Chapter 2

Breeding Program Design Principles for Royal Jelly

Katia Ostrovski-Tomporoski, Patrícia Faquinello,
Fabiana Martins Costa-Maia,
Maria Claudia Ruvolo-Takasusuki,
Pedro da Rosa Santos and Vagner Arnaut de Toledo

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Abstract

This research was carried out to infer the genetic value to produce royal jelly in Africanized *Apis mellifera* L. honeybees with the compilation of data collected from 2006 to 2011. Genetic information of the selected and accessed colonies was obtained using the total DNA extraction techniques of nurse honeybees' thorax with molecular markers for MRJP3 protein and characterized in *Apis mellifera* L. From the information on the colonies and genealogical structure were predicted genetic values of the colonies and queens for the larvae acceptance trait (%), royal jelly per colony (g), and royal jelly per cup (mg). Animal model with Bayesian Inference was used from Multiple Trait Gibbs Sampling software in Animal Models, Gibbs chains 58,500 cycles resulting from 650,000 cycles with intervals and disposal of 65,000 and 10 withdraw, respectively. From the predicted values, the colonies were classified into upper and lower. To compare the average of the genetic values according to the genotypes, the average multiple comparison tests were proceeded and implemented in routine PROC GENMOD from the Statistical Analysis System. Environmental effects were considered, time and hive type (standard Langstroth) as having flat distribution and collection as chi-square distribution. The studies presented an increase in the alleles C and D and the alleles D and E—referring to MRJPs—found in the highest genetic value for royal jelly production. Alleles D, E, and C are important when evaluating the parameters larvae acceptance, royal jelly per colony, and royal jelly per cup and, occasionally, it was the DE genotype that stood out royal jelly production. Genotypes DE, DC, and EC are those that should be kept in this evaluation system for royal jelly production, and the other genotypes should be discarded because they had the worst performance for the parameters evaluated.

Keywords: *Apis mellifera* L., MRJP3, Bayesian inference, genotypes, genetic evaluation

1. Introduction

Despite the growing number in honey export figures in recent years, beekeeping in developing countries goes through a period of technological stagnation; that is, the genetic quality of the honeybees just has not shown significant progress and also has not developed new effective management techniques with increased productivity. The great demand for high performance of honeybee colonies with desirable behavioral characteristics contributes to changing the natural biodiversity through mass importation of queens [1]. From this point, Almeida and Carvalho [2] reported that to enter the increasingly competitive market of bee products, it is important that beekeepers innovate in management and use of technologies, going to observe beekeeping by a business vision, and contribute to maintaining the genetic biodiversity of honeybees. In Brazil, beekeeping activity has not received major financial support from the federal government or the service companies have technical training and knowledge to pass on technologies, new or traditional, to beekeepers. However, with the devaluation of the country's currency, the real against the dollar in recent years, every day beekeeping has become more rewarding and competitive in relation to other agricultural activities; however, we note that there has been an increase in the number of managed hives, but there is no significant increase in the number of beekeepers.

One way to improve production is through genetic breeding. Animal breeding programs select the best individuals to be used as breeding to the next generation [3] and evolved over the past decades [4] because science and technology have come to assist in better identification of genetic information available. Breeding programs have calculations, scientific principles, biotechnology, and advances in computing and information technology, which together enable the almost total of process efficiency. The honeybee improvement is very important for beekeepers, but for this improvement, it is necessary join honeybee adaptation to the environment, be productive, and be economically sustainable for beekeepers [4]. However, Kinghorn et al. [5] reported that the adoption of applied techniques to the breeding depends on the balance between what is possible from a technological point of view and what is acceptable in socio-economic context of the production system.

The Africanization process led to significant changes in the rearing and production, which combined with the research, led advances in instrumental insemination, queen production, genetic breeding with determination of strains for the production of honey, royal jelly, and hygienic behavior. Thus, the need for greater professionalism among beekeepers spurred the search for information and the inclusion of genetic breeding programs.

Among several factors that provide improvements in production, we can highlight the role of animal breeding. Many are the work of initiatives in this area in our country, all focused on strategies that will generate genetic progress proven to beekeeping. For instance, the continuous production of selected queens allows the beekeeper to immediately substitute dead or old queens by young queens, with desirable genetic and phenotypic characteristics. Moreover, the perfect development and productivity of the colony depends mainly on the age and quality of its queen [6], once the progeny inherits half of characteristics from the mother queen. The

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quality of the queens is affected by the genotype, nutrition, production methods, time of production, and the age of the larvae, among other factors [7].

