Original Article

EFFECTS OF QUEEN CELL SIZE AND CAGING DAYS OF MOTHER QUEEN ON REARING YOUNG HONEY BEE QUEENS *APIS MELLIFERA* L.

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Abstract

This study aims to investigate the effect of queen cell size (9.4mm, 9.6mm, 9.8mm and 10.0mm) and mother queen caged time (0 day, 2 days and 4 days) on rearing young queens without grafting larvae. The birth weight, ovarian tubes, thorax length and width were significantly increased with the increasing diameter of queen cell size. The expression level of Vitellogenin (Vg) in young queen ovaries was also up-regulated with the increased queen cell size diameter. These results indicate that the queen cell size can strongly affect the rearing queen quality and reproductive ability. Moreover, the weight, length and width of laying eggs rose with the mother queen caging time, and young queens reared with the hatched larvae from these eggs were also increased in terms of birth weight, ovarian tubes, thorax length and width. Furthermore, the expression level of Vg in reared queen ovaries was also up-regulated with the caged time. These results reveal that the caged time of queens could significantly influence egg size and their relative queen quality.

Keywords: bee queens, queen cell size, imprisoned days of mother queen, quality of reared queen

INTRODUCTION

Queens are vital for the survival of honey bee colonies, not only because of their ability to lay a large numbers of eggs but also because of the social coherence of their pheromones (Amiri et al., 2017). Queens with high reproductive potential produce colonies that exhibit high growth and survival (Rangel, Keller, & Tarpy, 2013). Beekeepers have developed techniques to rear large numbers of young queens and typically replace their old queens, because of the critical importance of a vigorous queen to colony survival and productivity (Büchler et al., 2013; Amiri et al., 2017). When rearing young honey bee queens, 1st star larvae are usually grafted into queen cells and are inserted into a queenless colony. However, the quality of reared queens are affected by many such factors as genetic background, rearing season, queen breeding methods and the age of larvae used for rearing queen (Gilley, Tarpy, & Land, 2003; Koç & Karacaoglu, 2004; Liu et al., 2011; Zhang et al., 2013; Hatjina et al., 2014; He et al., 2017). Beyond that, external environmental factors also affect the quality of reared queens, such as the intensity of the colony, food-storage and weather (Nabors, 2000; Liao et al., 2016).

Queen cells are used to rear new queens in a honeybee colony and the natural size of the swarming queen cells of *Apis mellifera* ranges between 8-10 mm. Büchler et al. (2013) claimed that queen cells should be 8-9 mm in diameter at the rim and the most common diameter of a plastic queen cell for queen rearing s is about 9.4mm. However, a cell could be 9.6, 9.8 or 10.0 mm, the size has been reported to affect queen-worker differentiation (Shi et al., 2011). Moreover, when the mother queen was prohibited to lay eggs for a few days in a queen cage and released for laying, increased weight and size of laid eggs were observed,

which then developed into better quality queens with more ovarian tubes (Liu et al., 2012; 2014). We reared young queens with different queen cell sizes in the same colonies at the same time to determine the effect of different size of queen cell on the quality of reared queens. Meanwhile, we reared queens with hatched larvae from the first eggs which had been laid by the mother queen who was forbidden laying in queen cage with different caged time and released for laying, to determine the effect of caged time of mother queen on the quality of reared queens.

MATERIAL AND METHODS

Experimental honey bee colonies

With the use of standard beekeeping techniques, the *Apis mellifera ligustica* colonies were kept at the Honey-bee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46 °N, 115.49 °E).

Rearing queens

According to the new method for queen rearing without larvae grafting as described by Pan QZ et al. (2013), the mother queen was restricted from laying eggs for six hours on a built-up comb and then was removed to a normal comb. After three days, the eggs hatched into larvae and were inserted into queen cells with an inner diameter of 9.4 mm, 9.6 mm, 9.8 mm and 10.0 mm, respectively. The larvae-containing queen cells were then inserted into queen rearing frames according to the layout shown in Fig. 1. Next two frames were placed in a breeding colony to be further looked after by nurse bees for rearing queens, and so the effect of queen cell distribution was avoided. When the newly reared queens emerged, their birth weight, ovarian tube number, thorax length and width, and Vitellogenin (*Vg*) expression level in ovaries were tested. The effect of queen cells with different diameters on the quality of rearing queens was analysed to determine the best inner diameter. All the experiments were replicated three times on three bee colonies.

