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Horizontal and vertical colonies for royal jelly production in Brazil

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ABSTRACT - The potential of royal jelly production in Africanized honeybee colonies was evaluated using techniques involving two queens per colony in horizontal and vertical systems during the spring of 2019. The techniques were tested for their effect on cell acceptance (%); royal jelly production per colony (g), per harvest (g), and per position of cell bar (g) and compared with standard techniques (five and ten-frame hives) in southern Brazil. Results showed statistical significance in royal jelly production between methods. Vertical colonies produced a significantly greater amount of royal jelly per colony and per harvest (8.26 and 53.28±4.98 g) compared with single-queen colonies in ten-frame hives (4.30 and 32.76±3.57 g) and five-frame hives overlapping (2.03 and 14.45±2.48 g), but did not differ from two-queen horizontal colonies (8.09 and 46.81±4.90 g). In contrast, there was no significant difference in queen cell acceptance rate within vertical, horizontal, and ten-frame colonies. Royal jelly yield of two-queen vertical colonies increase as compared with standard colonies.

Keywords: five-frame nucleus hive, horizontal colony, ten-frame Langstroth hives, vertical colony

1. Introduction

Bee products have been used for millennia as food and medicine because of their nutraceutical properties and high nutritive value (Kuropatnicki et al., 2018; Ramanathan et al., 2018). In addition to honey, products such as beeswax, bee pollen, and royal jelly are highly coveted for possessing cosmetic and health-promoting compounds. Nevertheless, the demand for these ingredients far exceeds supply (Zheng et al., 2018).

Royal jelly is a complex compound containing nutrients such as water, proteins, sugars, lipids, minerals, water-soluble vitamins, and free aminoacids among others (Wytrychowski et al., 2013), and is produced by the hypopharyngeal and mandibular glands of nursing bees (Rembold and Dietz, 1966). Factors such as colony strength, food resources, temperature, humidity, age of grafted larvae, tools, and hive management techniques are key to obtaining a good yield of royal jelly (Li, 2000).

Management techniques with multiple queens (polygyny) in a colony have been implemented in many apiaries to maintain strong colonies throughout the year and used in production or as support for the rapid growth of other colonies to increase royal jelly production (Cao et al., 2016). Polygyny may occur naturally in *Apis mellifera* colonies when virgin queens are prepared for breeding swarms or when the queen will be replaced (Gilley and Tarpy, 2005).

Experimentally, it is possible to maintain colonies with multiple queens by using queen excluders to avoid aggressive behavior and consequent queen losses, or by mandible ablation of egg laying queens older than six months so that they can cohabit the same nest (Human et al., 2013). Two-queen systems designed to manage and increase honey production have been used extensively on a small scale, but have not been adopted by large commercial beekeepers. Horizontal and vertical colonies used in honey production result in 60 to 75% higher production when compared with ten-frame hives and a single queen; lower production cost, time, and equipment used per kg honey but extra labor is essential, and it may be difficult for some beekeepers to find qualified assistance (Peer, 1969; Walton, 1972; Tegart, 1984).

On the other hand, the method of mandible ablation has been used for more than ten years in China, and research shows that queens did not kill each other and coexisted peacefully, and given proper management, the advantages of multiple queen colonies such as providing larvae for royal jelly production, capped brood for the rapid build-up of production colonies, and workers for package bees outweigh the amount of work needed for their maintenance (Dietemann et al., 2008; Zheng et al., 2009).

Other advantages of these techniques include higher foraging activity, disease resistance, better division of labor among worker honeybees, higher production efficiency, and high egg laying by the queens, resulting in strong colonies (Gris Valle et al., 2004; Zheng et al., 2009; Delaplane, 2015; Hesbach, 2016). Despite the fact that systems with two or multiple queens can improve honey bee colony performance, there are no published studies reporting the use of these systems in Africanized bees in Brazil, and researchers report only the behavior of Italian breed (*Apis mellifera ligustica*) in countries such as China, New Zealand, Canada, the United States, and Mexico.

