarding the amount of the diet for real and false queen cells (Spring F=0.002, p=0.967. Autumn F=0.031, p=0.861).

The Fate of False Queen Cells

The sealed queen cells with drone larvae have a characteristically elongate tip that differentiates them from the real queen cells (Fig. 1). This different shape of false queen cells is not typical and some of them do keep the usual shape of the queen cells. In practice, all the queen cells with elongate tips that we have seen in bee colonies, were constructed over drone cells.

The 60 accepted false queen cells (Fig. 2), had two-day-old larvae, as they were chosen one day after grafting. Their destruction started from the third day of larvae age, and maximised between the 6th and 9th day when 41 cells (68.3%) were destroyed. From a total of 60 cells, 13 (21.6%) were sealed the sixth day, and 8 (13.3%) the seventh day of larvae age. Since the grafted larva was between 6 and 24 h of age, the precise day of sealing is difficult to trace, but surely was completed by the eighth day. After capping, the bees continued to tear down the false queen cell, but finally they kept 5 sealed false cells till the end. Since no emergence was observed from those cells, we tore them open

Fig. 1. False queen cell grafted with drone larvae. Usual but not typical elongated shape.

and found dead larvae in all of them. Obviously these larvae died before pupation and for some reasons the bees kept their cells longer.

Out of 92 protected false queen cells, 14 drones emerged from the incubator and 4 from the push-in cages. All wrapped queen cells in the colony were destroyed by opening a small hole at the tip of the cell. Reversed false queen cells were also all destroyed right after sealing; between eight and twelve day after grafting. Placing the false queen cells in a horizontal position did not help since they were destroyed within two days after sealing.

The six drones (out of twenty) that were found dead in the incubator were at the larval stage.

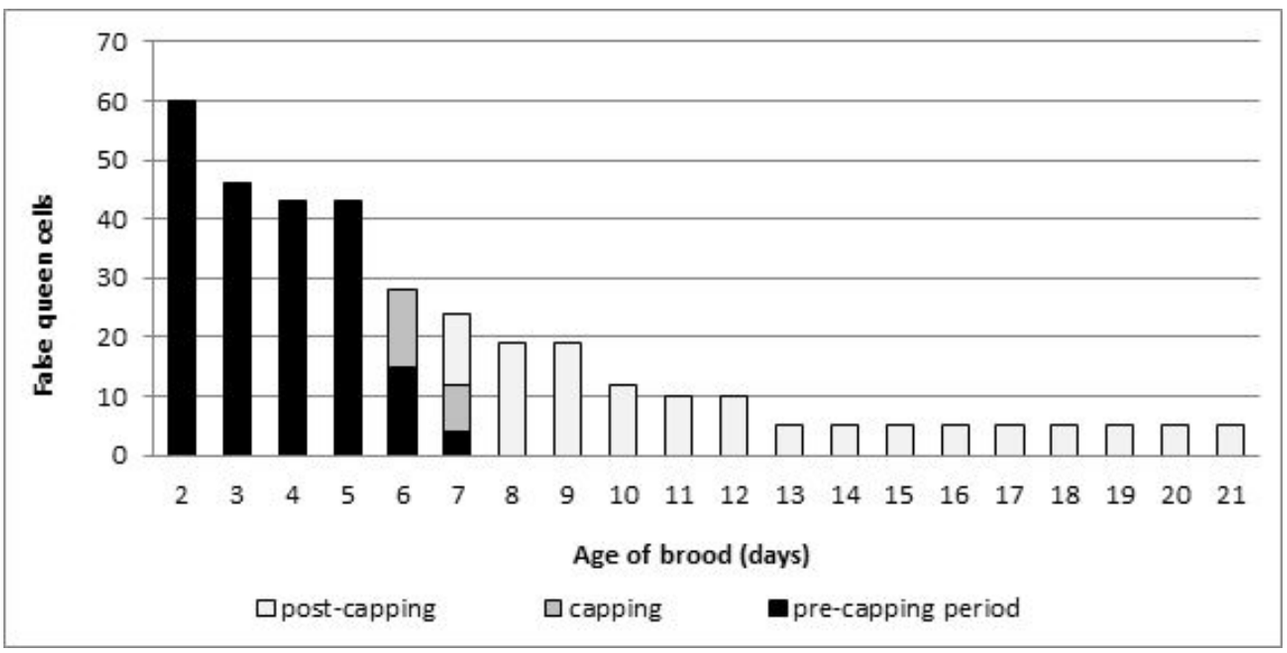

Fig. 2. The destruction of false queen cells containing drone larvae

Table 2

Morphological characteristics of drones that emerged from queen cells and drones and workers that emerged from regular cells