Queens were caged and prohibited for laying eggs for four days, and released to lay eggs on the built-up comb for six hours, and then removed to a normal comb. At the same time, thirty newly laid eggs on the built-up comb were immediately measured for their weight, length and width with an analytical balance and microscope system, while the built-up combs with the remaining newly laid eggs were transferred to the hatching area without the queen. Three days later the supporting devices with newly hatched larvae were inserted into queen cells with an inner diameter of 10.0 mm and then were added to the breeding colonies for queen rearing. The birth weight, thorax length and width, ovarian tubes numbers and the *Vg* expression level of reared queens were measured. Similarly, the mother queens were kept in a queen cage and forbitten to lay eggs for 0-2 days and released to lay eggs for six hours. The weight, length and width of newly laid eggs were recorded and new queens were reared with the hatched larvae from the remaining newly laid eggs with the same method. When the new queens emerged, their birth weight, ovarian tube number, thorax length and width

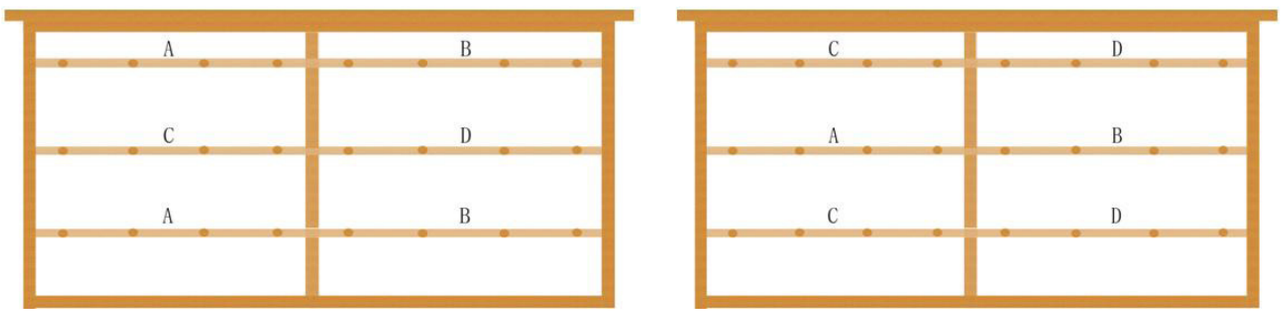

Fig.1 Layout of different queen cell diameters in the queen rearing frames. A, B, C and D indicate a queen cell with a diameter of 9.4, 9.6, 9.8 and 10.0 mm respectively and the points represent the position of queen cells.

Table 1.

Gene-specific primers used in real time quantitative PCR

Target gene	Type Name	Primer sequence (5'-3')
Vg	Forward Primer	CGCATCACGAATACGACTAAGA /
	Reverse Primer	ACGCTCCTCAGGCTCAACTC
β-actin	Forward Primer	GCTGGTTTCATCGATGGTTT/
	Reverse Primer	ACGATTTCGACCACCGTAAC

and the *Vg* gene expression level in reared queen ovaries were measured**.** The effect of caged time of mother queen on the quality of newly reared queens *Apis mellifera* L. with the best queen cell diameter was analysed.

The birth weight

On the first day of emerging, queen cells were transferred to a box of at a constant temperature of 35°C and humidity of 80%. The birth weight of twelve newly emerged queens in each group was weighed with an electronic scale.

Ovarian tubes numbers

Nine newly emerged queens from each group were starved for five hours and their abdomens were removed with scissors. The abdomens were fixed in 10% paraformaldehyde for four hours. The ovarian tissues were then taken out and fixed in 10% paraformaldehyde for twenty hours with embedding cassettes, washed with phosphate buffered saline (PBS), dehydrated in ethanol and embedded in paraffin. Five-micrometer sections were cut on a microtome and after the removal of paraffin stained with hematoxylin & eosin (H&E) for general morphology. After staining, the slides were analyzed with a light microscopy and the ovarian tubes were counted (Gan, Tian, & Yan, 2012; Zhang et al., 2015).

Thorax length and width

After the ovarian tissues were taken out, the queen's head and abdomen were removed with scissors and the thorax length and width of the queens were measured.

The expression level of Vitellogenin gene

The ovarian tissues of nine newly emerged queens in each group were taken out with scissors and rinsed with DEPC water. The ovarian tissue was put into a 1.5 mL EP tube after being rinsed, and kept in liquid nitrogen until used for RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA with Prime Script™ RT Master Mix kit (TaKaRa). Real-time quantitative PCR was performed on real-time PCR system with the Real Time SYBR master mix kit. Gene-specific primers were listed in Tab. 1 and the β-actin gene was used as an internal control. All samples were analyzed in triplicate.

Data analyses

Data was analyzed by analysis of variance (ANOVA) through StatView (v 5.01, SAS Institute, Gary, NC, USA). Multiple comparisons of the means were carried out using Fisher's protected least significant difference only after ANOVA showed a significant effect ($P < 0.05$). Data are Means ± SD.