We tested the hypothesis that the use of two queens influences the royal jelly productive performance of Africanized colonies. Thus, the objective of this research was to analyze the effects of vertical and horizontal management systems on royal jelly production and compare them with the standard systems of Brazil (ten-frame hives and five-frame nucleus overlapping).

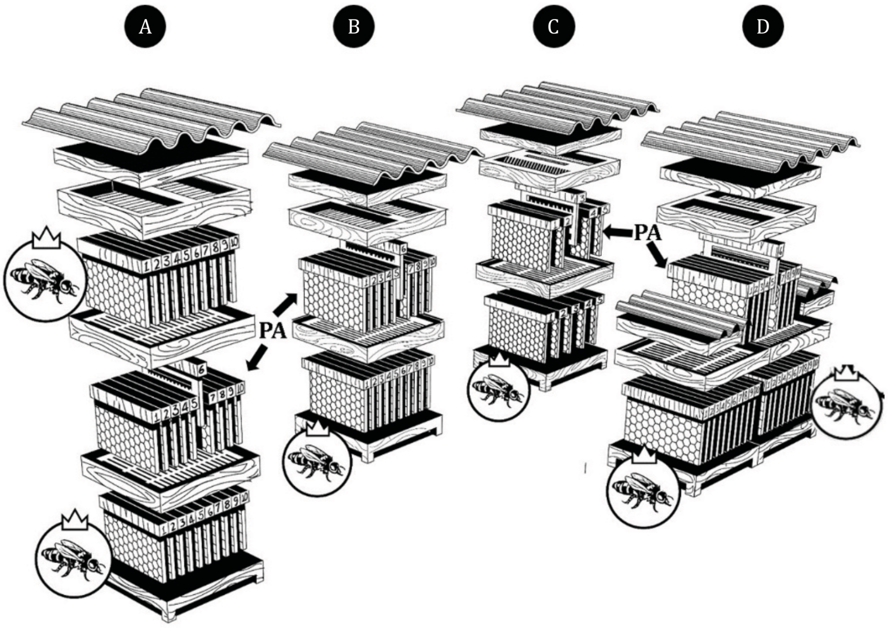
2. Material and Methods

This study was carried out in Maringá, Paraná, Brazil (23°21' south, 52°04' west, and average altitude of 555 m).

Twenty-four experimental colonies were established in Langstroth beehives and divided into four groups: two-queen vertical colonies housed in three ten-frame hives separated by excluders (Gris Valle et al., 2004; Delaplane, 2015); two-queen horizontal colonies housed in three ten-frame hives forming a pyramid separated by excluders (Hesbach, 2016); ten-frame hive colonies with one queen and two ten-frame hives separated by an excluder; and the last group (five-frame hives) comprised colonies with one queen and two five-frame nucleus hives separated by a queen excluder (Figure 1). One month before starting the experiment, the two-queen colonies were established (Farrar, 1958).

Colonies of the breeding program for high royal jelly production (Baitala et al., 2010) were used for reared queens before the start of treatments. Reared queens were introduced into experimental colonies for natural mating. The colony structure was divided into a propagation area (with a queen) and a production area (without a queen) (Hu et al., 2019). Twenty-four hours before grafting, beehives were managed and standardized; the production area consisted of nine combs, including two honeycombs, two bee bread combs, three sealed brood combs, and two open brood combs (Figure 1 – PA), and the propagation area consisted of three honeycombs, two bee bread combs, two open brood combs, two sealed brood combs, and one empty comb for egg laying (Figure 1 – Queen). Subsequently, this management was undertaken every ten days.