The structuring of a breeding program involves the planning issues that are critical to its success [5], especially in honeybees [8]. Definition of the strategies is shown in **Figure 1**, and the training of personal for their development is a key factor to the success of the program [8].

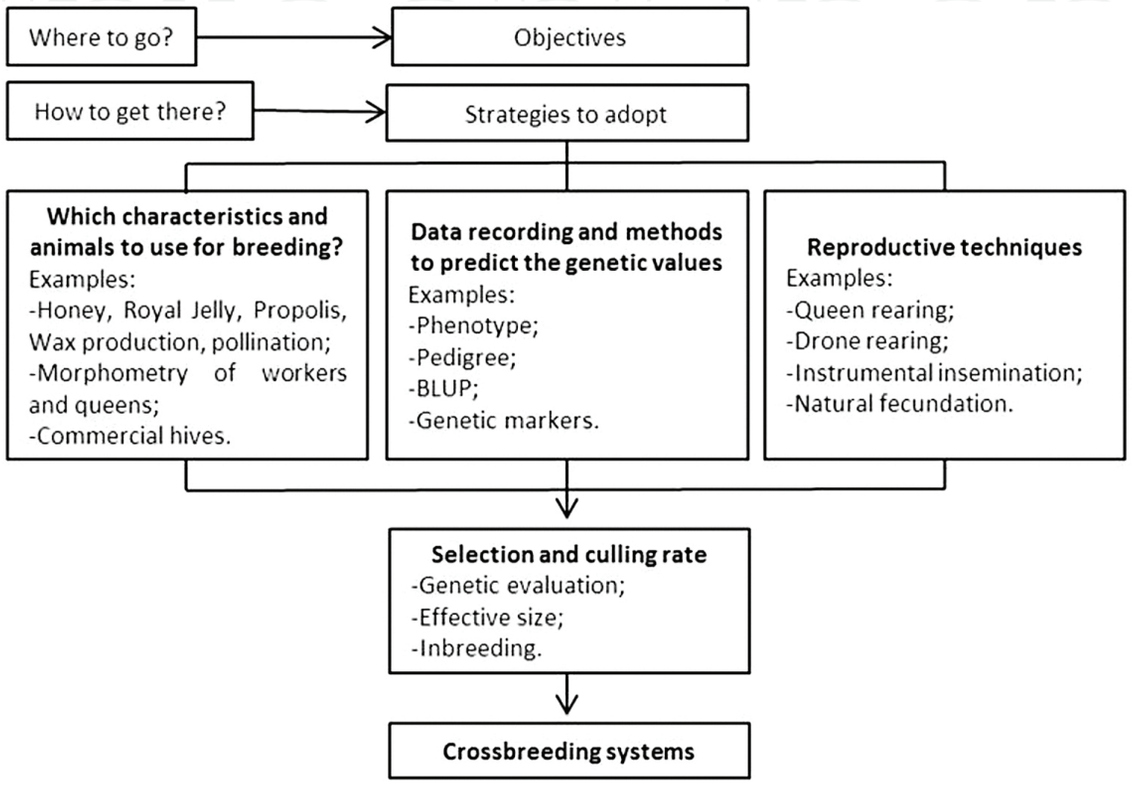

Figure 1. Strategies for honeybee breeding programs. Source: Ref. [8] adapted from Ref. [5].

The interest in establishing a breeding program with Africanized honeybees in the country is growing as well as the search for adequate and accurate methods for estimating genetic parameters for economic interest. Studies such as Costa-Maia et al. [9], Faquinello et al. [10], Wielewski et al. [11], Garcia et al. [12], and Padilla et al. [13] currently make up part of the framework of publications related to estimate genetic parameters of Africanized honeybees in Brazil, through the method of mixed models and Bayesian inference.

Faquinello et al. [10] estimated parameters of variance and covariance genetic, heritability, and genetic correlations for royal jelly production in *Apis mellifera* L. Africanized through Bayesian inference and concluded that royal jelly production is greatly influenced by external environment and possibly by internal environment by promoting low values of heritability to the analyzed characteristics. Moreover, these factors to be identified should be controlled by the beekeeper as much as possible; otherwise, the acquisition of queens with fitness for royal jelly production no longer will ensure maximum production. The authors [10] found values of positive genetic correlations between royal jelly production by colony and per cup of 0.29, for

larvae acceptance and royal jelly production by colony were 0.42, and low 0.06 for larvae acceptance and royal jelly production per cup. Selection based on the characteristic of royal jelly production per colony presented a positive trend of genetic gain for the larvae acceptance and royal jelly production per cup.