Characteristic (in mm)	Drones from queen cells	Drones from drone cells	Workers from worker cells
Front Wing length	8.92[b] (8.22-9.38)	9.57[a] (9.34-9.95)	7.12[c] (6.61-7.46)
Front Wing width	3.21[b] (2.95-3.50)	3.41[a] (3.22-3.59)	2.63[c] (2.33-2.82)
Marginal cell	3.46[b] (2.35-3.74)	3.75[a] (3.63-3.87)	2.64[c] (2.51-2.78)
Wing hooks	22.5[a] (20.0-25.0)	22.3[a] (20.0-27.0)	21.6[a] (20.0-23.0)
Length of femur of hind leg	2.61[a] (2.45-2.82)	2.59[a] (2.42-2.74)	2.11[b] (1.99-2.39)
Length of tibia of hind leg	2.98[b] (2.70-3.20)	3.16[a] (2.82-3.35)	2.61[c] (2.44-2.90)
Length of metatarsus	2.02[b] (1.77-2.16)	2.16[a] (2.02-2.29)	1.72[c] (1.51-1.92)
Width of metatarsus	1.12[a] (1.02-1.23)	1.08[a] (0.99-1.30)	0.95[b] (0.79-1.11)
Proboscis	2.50[c] (1.68-3.00)	3.15[b] (2.27-3.55)	4.85[a] (4.21-5.22)

In each line, values followed by different letters were significantly different (α=0.05, one – way ANOVA, Duncan's test, where a>b>c)

Table 3

Chemical composition of royal jelly produced from queen cells with drone and worker larvae

Physicochemical parameters	Drones RJ (false queen cells)	Workers RJ (real queen cells)
Moisture (%)	69.3[a] (67.8-71.8)	70.3[a] (68.7-71.8)
Crude Protein (%)	14.1[a] (11.6-15.5)	14.6[a] (12.9-16.8)
10-HDA (%)	3.0[a] (2.7-3.3)	3.0[a] (2.3-3.4)
Fructose (%)	2.8[a] (2.3-4.1)	3.2[a] (2.9-3.5)
Glucose (%)	4.0[a] (2.5-5.0)	4.7[a] (4.3-5.0)
Sucrose (%)	1.6[a] (1.0-3.2)	1.9[a] (1.0-3.7)

In each line, values followed by the same alphabet letter do not differ significantly (α=0.05, t-test)

The remaining fourteen from the incubator and four from the push-in cages, uncapped the queen cell themselves and emerged. Eleven drones (61%) emerged on the 23rd day after the egg laying and the rest on the 24th. All imagoes were drones and the only apparent difference between the emerged drones and the normal drones was their wrinkled antennae. The antennae were probably wrinkled because of their falling to the tip of the cell. The surplus of royal jelly weighed from 0.43 g to 0.49 g and was located in all of the false queen cells.

Ten "king-drones" were used to record their morphological characteristics. We used only the drones emerged in the incubator as we were sure about their age and days of feeding. As can be seen in Table 2, the front wings of drones from queen cells, when compared to regular drones,

were smaller in length (F=229.872, p=0) and width (F=79.628, p=0). Drones from false queen cells also had smaller marginal cells (F=51.633, p=0), length of tibia (F=37.868, p=0), and length of metatarsus (F=49.50, p=0) than those from regular cells, but all were bigger than those of workers. The length of the femur (F=58.608, p=0) and the width of the metatarsus (F=12.38, p=0) were similar for all drones, but different for workers. The proboscis was smaller in drones emerged from queen cells than drones emerged from drone cells, but for both cases the proboscis was smaller when compared to the proboscis of the workers (F=111.421, p=0). Finally, the number of hooks in the hind wings, was similar in both workers and drones emerged from regular or queen cells (F=0.744, p=0.484).

