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Queen Bee (*Apis mellifera* L.) Production Using Grafting Method and Non-Grafting System

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Abstract

Currently, most beekeepers replace queens artificially by employing selection and genetic improvement through methods (grafting) or systems (non-grafting) to achieve higher yield and productivity in colonies. However, there is a lack of information regarding the acceptance and oviposition rates of queens reared by both methods in the tropical region. The study aimed to evaluate the effect of queen bee rearing methods, non-grafting (Nicot), and grafting (Doolittle) on the reproductive phenotypic characteristics of *Apis mellifera* L. A completely randomized design was used to evaluate six colonies with two rearing methods for queen bee production with three replicates per method from October to December 2024. Colonies were supplemented with 1 L of sugar syrup and 50 g of a protein supplement in paste form. The results showed that the Doolittle method was superior ($P < 0.001$) in larval acceptance (90 % vs. 70 %), queen cell size (20 mm vs. 19 mm), emerged queens (80 % vs. 45 %), weight (195 mg vs. 180 mg) and laying (677 eggs vs. 441 eggs), compared to the Nicot system (70%; 19 mm; 45%; 180 mg; 441 eggs, respectively). In conclusion, the Doolittle method proved more effective in queen bee rearing, optimizing parameters such as weight and larval acceptance. These findings underscore the importance of adapting beekeeping strategies to environmental conditions, including implementing supplemental feeding to ensure productivity and quality in queen bee rearing.

Subject Areas

Reproductive Biotechnology

Keywords

Apis mellifera, Queen Bees, Queen Rearing, Doolittle Method, Nicot System

1. Introduction

Honeybee colonies (*Apis mellifera*) rear queen bees in response to specific conditions, such as replacing an aging queen or when the queen exhibits poor performance, whether due to infertility, poor mating, low egg-laying rates, or the development of a reproductive swarm [1]. The mated queen gradually decreases egg-laying after her first productive year, making queen replacement essential to prevent colony loss [2].

Regardless of the method used, queen bee rearing can be affected by environmental factors [3]. These conditions influence the diversity and abundance of flowering species [4], impacting the flow of nectar and pollen that bees collect and store in the hive. Food scarcity contributes to the reduction of the colony population [5]-[7]. In the case of queen rearing, this situation affects the acceptance and development of queen cells, factors that will directly influence the development and reproductive behavior of the queens.

Currently, many beekeepers are turning to the use of improved biological material for queen rearing [8], which is primarily reproduced through two methods: the grafting method, known as the Doolittle method, and the non-grafting method, referred to as the Nicot system. The Doolittle method involves manually transferring larvae less than 24 hours old into artificial queen cells, which are then placed in a frame inside an initiator hive, allowing the worker bees to continue their development [9].

On the other hand, the Nicot system, introduced recently, uses a modular plastic box designed for the queen to lay eggs directly into the artificial queen cells, thus eliminating the need for manual grafting [10]. Given the increasing use of the Nicot system, analyzing its performance under different environmental conditions and management practices is pertinent. This would allow for optimizing its application in modern beekeeping, contributing to the sustainability and productivity of *Apis mellifera* colonies.

In addition, energetic and protein-based supplemental feeding plays a key role in queen rearing, especially during periods of low flowering. Previous studies [5] [7] have shown that supplementation with sugar syrup and protein patties ensures an adequate supply of essential nutrients, promoting optimal queen development.

Therefore, the objective of this study was to evaluate the effect of queen-rearing methods, the non-grafting system (Nicot), and the grafting method (Doolittle) on the reproductive phenotypic characteristics of *Apis mellifera* S.

2. Materials and Methods

2.1. Study Site

The study was conducted at the apiary of the Mocochá Experimental Field, belonging to the National Institute of Forestry, Agricultural and Livestock Research

(INIFAP). The experimental site is located at kilometer 25 of the old Mérida-Motul highway, at 21˚06'18"N and 89˚27'12"W, with an altitude of 9 m above sea level [11]. Meteorological data for the study period (October-December 2024) were obtained from the National Water Commission (Comision Nacional del Agua CNA) database for the Yucatán Peninsula. Data were sourced from the Mocochá meteorological station, located at the experimental site. Mean monthly temperature, relative humidity, and total precipitation are summarized in **Table 1**. Floral-resource availability was assessed weekly by recording the number of blooming plant species within a 500 m radius around the apiary.