The research of genetic parameters in a given population to establish a selection process becomes a possibility for the characteristics of economic interest. Therefore, there is an increase in the frequency of desirable genes of the locus of economic importance. Therefore, the genetic progress is due to increasingly correct use of information on individuals applying for selection, resulting from the growing momentum in the methodological knowledge of genetic evaluation and the use of molecular genetics using molecular markers.

To further improve the quality of information, molecular genetics plays a key role because it locates and explores genes that have the greatest effect on the expression of quantitative traits. Lino-Lourenço and Costa-Maia [8] reported that the main livestock goal of genomic analysis in domestic animal species has been the dissection of the genetic architecture of the characteristics of economic interest, determining the number of genes and the contribution of each one for the expression of the phenotype and enabling the understanding that beyond the various genes of small effect, there are those few who print large effect.

Selection based on phenotype was complemented by information based on the genetic value, and then the influence of molecular genetics came to add and improve the quality of information in animal breeding programs. It is expected in future, an increase in gain per selection because the genetic merit of the animal can be obtained directly in its genome in a genetic evaluation program featuring all and any existing polymorphism. Therefore, studies on royal jelly production in the sphere of quantitative genetics associated with molecular genetics have great importance for the establishment of breeding programs of *Apis mellifera* L. This research line demands beyond the laboratory work, it requires knowledge of management production and the royal jelly quality.

Royal jelly is a heterogeneous substance secreted by cephalic glands (hypopharyngeal and mandibular glands by nurse honeybees of *Apis mellifera* L.), which feed the larvae in the first 3 days of life, while the queen is fed with royal jelly throughout all its existence. Royal jelly is yellowish product, viscous, and creamy consistency, having a characteristic odor and slightly spicy flavor [14]. Its secretion is promoted by pollen intake with added regurgitated solutions of the nursing workers, consisting primarily of sugars [15].

Fert [16] reported that royal jelly is a product that regenerates cells and tissues, normalizes blood pressure, and stimulates the production of red blood cells. Royal jelly has numerous functional properties and has been widely used in medicines, cosmetics, and healthy food in many countries [17]. In general, it consists of 60% water, proteins (41–42% dry matter), carbohydrates (30% dry matter), and small amounts of minerals, polyphenols, and vitamins [18]. Due to its composition, royal jelly is a key factor in the development of queen honeybees by the potential to increase in fertility, longevity, and body size [19].

For royal jelly production, especially during natural food shortage season, honeybees will need more protein provided by pollen, which can also be obtained from dairy products or brewer's

yeast. Royal jelly production is induced with artificial cells for queen rearing, containing newly hatched larvae, so the worker honeybees are encouraged to deposit royal jelly to feed the developing larvae [20].

Italian honeybees were introduced in China at the beginning of twentieth century [21], and royal jelly is one of the most important products to the Chinese beekeepers, producing between 200 and 3500 tons/year corresponding to 90% of total world production [22]. Initially, royal jelly production in China was only 0.2–0.3 kg/colony/year. Beekeepers of Zhejiang province began the process of selecting honeybees, and after 20 years, the production increased to 2.0–3.0 kg/colony/year. Since 1980, the Chinese government noticed the importance of this product to the country and started to invest in honeybee selection for royal jelly production in the same province and the other regions of the country, with production reaching from 6.0 to 8.0 kg/colony/year in the 2000s, and currently this value exceeds 10 kg/colony/year [23]. To reach such high production values, Chen et al. [24] developed a production system, which involves eight steps to obtain these high yields, including dietary supplementation, the adaptation or adequacy of equipment, manipulation abilities, and high number of cups per colony.

The main protein found in royal jelly—known as Major Royal Jelly Proteins (MRJPs) account for over 90% of total soluble protein composition [25]. The genes encoding key proteins of royal jelly began to be identified in studies made by Klaudiny et al. [26] and Albert et al. [27]. After these pioneering studies, several researches aim to characterize new genes encoding the MRJPs [25, 28, 29]. The locus Mrjp is part of an arrangement of nine genes encoded on an array of 65 kb. The MRJP family appears to have evolved from a single ancestral gene encoding a member of the yellow protein. Five genes encoding the proteins of yellow family are located in the genomic region containing the genes encoding MRJPs [30].