Composition of Royal Jelly

No statistically significant differences were observed for any of the examined parameters concerning chemical synthesis of royal jelly produced from false and real queen cells (Tab. 3). The moisture percentage of royal jelly produced from false queen cells, ranged between 67.8% and 71.8%. The total protein content ranged between 11.6% and 15.5%, and the percentage of 10-HDA ranged between 2.7% and 3.3%. Regarding sugars, fructose ranged from 2.3% to 4.1%, glucose from 2.5% to 5.0%, and sucrose from 1.0% to 3.2%. Drone larvae in queen cells fed on the same composition of royal jelly as that fed to the worker larvae.

DISCUSSION

The results of this experiment indicate that the bees accept male larvae in queen cells and rear them as queens. Similar results were observed even when the bees of a colony had the option to choose between drone and worker larvae, in spring or in autumn. These findings confirm Woyke's (1965) statement that workers accept and rear male larvae in queen cells, in the presence of female larvae in adjacent queen cells. The drone larvae in the queen cells received the same quantity and quality of royal jelly which is given to normal queen larvae. The different food did not have a detrimental effect on larva development. Adult drones can develop on a mass provision of royal jelly as indicated also by Smith (1961) and Woyke (1971).

Naulleau (1962) recorded that drone larvae died after the cells were sealed, while Fell and Morse (1984) stated that all emergency false queen cells with drone larvae were destroyed immediately before or shortly after capping. Woyke (1965) found that 5 out of 8 queen cells (62.5%) with drone larvae were sealed. We found that the destruction of false queens started from the third day of larva age, and maximised between the 6th and 9th, then continued after capping. Out of 60 accepted false cells, 21 (35%) were capped between the sixth and seventh day after grafting, which means 9 to 10 days after egg laying. Woyke (1965) also found that many of the drone larvae in queen cells capped 6-7 days after grafting with one-day-old larvae. According to Winston (1987), the average capping time for drones is 9.3 days after egg laying with a minimum and maximum of 7 to 10 days, respectively. In spite of the variation that exists in the development time of larvae (Winston, 1987; Tofilski & Czekonska, 2004), the bees sealed the queen cells in time that coincide with that of the regular drones, even though they fed them as queens.

Some false queen cells with dead brood inside, remained in the colony for a longer time without being destroyed by bees. Woyke (1971) explained that the destruction of the false cells containing drone larvae is due to the falling of drone larvae to the bottom of the queen cells. He noted that false queen cells with drone larvae are lengthened, probably to hold the falling larva. We also noticed this extension to the tip of some of these false queen cells (Fig. 1 and Fig. 3). We believe that besides falling, worker bees may play a significant role in the destruction of the cells and of the killing of the drone brood inside the cell. False cells that were in contact with bees (wrapped, in a horizontal position, upside down) were torn down while those that were protected in the incubator (20 in total) and in push-in grid cages (12, in total) without bees, produced 14 (70%) and 4 (33.3%) healthy adult drones, respectively. Smith (1961) also observed that a normal adult drone emerged in an incubator from a false queen cell.