Table 1. Floral species recorded and environmental conditions during the scarcity flowering period (October-December 2024).

Family	**Species**		
Asteraceae	*Ipomoea crinicalyx*		
Convolvulaceae	*Jacquemontia pentantha*		
Leguminosae	*Mimosa bahamensis*		
Verbenaceae	*Lantana camara*		
Environmental Parameters	**October**	**November**	**December**
Mean Temperature (˚C)	25.5	24	24
Maximum Temperature (˚C)	30	29	29
Minimum Temperature (˚C)	21	19	19
Mean Relative Humidity (%)	65	70	75
Monthly Precipitation (mm)	100	50	25

2.2. Ethics Committee Approval

The honey bees used for the experiments in this manuscript are the property of INIFAP. They were treated and cared for by professional personnel, following good management and sustainable production practices established in the Official Mexican Standard NOM002-SAG/GAN-2016.

2.3. Experimental Colonies

For both rearing methods, three colonies housed in 8-frame Langstroth nucleus hives from a hybrid genetic line (a cross between African and European bees) were used. In each method, 48 queens were reared. During the study period, each colony received 1 L of sugar syrup and 50 g of a protein supplement in paste form, composed of 20% pollen and 80% brewer's yeast [12]. This was administered during September and October, the time of year when the availability of food sources in the field is lowest.

2.4. Experimental Design

A completely randomized design was implemented with three colonies per rear-

ing method (six colonies in total). Although several larvae and queens were produced within each colony (approximately 16 grafted larvae and up to 48 queens in total), these individuals represent pseudo-replicates nested within each source colony. Consequently, the experimental unit for statistical comparison was the colony (n = 3 per method).

2.5. Selection of Biological Material

The brood used for both treatments was obtained from a single mother queen, selected according to the methodology described by García *et al.* (2013) [9]. This procedure included the evaluation of her population strength, foraging behavior (number of bees returning with pollen per minute), defensive behavior (number of stingers left on a piece of cloth exposed at the hive entrance for one minute), hygienic behavior (amount of capped brood removed from the comb within 24 hours), and the number of queen cells that reached the capping stage.

2.6. Doolittle Method

The queens were reared using the larval grafting method described by Dhaliwal *et al.* (2019) [13]. The procedure involved extracting larvae between 12 and 24 hours old from the brood comb and placing them into plastic queen cell cups. Each cell cup was pretreated with a droplet of royal jelly. Twenty-four hours before grafting, the cell cups were introduced into the starter colony for cleaning and acclimatization to the colony's specific odor. The cell cup bar (containing the cell cups) was secured into a grafting frame, which was then placed into an orphaned hive. The starter colony was composed of three combs: one of capped brood, one of honey, and one of pollen. The grafting frame with the cell cup bar was positioned in the center of the colony to ensure optimal temperature and humidity conditions for queen development.

2.7. Nicot System

For queen-rearing non-grafting, the system described by Büchler *et al.* (2013) [10] was used. The queen cell cups were placed in a cassette attached to a comb frame, which was introduced into the mother colony for cleaning and odor familiarization over 24 hours. After this period, the queen was located and confined to the cassette to acclimate and initiate egg-laying into the cell cups. Following 48 hours of queen introduction, the presence of eggs was verified. If eggs were observed, the queen was released, and the cage was removed to access the cell cups containing larvae (12 - 24 hours old if hatched). These cell cups were then secured into a frame with a grafting bar, which was introduced into an orphaned hive containing one comb of capped brood, one comb of honey, one comb of pollen, and the grafting frame holding the cell cups.

2.8. Measurement of Queen Rearing Parameters

Standardized rearing protocols described by Abou-Shaara *et al.* (2024) [14] were

followed to evaluate larval acceptance, queen cell size, queen emergence, and queen weight at emergence.

2.8.1. Acceptance Percentage

The accepted cell cups were counted 72 hours after the queen cell cups were introduced into the starter colony.

2.8.2. Queen Cell Size

The size of the queen cells was measured using a millimeter ruler. The data obtained were used to calculate each method's percentage of emerged queens.