The five main representatives of this family are MRJP1, MRJP2, MRJP3, MRJP4, and MRJP5 [25, 31]. Although it has been proposed that the royal jelly has substances that induce differentiation of the queen, little is known about the function of its components, especially the protein portion [32]. The MRJPs were characterized in *Apis mellifera* L. [29, 30]. However, Baitala et al. [33] reported that data in the literature on the use of MRJPs as molecular markers in genetic structure of population studies and as selection markers associated with the improvement of royal jelly production are still scarce.

1.1. Factors influencing royal jelly production

Among the factors that affect royal jelly production are the internal factors of the colony, such as posture and acceptance of larvae, and external factors, such as nutrition, climate conditions, temperature, precipitation, humidity, and even genetic factors [10, 34]. Toledo and Mouro [35] observed the same environmental variables and reported that the maximum external temperature and relative humidity interfered positively, i.e. increased royal jelly production, while the rainfall had a negative influence.

Studying the effect of environmental variables in the royal jelly production, Toledo et al. [34] stated that precipitation did not affect the royal jelly production as well as the addition of protein supplement (35%). Minimum relative humidity and maximum temperature negatively

affected the number of accepted larvae and maximum relative humidity positively influenced the amount of accepted larvae [34]. Garcia and Nogueira-Couto [36] reported that there are differences in the larvae acceptance for the royal jelly production performed at different times during the year.

Royal jelly production is influenced by the number of collections per season, geographical location of the apiary, the experience of the beekeeper, and the genetic origin of honeybees [37]. Albarracín et al. [38] reported that larvae acceptance between genetic groups of Italian and Africanized origin and found no significant differences between them; the average production of royal jelly per colony was similar for both.

Over a month, the time collection 48 h after the larvae grafting presented higher income than the time collection 72 h after the grafting. van Toor [20] recommended a technique of two collections, the first being after 72 h after the larvae grafting and the second performed 48 h later; colonies producing 180 mg or less of royal jelly per cup should be replaced. However, Zheng et al. [21] recommended that the new standard assessments for royal jelly quality in China consider the collection time, because they found significant differences in the sample quality after 24, 48, and 72 h the larvae grafting. Sereia et al. [39] concluded that the average amount of royal jelly deposited every 68 h per cup was higher when supplemented with isolated soy protein, brewer's yeast, palm oil, and linseed oil. Some studies reported variation in royal jelly production of *Apis mellifera* L. Africanized honeybees from 188 to 234 mg/cup [10, 35, 40]. Toledo and Mouro [35] stated that the amount of royal jelly obtained per cup varies with the time that is left within the colony and obtained average of 253 mg/cup for Africanized and 198 mg/cup for Carniolian honeybees. Sereia et al. [39] observed differences for the percentage of total acceptance of grafted larvae with a mixture of linseed oil + palm oil and isolated soy protein + brewer's yeast that had, respectively, 63.45% and 63.75% of accepted cups when compared with palm, linseed, isolated soy protein, yeast, and controls I and II (45.80%, 49.71%, 50.32%, 50.95%, 49.60%, and 52.17%, respectively).

Sereia et al. (2010a) [41] studied the supplements with different nutrients in honeybee diet and found that by having a glandular origin, royal jelly production varies with the nutritional quality of the available sources and recommended protein supplementation to royal jelly production in honeybee colonies, still being economically viable the beekeeper [42]. Furthermore, the use of dietary supplementation on the royal jelly production is a matter that is part of the quality specifications of this product in France [37]. Sereia et al. [41] evaluated the nutritional quality supplements containing six different sources of oil and protein and concluded that the use of linseed and palm oils combined with brewer's yeast to prolong the longevity of Africanized honeybee workers, and the sunflower oil increases the royal jelly production [43]. Royal jelly production involves biological and behavioral interactions intrinsic to the honeybees, and its variability has important genetic and environmental components internal and external to the colonies [34, 36].

1.2. Chronology of selection for royal jelly production

As an agribusiness activity in development in Brazil [34], beekeeping has great potential for marketing products in addition to honey, such as royal jelly, for example, although it is not

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considered a product of the conventional beekeeping [42]. Besides these products, the Brazilian beekeepers are increasing the production of propolis mainly and pollen because their prices are rising too.