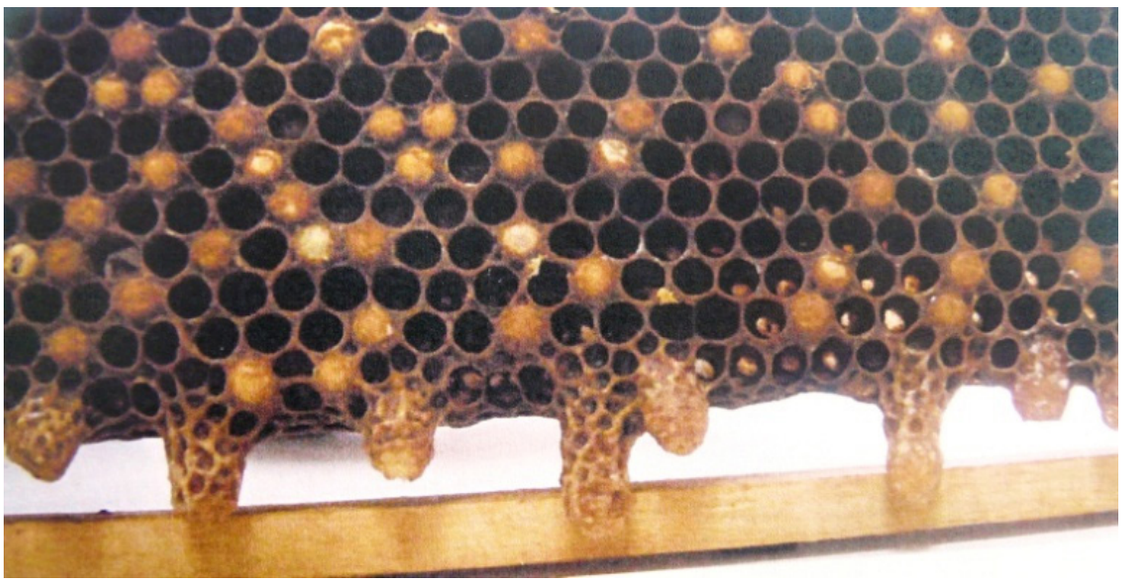

Fig. 3. Natural constructed false queens with drone larvae

The acceptance of drone larvae in queen cells, is not a conclusive evidence that bees do not recognize the sex of the larvae. The nurse bees may finished the queen cells that had been given to them with the larvae inside. But the construction of queen cells over drone larvae in the worker's comb containing unfertilised eggs (Woyke, 1956), and the naturally found queen cells over drone larvae, support the conclusion of Woyke (1956) that bees do not recognise the sex of bee larvae. Earlier beliefs that nurse bees recognise the sex of bee larvae and feed them accordingly (Haydak, 1958; Naulleau, 1962) seems unlikely, at least at the early state of larval development. Haydak (1956) based his assumption on larval food of older drone larvae. Indeed, Sasaki et al. (1995) postulated that bees may be able to identify male larvae at their late state of development (4-5 days of larval age) by their cuticular compounds. Le Conte et al. (1995) showed that 9-day-old larvae produced large amounts of chemical stimulus by which bees recognised the larval age. Whether these stimuli are associated with the destruction of queen cells with drone larvae, remains unknown.

The emerged imagoes had the morphological characteristics of the drones. The earlier hatching of 61% of them, could be attributed to different nutrition but also to the rearing conditions, when compared to the drones that hatched in normal bee colonies. Factors like temperature and humidity could have influenced the development duration. However, the fluctuation of the developmental period is within the natural variability mentioned by Winston (1987). The changes in the diet of the drone larvae affect the morphological characteristics of adults. Drones from false queen cells had smaller wings and legs than regular drones but bigger than regular workers. There were no differences in the number of hooks. Takeuchi, Terunma, & Sakai (1971) also indicated that the nutrition of drone larvae produced by laying workers may alter morphological characteristics like length of tongue, length of forewing, and width of metatarsus. Such changes were also recorded for worker bees by Ruttner (1988). It is well known that royal jelly has active biological substances that directly affect the external features of workers and queens. Recently, it was found that a protein called royalactin is responsible for these changes, as it increases the body size and shortens the development time in honey bees (Kamakura, 2011). In our study statistically significant differences were observed in the morphological characteristics of drones nourished with royal jelly. This could be due to the royalactin or other biological substances that play a significant role in morphogenesis of the evolution stages of male larvae.

Obata and Nonogaki (1965) found that bees supplied queen cells with slightly more royal jelly when drone larvae were grafted instead of worker larvae. In our experiment, we did

not find differences in the quantity of royal jelly even during the fall trial, when bees tend to reject drones. The composition of royal jelly that is produced from false queen cells was similar to that produced in real queen cells. The continuous feeding of drone larvae leads us to consider the earliest hypothesis about communal brood rearing. The drone larvae from the false queen cells were continuously fed while drones in regular drone cells were fed only when they needed it. This suggests that if a signal and response mechanism between larva and nurse workers exists, this is not related to the need of larva for the amount of food present in the cell (Free, Ferguson, & Simpkins, 1989) or to a pheromone that discourages further visits (Simpson, 1966).

The phenomenon of feeding drones in queen cells as if they were queens, can also have a practical use in the production and harvesting of royal jelly by using drones instead of worker larvae for grafting. Although this is not an important impediment to the process, since worker larvae are not in short supply, the biggest drone cells can facilitate a grafting procedure. The knowledge itself can have a practical application in finding suitable larvae for grafting.

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