Two royal production harvests were made per week during the spring period of 2019 by using a modified Doolittle (1889) method, which consisted of transferring larvae between 12 and 24 h of age from the comb to commercial plastic cells. Before grafting, the grafting frames were dipped in sugar



A - vertical colonies; B - single-queen colonies housed in a ten-frame nucleus hive; C - single-queen colonies housed in five-frame hives; D - horizontal colonies.
Combs 1 and 10: honeycombs; 2 and 7: bee bread combs; 3, 4, and 9: sealed brood; 5 and 8: open brood; 6: grafting frame in the production area (PA); Queen: empty comb for egg laying in the propagation area.

Figure 1 - Design of the treatments and internal view of the colony.

syrup and given to experimental colonies so that the bees would clean and prepare them for larvae. A grafting frame of three bars carrying 33 artificial cell cups was used and positioned in the middle of the production area. Hive systems were supplemented in each grafting cycle with 40 g of protein feed with a mixture of linseed oil, palm oil, isolated soy protein, brewer's yeast, honey, sugar, pollen, lecithin, and vitamin nucleus developed by Sereia et al. (2013) and 600 mL sugar syrup (1:2).

At 68-72 h after grafting, the grafting frames were removed from the colonies. The ring of wax at the opening of each cell was removed to expose the queen larva and royal jelly. Each larva was removed, and the royal jelly extracted, stored, and frozen in 50 g plastic jars.

Data normality was tested using the Shapiro–Wilk normality test, and to verify the homogeneity of variances Bartlett's test was used. When these assumptions were satisfied, an analysis of variance (ANOVA) procedure using a randomized block experimental design was performed using cell acceptance and royal jelly yield as the dependent variables, and vertical management, horizontal management, ten-frame Langstroth hives, and five-frame Langstroth nucleus, with six replicates each, as the independent variables.

The following statistical model was used in data analysis:

$$\hat{Y}_{ij} = \mu + A_i + B_j + \varepsilon_{ij},$$

in which $\hat{Y}_{ij}$ = observed value of the variable; μ = overall mean; A_i = effect of hive management; B_j = block or harvest; and ε_{ij} = random error associated with each $\hat{Y}_{ij}$ observation.

Cell acceptance (%) was evaluated by counting the number of larvae that were accepted in the colony related to the number of grafting larvae. The royal jelly production per colony (g), per treatment or collection period – three days (g), and per position of cell bar (g) were weighed on a digital scale (Shimadzu AY220, Brazil, accurate to 0.001 g). Cell bars were numbered from one to three to identify their position, with 1 = the bar closest to the brood area and 3 = the farthest bar from the brood area. Grafting and royal jelly harvesting was repeated ten times, each rearing episode consisting of three days between grafting and harvest.

For royal jelly yield per cell bar data, a nested analysis of variance (cell bar nested within the treatments) was used. In cases where the requirement of normality and homogeneity of variance between groups was not met, the non-parametric Kruskal–Wallis test was used to identify differences among treatments. *Post hoc* tests (Tukey's and Dunn's test) were used to detect significant differences among groups. All statistical analyses were performed with R statistical software for Windows (R Development Core Team, 2019).