RESULTS

Effect of queen cell diameter on birth weight, ovaries and thorax length and width of reared queens

The results showed that the method of queen rearing without grafting larvae is feasible and more than 85% of larvae were accepted. As shown in Tab. 2, birth weight, ovarian tube number and thorax length and width were all significantly increased with larger queen cell diameters ($P<0.05$).

Effect of queen cell diameter on expression level of Vg gene in reared queen's ovarian tissues

As shown in Fig. 2, the Vg expression level in reared queen ovarian tissues was higher as the queen cell size increased.

Table 2.

Effect of queen cell diameter on birth weight, ovaries and thorax length and width of reared queens

Queen cell diameters（mm）	Birth weight (mg) N=12	Ovaries N=9	Thorax length (mm) N=9	Thorax width (mm) N=9
9.4	194.83 ± 12.25^{a}	201.00 ± 6.87^{a}	4.52 ± 0.05^{a}	4.38 ± 0.07^{a}
9.6	215.33 ± 21.45^{b}	213.83 ± 6.74^{b}	4.61 ± 0.04^{b}	4.48 ± 0.03^{b}
9.8	231.50 ± 6.63^{c}	237.17 ± 7.03^{c}	4.69 ± 0.04^{c}	4.59 ± 0.03^{c}
10.0	247.67 ± 6.71^{d}	251.33 ± 6.65^{d}	4.79 ± 0.07^{d}	4.69 ± 0.03^{d}

Note: Data are reported as mean±SE. Values in the same column with different letter superscripts indicate significant differences (P<0.05). The same notation is used in Tab. 3 and Tab. 4.

Table 3.

Effect of mother queen caging days on weight and dimensions of laid eggs

Caged days （d）	Weight of laid eggs （μg）N=30	length of laid eggs （mm）N=30	Width of laid eggs （mm）N=30
0	160.17 ± 2.48^{a}	1.58 ± 0.01^{a}	0.30 ± 0.02^{a}
2	177.33 ± 2.34^{b}	1.62 ± 0.02^{b}	0.32 ± 0.01^{b}
4	183.33 ± 4.63^{c}	1.71 ± 0.01^{c}	0.33 ± 0.02^{c}

Table 4.

Effect of mother queen caging days on birth weight, ovaries and thorax length and width of reared queen

Caged days（d）	Birth weight (mg) N=12	Ovaries N=9	Thorax length (mm) N=9	Thorax width (mm) N=9
0	238.60 ± 6.80^{a}	225.57 ± 6.63^{a}	4.67 ± 0.04^{a}	4.56 ± 0.07^{a}
2	248.00 ± 4.53^{b}	249.00 ± 7.28^{b}	4.74 ± 0.06^{b}	4.68 ± 0.06^{b}
4	262.20 ± 4.87^{c}	264.29 ± 4.68^{c}	4.84 ± 0.03^{c}	4.79 ± 0.04^{c}

Effect of mother queen caged days on weight, length and width of laid eggs

As shown in Tab. 3, an increase in the number of days that the mother queen was caged was associated with a significant increase in the weight and dimensions of the laid eggs (P<0.05).

Effect of mother queen caging days on birth weight, ovaries and thorax length and width of reared queen

As shown in Tab. 4, birth weight, ovarian tubes number and thorax length and width all significantly increased for the greater duration of mother queen caging (P<0.05).

Effect of mother queen caging days on *Vg* gene expression level in reared queen's ovarian tissues

As shown in Fig.3, increasing the number of mother queen-caging days led to an increase in the Vg expression level in reared queen ovarian tissues.

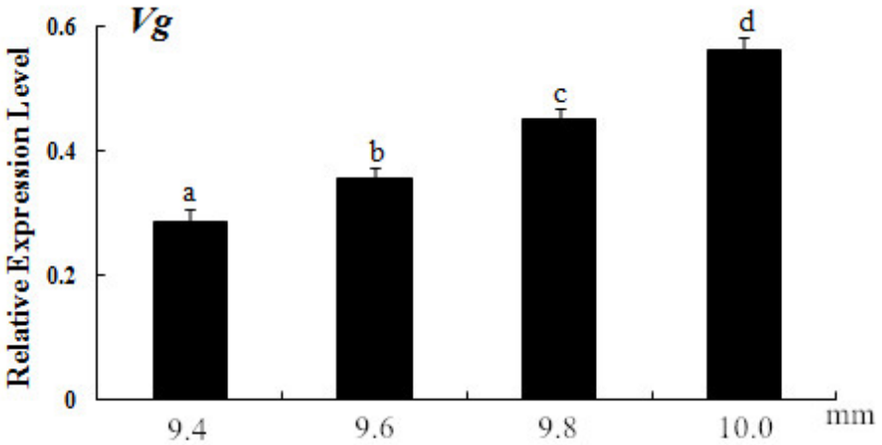

Fig. 2 Effect of queen cell diameter on gene expression of Vg in queen's ovarian tissues. Histogram shows the relative Vg expression level. Each bar corresponds to a single group and shows the mean±SE of three biological replicates. N=9. Different letters above bars mean a significant difference between groups ($P < 0.05$). The same as below.

