




Royal jelly production with queens produced by single and double grafting in Africanized honeybee colonies

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ABSTRACT. The objective of this research was to evaluate royal jelly production from Africanized honeybee queens of different lineages (lineage selected for honey production, lineage selected for royal jelly production and unselected) produced by single and double grafting and to compare royal jelly production among their offspring. Data were tested by double factorial analysis of variance and the means were compared by Tukey test at 5%. The parameters evaluated were: queen weight at emergence, percentage of larvae acceptance in the upper and lower bars, royal jelly per cup (mg) and royal jelly per colony/collection (g). Queens selected for honey presented greater weight at emergence, while the unselected queens were the lightest. Double grafting was better than simple grafting, since the queens were born 2.38% heavier. There was no difference ($p > 0.05$) in relation to royal jelly production according to neither the lineage nor even comparing the method by which the queens were produced. The cost of labor to produce queens by double grafting was much higher, as by the simple grafting about 170% more queens were born. The potential of Africanized honeybees with adequate production management and favorable environmental conditions favors the production of royal jelly.

Keywords: queen selection; manipulation; weight at emergence; genetic parameters; queen production.

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Introduction

To improve genetic parameters raising the quality of animal production, genetic selection and breeding become essential tools to achieve the goal. Among the methods used for genetic improvement, molecular markers are widely used to genetically map similarity between individuals. Stuchi, Toledo, Lopes, Cantagalli, and Ruvolo-Takasusuki (2014) used molecular markers to distinguish two species of *Tetragonisca* Moure, 1946 bees, demonstrating that EST-1, EST-2 and EST-3 are efficient markers for differentiating these species. Baitala, Nakasugui, Cantagalli, Toledo, and Ruvolo-Takasusuki (2013) observed that in order to establish a genetic breeding program with Africanized honeybees (*Apis mellifera scutellata* Lepeletier, 1836) for royal jelly production, the polymorphism of MRJP proteins can be used as a molecular marker. Baitala et al. (2010) found that selection of individuals for high royal jelly production can be performed using the loci *mrjp3*, *mrjp5* and *mrjp8*, since the three loci produced 17 alleles, indicating high allelic polymorphism for the loci studied.

Therefore, knowing the genetic resources of the selection candidates and using the tools of computer science help in this process (Khan, Matos, & Lima, 2009); it is not enough to have knowledge of the individuals, being also necessary to know the environmental variables that can affect their performance. Schafaschek, Hickel, Pereira, Oliveira, and Toledo (2016) found that local queens presented better performance for honey storage than queens introduced from other regions, possibly because queens introduced from other regions were brought virgin and were not acclimated to the environment, which ultimately affected their productive performance.

In continents or countries where there is more than one subspecies of honeybees, although they may be morphologically similar, their adaptations to the same environment may be completely different. Alqarni, Balhareth, and Owayss (2013) found morphological and reproductive differences in *Apis mellifera jemenitica*

Ruttner, 1976 and *Apis mellifera carnica* Pollmann, 1879 queens in Saudi Arabia, where the first subspecies is endemic and possibly its reproductive characteristics allowed for better adaptation to local conditions.

The queen weight at emergence is an important feature for genetic improvement in honeybees. Queens with undeveloped ovaries have a smaller volume of abdomen and are lighter, thus they are easier to fly (Harano, Sasaki, & Sasaki, 2007), which facilitates mating and fertilization. Berger, Poiani, and Cruz-Landim (2016) reported that the contact of the virgin queen with the workers within the colony is fundamental for complete ovarian development and therefore the queen bank was the most appropriate method to maintain the queens without harming their fertility. The same authors noted that there is a more rapid decline in queen fertility when they are kept in BOD incubators, probably due to the lack of pheromone interaction. Success in modern beekeeping depends on the use of computer tools, genetic selection, and proper colony management. Beekeepers are increasingly becoming professionalized and seeking to increase productivity. Among the management techniques used for queen production, single and double grafting were long compared in terms of quality of emerged queens, but the published works are conflicting. Montagner (1962) reported that queens produced by double grafting presented heavier weight at emergence and more ovarioles in each ovary, while Delaplane and Harbo (1988) concluded that double grafting did not produce heavier queens by comparing weight at emergence with queens produced by the single grafting method.