3. Results

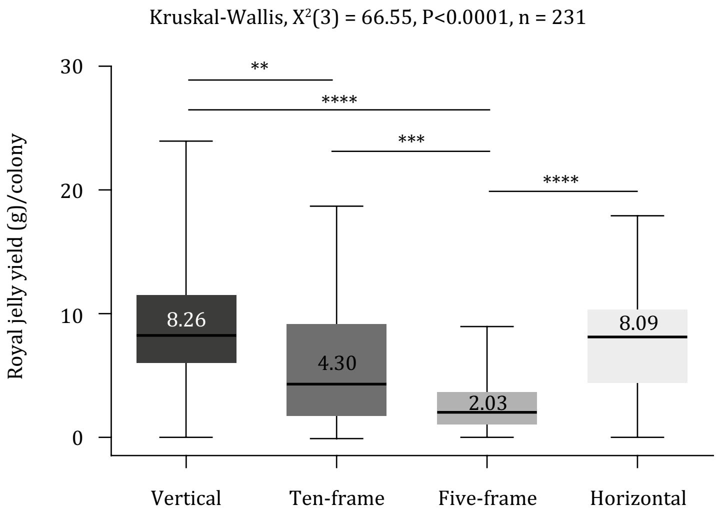
A total of 22869 cells were grafted over the study period, of which 7869 were accepted (34.4%). The Kruskal–Wallis test indicated that there was a difference between the groups for the variables: cell acceptance (X^2 = 46.24, df = 3; P<0.0001) and royal jelly yield per colony (X^2 = 66.55, df = 3; P<0.0001). For cell acceptance (Table 1), Dunn's *post hoc* indicated that the five-frame Langstroth hive group was lower than the vertical (vertical vs. five-frame, Z = 5.83, P<0.0001), horizontal (horizontal vs. five-frame, Z = 6.0, P<0.0001), and ten-frame Langstroth hive groups (ten-frame vs. five-frame, Z = 3.67, P = 0.00145).

Dunn's *post hoc* indicated that royal jelly production was significantly higher on vertical colonies than in five-frame (Z = 7.49, P<0.0001) and ten-frame colonies (Z = 3.59, P<0.0001) (Figure 2). However, colonies in ten-frame hives presented advantages such as easy and fast management, less labor, ease of feeding, and removal and return of the grafting frame. Vertical and horizontal systems were not different, and five-frame colonies produced the least amount of royal jelly.

Table 1 - Hive management for royal jelly production in Africanized bees (Median [$P_{2.5\%}$ – $P_{97.5\%}$] / Mean±SE)

Variable	Experimental group				P-value
	Vertical (A) (n = 6)	Ten-frame hive (B) (n = 6)	Five-frame nucleus hive (C) (n = 6)	Horizontal (D) (n = 6)	
Cell acceptance (%)	41.41a* [3.51–80.47]	28.28a [0.35–83.38]	15.15b [0.38–42.04]	43.43a [2.98–88.46]	<0.0001
Royal jelly yield/harvest (g)	53.28±4.98a#	32.76±3.57b	14.45±2.48c	46.81±4.90ab	<0.0001

* In the row, different letters show significant differences between them (Dunn's test, P<0.05).
# In the row, different letters show significant differences between them (Tukey's test, P<0.05).
Letters inside black circle indicate labels from Figure 1.



Dunn's test: **** P≤0.0001; *** P≤0.001; ** P≤0.01.

Figure 2 - Royal jelly yield by experimental group.

Royal jelly produced per collection period was significant (F = 21.64, P<0.0001), and Tukey's *post hoc* test indicated that it was significantly higher in vertical colonies than in single-queen colonies [vertical vs. five-frame (t = 38.82, P<0.0001) and vertical vs. ten-frame (t = 20.52, P = 0.0029)], but did not differ from horizontal management (vertical vs. horizontal, t = 6.47, P = 0.6096). There was no difference between ten-frame and horizontal management (t = −14.05, P = 0.0556) (Table 1).

The position of the cell bar within the colony did not influence the variables as the nested ANOVA did not indicate a difference between groups [acceptance (F = 0.35, P = 0.94) and royal jelly per colony (F = 0.37, P = 0.93)].

An overall decrease in royal jelly production per colony was observed during the fourth collection harvest (Figure 3), probably because there were five colonies with no larval acceptance: two colonies from the five-frame system, and one from each of the other systems. In vertical colonies, production increased during the first three harvests, dropped in the fourth and fifth harvests, remained constant during four harvests, and peaked in the last harvest.