2.8.3. Queen Weight

Upon emergence, the queens were weighed using a digital scale and classified according to the criteria proposed by Akyol *et al.* (2008) [15]. The three classification categories were: light (<190 mg), medium (190 - 200 mg), and heavy (>200 mg).

2.8.4. Egg-Laying Estimation

After emergence, queens were introduced into nucleus colonies for natural mating. Among all emerged queens, 20 successfully initiated oviposition (Doolittle = 12; Nicot = 8). For the detailed egg-count analysis, six laying queens were selected three per rearing method, one from each breeder colony to ensure balanced representation of source colonies. Mating success was confirmed by observing continuous egg-laying for seven consecutive days after introduction, a criterion widely accepted as a reliable indicator of successful mating in *Apis mellifera*. Egg counts were performed following the method described by Delaplane *et al.* (2013) [16], using an acetate sheet divided into 5 × 5 cm quadrants placed over the brood comb to record the number of eggs laid per day. The remaining laying queens were donated to local beekeepers in Mocochá, Yucatán, to strengthen colonies and promote sustainable apicultural practices in the region.

2.9. Statistical Analysis

Differences between methods were determined using Student's t-test applied to the colony means (n = 3 per method). The reported means and standard errors represent the average performance per colony, ensuring statistical independence among observations. Percentage variables (larval acceptance and queen emergence) were arcsine-square-root transformed before to analysis in order to stabilize variances. Normality of transformed variables was assessed using the Shapiro-Wilk test, and homogeneity of variances was tested with Levene's test. When assumptions were not met, results were verified using Mann-Whitney U tests, yielding consistent results. The values presented in **Table 2** correspond to untransformed means ± SE for ease of interpretation. Statistical significance was considered as follows: ns = non-significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

3. Results

The Doolittle method demonstrated significant superiority ($P < 0.001$) in larval

Table 2. Comparison of the Doolittle Method and Nicot System on queen bee rearing parameters during scarcity flowering season (Mean ± SE).

Parameters	Queen Rearing Method		Significance Level
	Doolittle Method (N = 48)	Nicot System (N = 48)	
Larvae acceptance (72 h)	43 ± 2.10[a]	34 ± 3.20[b]	***
Real cell size (mm)	20 ± 3.25	19 ± 2.80	ns
Emerged Queens	34 ± 3.14[a]	15 ± 2.90[b]	***
Weight (mg)	195 ± 3.40[a]	180 ± 2.20[b]	***
	(N = 3)	(N = 3)	
Egg-laying	677 ± 7.00[a]	441 ± 6.00[b]	***

[a,b]Different superscript letters within the same row indicate significant differences between the grafting method (Doolittle) and the non-grafting system (Nicot) (***P < 0.001).

acceptance at 72 hours, with a 90% acceptance rate (43 queens). Of these, 80% emerged from the cells (34 queens), with an average weight of 195 mg, classifying them as medium quality. Additionally, the average egg-laying capacity was 677 eggs in 24 hours. In contrast, the Nicot system showed lower acceptance (P < 0.001), with a 70% acceptance rate (34 queens). Of these, 45% emerged (15 queens), with an average weight of 180 mg, classifying them as low quality, and an egg-laying capacity of 441 eggs in 24 hours. Regarding queen cell size, no significant differences were found between the two methods.

Environmental data (Table 1) indicated that mean monthly temperature ranged from 24.0˚C to 25.5˚C, mean relative humidity from 65% to 75%, and total monthly precipitation decreased from 100 mm in October to 25 mm in December, consistent with the scarcity flowering season. Floral surveys during this period recorded low floral abundance, dominated by *Ipomoea crinicalyx*, *Jacquemontia pentantha*, *Mimosa bahamensis*, and *Lantana camara*, which together represent the principal pollen and nectar sources available under nutritionally restrictive tropical conditions.

Environmental data correspond to mean monthly values corresponding to the scarcity flowering season characterized by stable warm temperatures, decreasing relative humidity, and declining precipitation.