Few investigations were conducted to evaluate the performance of queens produced by single and double grafting for Africanized honeybees. Based on this, the objective of the present study was to evaluate royal jelly production of Africanized queens (*Apis mellifera scutellata* Lepeletier, 1836) of different lineages, produced by single and double grafting and to compare the production of royal jelly among their offspring.

Material and methods

The research was carried out at the Experimental Farm of Iguatemi of the Universidade Estadual de Maringá (FEI – UEM), in the Beekeeping Sector, located at 554.9 m altitude, at the following geographical coordinates: 23° 25' South latitude and 52 20' of West longitude, from June to December 2016.

In the first stage, three groups of queens of different genetics were selected. The first group consisted of a royal jelly producer lineage while the second group was composed of a honey producer lineage; both lineages were selected in the same apiary of this study since 2003 and 2010, respectively. The third group consisted of queens without genetic selection obtained from colonies collected in the northwest state of Paraná, Brazil. Therefore, there were two genetic groups from the same locality and another one of different origin from random locations.

In the second stage, F1 queens, daughters of the queens chosen above were produced. For the development of the F1 generation, 18 vertical mini-hives, initiator and terminator were used, each with two nucs of five overlapped combs separated by a queen excluder screen between the nucs. In the upper nuc of each mini-hive it was placed a frame containing 45 acrylic cups in which each bar had 15 cups. Ten days after larvae grafting, queen cells were removed from the mini-hives, placed in glass bottles of 20mL and the genealogy identified. Afterwards, they were placed in a BOD incubator with a mean temperature of $34 \pm 2^\circ \text{C}$ and relative humidity of $60 \pm 10\%$. The emergence of queens was followed up and the schedules noted to check their weight at emergence. The newly emerged queens were anesthetized with CO_2 and the live weight measurements (mg) were recorded on a digital scale accurate to 0.0001 g. After checking the weights, the queens were marked on the thorax with colors chosen according to the lineage and the method in which they were produced. In addition, they were transferred from the glass vial to JZsBZs type queen cages, along with five to eight nurse worker honeybees as companions. Candy food was provided so that the companions could feed their shelves and feed the queen; a small amount of water was sprayed on the cages once a day.

To produce the F1 queens of step two, 480 larvae were grafted from each of the selected lines at the beginning of the experiment, and from each lineage 240 larvae were grafted by single grafting and 240 by double grafting method, totaling 1440 grafted larvae. The method used for queen production by single grafting was described by Doolittle (1889), which consisted of grafting worker larvae from the comb to acrylic cups containing royal jelly, diluted to 50% in distilled water. The larvae used were from zero to 24 hours old. For the queen production by double grafting, the method described by Montagner (1962) was used, by means of which the larva is removed after grafting acceptance and a new larva with age between zero and 24hours is placed again in the same acrylic cup.

F1 queens born had a selection index of 5%, using weight at emergence as a parameter. Of the total, those which presented the highest body weight at emergence were pre-selected and stored in a BOD incubator with a mean temperature of $34 \pm 2^\circ\text{C}$ and relative humidity of $60 \pm 10\%$. Of the preselected queens, 18 were randomly selected and used to evaluate royal jelly production, six queens of each lineage, so that half was produced by single grafting and the other half by double grafting. That is, 18 colonies of Africanized honeybees *Apis mellifera scutellata* housed in vertical mini-hives. The mini-hives were composed of nine combs, five combs in the lower nuc and four in the upper nuc, plus a cell bar frame with two bars of 33 cups each. The lower and upper nucs were separated by a queen excluder. For supplementation on days of low feed flow, a cover feeder was used.