Horizontal colonies had a similar behavior from the first to the sixth and from the ninth to the tenth harvests to the vertical colonies. However, their production lowered in seventh and eighth harvests, because in the sixth harvest, one of the colonies lost the queen and we had to replace her, and in the eighth harvest, one of the colonies did not accept any larvae and, consequently, had no royal jelly production. The lowest values of royal jelly per harvest occurred in the five-frame colonies; this treatment decreased its production from first to fourth harvests, but recovered its production in the fifth cycle and maintained it constant until the end of the experiment. Ten-frame colony production ranged between 5 and 7 g of royal jelly during most of the experiment, peaking in the second harvest (Figure 3).

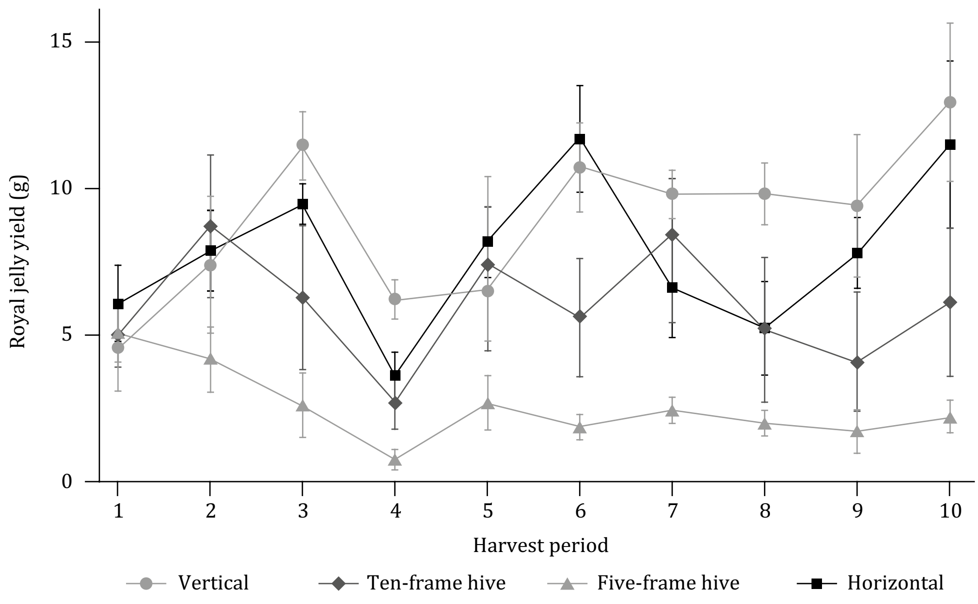


Figure 3 - Royal jelly yield per colony (mean±SE) over ten harvest points.

4. Discussion

The transformation of Brazilian traditional beekeeping into a modern enterprise is underway, and there seems a promising future for use of new management systems in production and marketing of high value products such as royal jelly. In our results, it was possible to demonstrate that vertical management systems in selected colonies of Africanized *Apis mellifera* bees supplemented with protein feed produce greater amounts of royal jelly as contrasted to conventional systems.

Faquinello et al. (2011) observed that selecting colonies to increase individual production also increased larval acceptance. Thus, larval acceptance of over 70% and average yields of over 25 g/colony in Turkey and 54.0 g/colony in China have been reported in selected bees (Şahinler and Kaftanoğlu, 2005; Şahinler et al., 2005; Hu et al., 2019).

In Brazil, variations in cell acceptance and royal jelly yield were observed in experiments conducted throughout the year with Africanized bees. When housed in ten-frame hives in the northeast of the country, colonies showed cell acceptance between 41 and 46% during June and August, respectively (Queiroz et al., 2001).

In the south of the country, colonies housed in ten-frame hives and supplemented with a mixture of linseed and palm oils from December to February produced 40.50% more jelly compared with colonies without supplementation, with values of 11.3 and 6.5 g/colony, respectively (Sereia et al., 2013). Similarly, colonies supplemented with a mixture of brewer's yeast and isolated soy protein from March to May increased royal jelly production from 7.0 to 11.7 g/colony (Sereia et al., 2013).