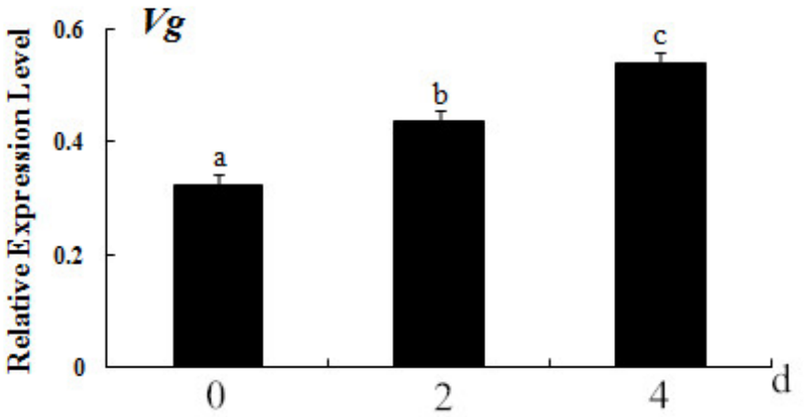

Fig. 3 Effect of mother queen caging days on Vg gene expression in queen's ovarian tissues. N=9.

DISCUSSION

Queen weight at emergence, thorax length and width, ovarioles and Vg expression level in queen ovarian tissues are critical physical characteristics for evaluating the quality of honey bee queens (Tarpy et al., 2012; Nunes et al., 2013; Amiri et al., 2017). The experimental results showed that birth weight, ovarian tubes number, thorax length and width and Vg expression level in ovarian tissues of reared queens all significantly increased when larger-diameter queen cells were used. The newly reared queens with 10.0 mm queen cells were clearly better than those reared with the other three cell sizes. 10.0 mm cells found to be better and the natural swarming cells were between 8.0-10.0 mm. The best swarming queens probably came from the 10.0 mm diameter cells. This might be because the larger queen cells had more space, which promoted rearing queen development and also the reared queen's birth weight, ovarian tubes number and other measured dimensions increased significantly. Shi et al. (2011) found different size of nest can significantly affect the DNA methylation level of larvae, affecting the Dnmt3 enzyme activity which plays an important regulating role in the development of larvae.

At the same time, we observed that the height of the royal jelly in different size queen cells was the same. The height of royal jerry is probably information for nurse bees to feed the larvae. So the 10.0 mm queen cell could contain more royal

jelly than the other three groups because of the large size cell. The nutrition and the amount of food given to young larvae cause the caste determination of worker and queen regulated developmental pathways. The more the larvae are given, the better the reared queen would be (Kucharski et al., 2008; Maleszka, 2008; He et al., 2017). However, when the larvae had enough royal jelly for food, the quality of food was very important for the development of larvae. With the increase of larvae, the food intake also increased. The bigger the queen cells were, the more fresh royal jelly was given. So the older larvae in bigger queen cells had enough fresh royal jelly for food everyday, while the older larvae in smaller queen cells did have enough who went on to ingest the remaining jelly left before. Guo et al. (2015) found that the composition of royal jelly from different harvesting times differed. This study found that queen cell diameter affects reared- queen growths which may be due to increased development space and more abundant food, but the specific molecular mechanism still requires further research.

Our results also showed that the weight, length and width of laid eggs significantly increased with an increase in mother queen-caging time, while birth weight, ovarian tube number and other dimensions of reared queens with larvae hatched from eggs also increased significantly. The quality of eggs or larvae affects the quality of reared queens (Woyke, 1971; Chuda-Mickiewicz & Prabucki, 1998). The results suggest that caging lets the queen lay larger eggs and thus the hatched larvae from the eggs are reared as better new queens. The longer the queen was caged, the more it accumulated such nutrients in the body as vitellogenin and other antioxidant enzymes (Alaux et al., 2011). Queen-delaying oviposition causes eggs to be bigger with more yolk protein (Torres, 1980). When the caged queen is released, her first eggs are large, and the quality of a queen reared from such eggs are better. We have shown that caging the queen can be used to increase the quality of fertilized eggs and improve the quality of queens reared from eggs laid by a caged queen.

In conclusion, our results indicate that in the conditions of our rearing colonies caging a mother queen for two to four days and then releasing increases the quality of eggs, which is conducive for queen rearing. When rearing new queens, we should use the bigger queen cell whose diameter is about 10.0 mm, which can increase the quality of reared queens.

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