For the queen introduction into the hives, colonies were orphaned 24 to 72 hours before. Upon introduction, the combs were inspected for the presence of queen cells. The queen cells found were destroyed for the purpose of increasing the acceptance of the queen introduced. After introduction, the acceptance of the queens was confirmed and the laying was verified 12 days after the introduction. The royal jelly production began after 50 days from the start of the new naturally fertilized queen, so all the honeybees present in the colony were daughters of the new queen (Terada, Garofalo, & Sakagami, 1975). As a result, the royal jelly production per colony was evaluated after this period.

For royal jelly production, the process was similar to that of queen production, in which case the process was interrupted 64 – 72 hours after larvae grafting and the royal jelly was collected. To obtain appropriate larvae for grafting, an empty comb was introduced, four days before grafting in the central region of support colonies, used only as donors of newly hatched larvae. After larvae grafting, the cell bar frames were marked with the colony number and carefully returned to their respective mini-hive.

Royal jelly collection was performed from 64 to 72 hours after grafting, using a vacuum pump suction system without manual contact with the royal jelly. The percentages of grafted larvae acceptance (%), royal jelly production per colony (g) and per cup (mg) were evaluated. The royal jelly collected was weighed in a digital scale accurate to 0.0001 g, wrapped in plastic pots and frozen. Then the cell bar frames were returned to the respective colonies for cleaning by the worker honeybees and the next day a new grafting was performed.

After the return of the cell bar frames with the grafted larvae to their respective colonies, energetic supplementation containing sucrose solution at the concentration of 1.46 moles/L was provided, in order to reduce their weakening and to improve the rate of larvae acceptance.

The climatic conditions were obtained through the weather station of the Experimental Farm of Iguatemi. Data of the analyzed variables were tested by double factorial analysis of variance (ANOVA) and the mean values were compared *a posteriori* by Tukey test at 5% significance. The analyses were performed using R statistical software (R Core Team, 2016).

Results and discussion

The mean queen weight at emergence for each lineage, according to the grafting method is listed in Table 1. There was statistical difference for the parameter weight at emergence for lineage and grafting method.

Table 1. Mean values with their respective standard deviations for queen weight at emergence of different genetic lineages and weight at emergence according to the grafting method.

	Genetic	Grafting method	Genetic x grafting method
		Weight at emergence (mg)	
Lineage selected for honey production (n = 152)		(185.30 ± 17.43) ^a	
Lineage selected for royal jelly production (n = 221)		(178.01 ± 20.82) ^b	
Unselected lineage (n = 172)		(173.79 ± 21.11) ^c	
Double grafting (n = 147)		(181.80 ± 19.49) ^a	
Single grafting (n = 398)		(177.57 ± 20.72) ^b	
Queens born in bank hives (Metorima et al., 2015)		165.61	
Queens born in BOD (Metorima et al., 2015)		157.04	

Means followed by different lowercase letters, in the same column, are statistically different by Tukey's test ($p < 0.05$).

Selected queens had a heavier weight at emergence than the unselected queens, and the ones selected for honey and royal jelly were 6.62 and 2.47% heavier, respectively. Probably, the queens with genetic selection were heavier than those not selected because they were part of a breeding program considering the weight at emergence as a selection criterion, which tends to increase the value of this parameter for the individuals. The colonies selected for royal jelly has been selected since 2003, while the honey producing colonies since 2011. Queens without genetic selection showed very large variation of weight within the same group, consequently the F1 generation of this group presented characteristics similar to those of the mother colonies, which reduced the mean weight at emergence. However, there was no difference ($p < 0.05$) for the genetic group interaction and the queen production method. The values of weight at emergence found in this study were higher than those found by Meteorima, Costa-Maia, Halak, Parpinelli, and Toledo (2015). Regarding the method of production, the double grafting method generated queens, on average, 2.38% heavier when compared to single grafting queens. In the present study, double grafted queens presented heavier weight at emergence due to the greater amount of food available for these larvae, because after the first grafting, the old larva was removed and another newly hatched was placed in the place, in a cup already containing a significant amount of royal jelly, unlike single grafting larvae, which are placed in cups containing royal jelly diluted in water (1: 1).