Apis mellifera bees housed in five-frame hives from November to May produced around 1.80 g/colony/harvest and larval acceptances between 29 and 43% (Garcia and Couto, 2005; Toledo et al., 2010; Pereira et al., 2019). From June to December in the same region, production of 3.6 g/colony/harvest was reported with an average of 136.5 mg per cell cup and 39.5% cell acceptance using five-frame colonies (Santos et al., 2019), values that agree with the results obtained in this experiment.

Differences in yields can be attributed to the production method used, presence of the queen in the colony (Şahinler and Kaftanoğlu, 2005), number and type of cell cup used (Garcia et al., 2000; Pereira et al., 2019), age of grafted larvae (Büchler et al., 2013), supply of supplements (Muli et al., 2005), feed supply during the experimental period, season of the year, and environmental variables such as humidity and temperature (Garcia and Couto, 2005; Toledo et al., 2010; Büchler et al., 2013).

Our results show that, despite benefits such as easy implementation, lower investment, and less labor obtained by using five-frame colonies, these obtained the lowest values in all variables; so, their use in commercial apiaries for royal jelly production is not recommended.

The height of the vertical colonies and the number of chambers used made management difficult, requiring at least three people to remove or return the grafting frame and taking more time to manage them, which could result in robbing. Values of royal jelly using horizontal and vertical management systems are not found in the literature. However, colonies with two queens in both management systems showed significantly higher royal jelly production per colony and harvest compared with the standard system. Their production was oscillating, but these colonies were strong throughout the experiment and showed an excess of 11 g in the last harvest production, which means that they can produce for periods equal to or superior to 32 days (duration of this experiment). In addition to their higher production, two-queen colonies are of great interest in providing young workers for producing package bees (Zheng et al., 2009). Walton (1972) also described an increase of 60 to 75% in honey production of colonies with two queens managed for two years.

One factor to explain these results is the queen bee pheromone, which modulates many aspects of the physiology of adult workers such as gene expression and behavior, and is critical for colony social organization (Le Conte and Hefetz, 2008; Kocher and Grozinger, 2011). Having two queens producing pheromones within the colony may increase the dispersal and amount of pheromone per worker, regulating gene expression in nurse and forager bees and, therefore, royal jelly production (Grozinger et al., 2003). Similarly, pheromone brood and β-ocimene stimulate behavioral maturation, hypopharyngeal gland physiology, and pollen foraging, increasing the size of the foraging force of the colony in the long term (Traynor et al., 2014; Ma et al., 2019).

In systems with two queens, the worker bees of each colony can move freely up and down the queen excluder, there is more efficient heat and pheromone transfer, and good food availability, facilitating standardization of the production area when needed. Moreover, these colonies need fewer boxes, covers, and frames and are characterized as having strong populations that allow better location and selection of food sources. On the other hand, they need more time to become established, and careful management is required to avoid a flow of queens between nest chambers.

5. Conclusions

Vertical colonies of Africanized bees show better production than one-queen colonies, showing that the implementation of advanced production techniques can increase the optimization of royal jelly production of Brazilian beekeeping.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: J.C. Camargo López and V.A.A. Toledo. Data curation: J.C. Camargo López and T.H.S. Souza. Formal analysis: J.C. Camargo López. Funding acquisition: J.C. Camargo López and V.A.A. Toledo. Investigation: J.C. Camargo López and V.A.A. Toledo. Methodology: J.C. Camargo López, D. Galhardo and T.H.S. Souza. Project administration: J.C. Camargo López, D. Galhardo, C.G.S.J. Pedroso and C.L. Figueira. Resources: V.A.A. Toledo. Supervision: V.A.A. Toledo. Validation: V.A.A. Toledo. Visualization: J.C. Camargo López and V.A.A. Toledo. Writing-original draft: J.C. Camargo López. Writing-review & editing: J.C. Camargo López, D. Galhardo, T.H.S. Souza and V.A.A. Toledo.

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