4. Discussion

The data obtained on larval acceptance are similar to those reported by Simbaña-Chorlango (2015) [17], where the Doolittle method showed superior larval acceptance at 72 hours and higher emergence rates compared to the Miller and double-grafting methods in *Apis mellifera* bees. Similarly, Dodologlu *et al.* (2004) [18] observed that the Doolittle method achieved a larval acceptance rate of 95%. Likewise, Cengiz *et al.* (2019) [19] demonstrated that the Doolittle method, combined with supplemental feeding, increased larval acceptance up to 82%. However,

Dhaliwal *et al.* (2017) [20] reported higher larval acceptance with the Nicot system than the Jenter and Doolittle methods during autumn (September to November).

Regarding queen cell size, Arias-Lagos (2019) mentions that the Nicot system produced larger queen cells compared to the Doolittle method. However, these results differ from those obtained in this study, where no significant differences were observed between the two methods. This discrepancy could be attributed to the specific characteristics of the predominant bees in the region, in this case, Africanized bees (*Apis mellifera* scutellata hybrids). These bees exhibit morphological and physiological differences compared to European bees (*Apis mellifera* ligustica or carnica) evaluated in previous studies, which may influence queen cell development and explain the lack of differences observed here. On the other hand, Dodologlu *et al.* (2004) [18] reported that with the Doolittle method, queen cells were longer (24.8 mm) compared to those naturally developed by bees (19.47 mm).

The weight values were similar to those reported by Akyol *et al.* (2008) [15], who, when applying the grafting method, obtained queens with an average weight of 192 mg, classifying them as regular quality. However, these values differ from those reported by Güler *et al.* (1999) [21], who registered an average weight of 177 mg in *Apis mellifera* anatoliaca and 150 mg in *Apis mellifera* caucasica. On the other hand, Arias-Lagos (2019) [22] reported a higher weight in queens reared using the Nicot system compared to the Doolittle method.

Regarding egg-laying performance, no previous studies were found to indicate the percentage associated with the different queen-rearing methods.

The results of this study reveal significant differences that underscore the need to optimize and adapt the established parameters for queen rearing. Currently, these standards are primarily based on studies conducted with European bees. However, the characteristics used to classify them by quality differ markedly from those of the Africanized bees predominant in the region, highlighting the importance of adjusting these parameters to local conditions.

Inadequate temperatures during queen rearing cause a metabolic imbalance by disrupting pupal thermogenesis and energy metabolism, which physiologically reduces mitochondrial respiratory efficiency and the synthesis of key structural proteins [23]. This compromises the development of the ovary and glandular system, resulting in queens with lower body mass, diminished lipid reserves, and reduced sperm viability [24] [25], which leads to loss of fertility [26].

This challenge is severely exacerbated by nutritional stress. Floral scarcity and the low availability of plants rich in pollen and nectar impose an energetic and protein restriction on the colony [27]. This reduces the quantity and quality of the lipids and proteins that nurse bees can destinate to the royal jelly [28], and consequently, to the feeding of the queen larvae. This nutritional deficit during the pupal stage intensifies the energy trade-off towards immediate survival rather than reproduction, collectively further reducing the reproductive efficiency and longevity of the queens, thereby gravely compromising colony stability [29].

Limitations. This study entails certain experimental constraints that should be acknowledged. The limited number of colonies (three per rearing method) re-

duces the statistical power to detect subtle differences and may restrict extrapolation to broader apicultural contexts. However, this design was intentionally standardized to minimize environmental and genetic variability, thereby allowing a controlled comparison between rearing techniques under homogeneous management conditions.

The study period, corresponding to the scarcity flowering season, provided a relevant ecological context for evaluating queen rearing under nutritionally challenging conditions typical of tropical apiculture. Nevertheless, the results should be interpreted within this specific seasonal and regional framework, as resource availability and climatic fluctuations may modulate larval acceptance and queen performance. Future research should replicate the experiment with a greater number of colonies, diverse genetic lines, and across different flowering periods to confirm the robustness and generality of the observed patterns.

5. Conclusion

Based on the results obtained, the Doolittle method ensured precise control in queen rearing, producing queens with optimal weight and high larval acceptance. In contrast, the Nicot system, which is more practical for mass queen production, prioritizes quantity over quality. Furthermore, confining the queen in a restricted space may induce stress, potentially leading to multiple egg-laying within the cells and complicating the uniformity of the process.

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Conflicts of Interest

The authors declare no conflicts of interest.

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