Table 2 presents the actual royal jelly production data according to the genetic lineage of the queens, and there was no difference ($p > 0.05$) for any of the evaluated parameters.

The smaller number of larvae accepted in the double grafting allowed the accepted larvae to be fed with more food, increasing the weight at emergence. Of the total of grafted larvae, the double grafting method generated only 147 queens, while in the single grafting, 398 individuals were produced, representing 170.75% more queens produced by the single grafting. Consequently, double grafting method increased labor and cost of production, since it requires larvae grafting to be performed twice, not just one, as in single grafting.

Alber (1965) observed that double grafting larvae received more attention from nurse honeybees and that this difference was greater over time, and some single grafting larvae were even neglected by nurse honeybees. Delaplane and Harbo (1988) found no difference in weight at emergence by comparing single and double grafting methods, however, they concluded that the use of diluted royal jelly in the cups increased the acceptance of the larvae in relation to the larvae grafted to cups without food.

Honeybee nutrition has become increasingly important in beekeeping worldwide and with this several works have appeared to improve the colony performance or to find substitutes for nectar and pollen for periods of food shortage. Kamakura (2011) observed that honeybee larvae fed royalactin protein had more developed ovaries and heavier body weight, besides shortening the period of larval development when compared to larvae that received diets containing 450-kDa protein and casein.

Table 3 lists mean values for percentage of accepted larvae, royal jelly per bar, royal jelly per cup and royal jelly per colony/collection according to the method by which queens were produced. There was no difference ($p > 0.05$) for any of the analyzed parameters.

Table 2. Mean values with their respective standard deviations for percentage of accepted larvae, royal jelly per bar, royal jelly per cup and royal jelly per colony/collection according to the genetic lineage.

	Lineage selected for royal jelly production (n = 6)	Lineage selected for honey production (n = 6)	Unselected (n = 6)
Percentage of larvae accepted in the upper bar	(41.91 ± 19.39) ^A	(39.21 ± 18.09) ^A	(40.91 ± 16.52) ^A
Percentage of larvae accepted in the lower bar	(40.91 ± 13.88) ^A	(36.94 ± 14.42) ^A	(37.36 ± 14.82) ^A
Royal jelly in the upper bar (g)	(1.98 ± 1.08) ^A	(1.77 ± 0.92) ^A	(1.77 ± 0.79) ^A
Royal jelly in the lower bar (g)	(1.95 ± 0.89) ^A	(1.72 ± 0.73) ^A	(1.70 ± 0.79) ^A
Royal jelly per cup in the upper bar (mg)	(127.82 ± 0.05) ^A	(139.92 ± 0.05) ^A	(127.36 ± 0.05) ^A
Royal jelly per cup in the lower bar (mg)	(137.80 ± 0.05) ^A	(143.31 ± 0.04) ^A	(143.15 ± 0.05) ^A
Royal jelly per colony/collection (g)	(3.93 ± 1.63) ^A	(3.49 ± 1.43) ^A	(3.47 ± 1.38) ^A

Means followed by equal uppercase letters in the same row do not differ statistically by Tukey's test ($p > 0.05$).

Table 3. Mean values with their respective standard deviations for percentage of accepted larvae, royal jelly per bar, royal jelly for cup and royal jelly per colony/collection according to the grafting method, independent of genetic group.

	Single grafting (n =9)	Double grafting (n = 9)
Percentage of larvae accepted in the upper bar	(39.47 ± 17.79) ^A	(41.36 ± 18.18) ^A
Percentage of larvae accepted in the lower bar	(38.30 ± 15.15) ^A	(38.51 ± 13.67) ^A
Royal jelly in the upper bar (g)	(1.84 ± 0.96) ^A	(1.84 ± 0.93) ^A
Royal jelly in the lower bar (g)	(1.78 ± 0.87) ^A	(1.79 ± 0.74) ^A
Royal jelly per cup in the upper bar (mg)	(135.79 ± 0.06) ^A	(127.62 ± 0.05) ^A
Royal jelly per cup in the lower bar (mg)	(141.06 ± 0.05) ^A	(141.74 ± 0.04) ^A
Royal jelly per colony/collection (g)	(3.63 ± 1.53) ^A	(3.63 ± 1.47) ^A

Means followed by equal uppercase letters in the same row do not differ statistically by Tukey's test ($p > 0.05$).

The mean value throughout the experiment for the percentage of accepted larvae was 39.5%. Similar value was reported by Mouro and Toledo (2004), who obtained a mean of 35.8% for Africanized honeybees and 55.5% for Carniolan honeybees. In the present study, royal jelly production per cup and royal jelly production per colony/collection were 136.56 mg and 3.63 g, respectively. These values were higher than the 29.2% larvae acceptance and 1.83 g royal jelly/colony/collection reported by Toledo, Alves, Oliveira, Ruvolo-Takasusuki, and Faquinello (2010), working with Africanized honeybees without selection. However, the amount of royal jelly per cup observed by these authors was approximately 56% higher than in the present study.

During the analyzed period, royal jelly production per colony/collection presented little variation, possibly because the climatic factors remained practically the same during the collection stages. Although one of the groups consisted of colonies of selected lineage to produce royal jelly, there was no difference in the production components of the royal jelly according to the queen's lineage. There was probably no difference ($p > 0.05$) in the royal jelly production because there was also no statistical difference in the percentage of accepted larvae after grafting and in the amount of royal jelly deposited per cup. Although the amount of royal jelly per cup was low, there was good acceptance of larvae, which increased the total amount of royal jelly produced, confirming the high genetic correlation (0.42) found by Faquinello et al. (2011) for these two characteristics. The accepted larvae (39.5%) can be explained due to the process of selection of the colonies producing honey and royal jelly, together with the environmental conditions that were provided to the colonies. Some important factor was missing so that the genes of these honeybees could be expressed in their fullness. Toledo and Mouro (2005) also found no difference in royal jelly production between Africanized honeybees selected for honey and for royal jelly. Faquinello et al. (2011) observed that colony internal conditions can influence the royal jelly production as well as environmental variables, so the importance of selecting individuals adapted to local conditions. Moreover, Meixner et al. (2014) verified that mass importations of individuals can harm the genetics of the local population and can still be a vector of pests and disease, but it is important to promote the heterosis and keep the honeybee diversity.

Possibly the queens selected for royal jelly and honey production presented productive parameters similar to queens without genetic selection because the double grafting larvae were removed 72 hours (Montagner, 1962) after the first grafting and did not with 20 to 30 hours. Delaplane and Harbo (1988) observed that Africanized queens produced by double grafting were born heavier and had a longer life span. On the selection of queens for royal jelly production, Parpinelli, Ruvolo-Takasusuki, and Toledo (2014) reported that when queens are selected for this trait presented the alleles C, D and E of locus *mrjp3* more frequently. In addition, a repetitive region of this locus indicated an importance of having a queen with good genetic quality for high royal jelly production, since the offspring will inherit this same characteristic (Baitala et al., 2010).

Although there are specific lineages for high royal jelly production in China, in some regions of the country, climatic factors prevent royal jelly production for much of the year, so many beekeepers chose to use hybrid queens, selected for both royal jelly and honey production allowing to increase the diversity of products produced by the colony (Cao, Zheng, Pirk, Hu, & Xu, 2016).

Conclusions

Double grafting queens present heavier weight at emergence than single grafting queens. However, regardless of the lineage and method by which the queens were produced, royal jelly production is similar.

This shows that selected lineage of Africanized honeybees is probably more demanding than unselected honeybees and therefore require a more favorable environment to be able to express their potential, such as protein food supplementation during the production period.

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