Currently, in Brazil, there is a poly-hybrid that emerged from the crossing of four subspecies of European (*Apis mellifera mellifera*; *A. m. caucasica*; *A. m. carnica*; *and A. m. ligustica*) with African honeybee *Apis mellifera scutellata* [44], who went through natural selection processes and adapted, resulting in what is called today of *Apis mellifera* L. Africanized, with great potential for selection by their genetic diversity [35]. Garcia and Nogueira-Couto [36] reported that African honeybees have adapted excellently in Brazil by the similarity of environmental conditions of the Brazilian territory with its homeland, the high adaptive capacity and to print this trait in their offspring crossings with other subspecies, ensured the expansion of Africanized honeybees throughout the Americas. Africanized honeybees present many differences from European honeybees, such as size and shape of the nest, life cycle (shorter for Africanized honeybees), colony growth and reproduction, production of males, swarming, defensiveness [45], and finally honey production, in tropical climate conditions.

Since 2006, at Maringa State University, studies intensified the evaluation system in Africanized honeybee colonies started in 1996, by joint operation of quantitative genetics with molecular genetics, aiming to provide grants to start a breeding program to select characteristics of economic interest, such as increased production of royal jelly. The utilization and production of technical evaluations in Beekeeping Division of the Experimental Farm of Iguatemi at the University, receiving support from the Laboratory of Genetics and Cellular Biology, started more effectively compiling data for the selection of *Apis mellifera* L. Africanized honeybees.

Mouro and Toledo [40] had higher royal jelly productions in hybrid Carniolan in relation to Africanized honeybee colonies. However, royal jelly production increased 109.19% in the first generation after performing the selection in Africanized honeybees. Therefore, it is essential to select the queens and recommended that the criteria to be adopted for the selection should be the production per colony for not occur losses in adaptive traits like disease resistance [40].

Africanized honeybees when selected, for royal jelly or honey production, were more effective in royal jelly production than Carniolian honeybees [35]. In addition, as an efficient measure to increase production, Toledo and Mouro [35] recommended selection of Africanized colonies because of the genetic diversity of wild swarms or natural colonies of these honeybees in Brazil, by presenting significant results and are applicable to the field reality. Toledo and Mouro [35] used Africanized honeybee colonies collected in nature for the royal jelly production, from August 1996 to March 1998. After initial results, the five most productive colonies were selected and compared with five Carniolan hybrid colonies with daughter queens from queens who came from Germany and observed that the Africanized honeybees produced more royal jelly when compared with Carniolan honeybees.

From August 2002 to February 2003, Toledo et al. [43] evaluated the royal jelly production in honeybees that received supplementation isoproteic (30%) and isolipid (5%) and concluded that honeybee food containing sunflower oil increased royal jelly production by colony at

28.79%. van Toor [20] reported that they should select the colonies with royal jelly production above average, considering the size of the colonies, high production of nurse honeybees, and favorable genetic predisposition.

Bayesian inference is a tool that contributes to efficient selection programs and has been used in the evaluation of animals to obtain more accurate estimates. The distribution of data allows the analysis of sets with varying sizes, providing accurate estimates of the variance components, breeding values, and credibility intervals [10, 46]. Interest in establishing breeding programs in Africanized honeybees in the country is increasing as well as the search for adequate and accurate methods for estimating the genetic parameters for economic interest.

Faquinello et al. [10] used Bayesian inference to estimate the variance components, covariance, and genetic parameters for royal jelly production in Africanized honeybees through the animal model. During the experimental period, these authors [10] evaluated several parameters of royal jelly production in colonies with daughter queen of matrix colonies. These parameters were larvae acceptance, royal jelly production per colony, and royal jelly production per cup in overlapped nucs (five frames each) and overlapped Langstroth hives (10 frames each). Selection based on genetic evaluation of queens contributed to the increase royal jelly production choosing parameters like larvae acceptance, royal jelly production by cup, and royal jelly production per colony [10]. Having a quality queen also means greater production of eggs which in turn will strengthen the colony optimizing production [47]. Sereia and Toledo [48] reported that there was genetic effect in the royal jelly production and concluded that descendants of other best royal jelly producing colonies presented higher number of accepted larvae and larger amount of royal jelly deposited by cup.

The number of nurse honeybees in the colony can directly influence the amount of royal jelly produced. Several surveys presented increased expression of *mrjp* levels or increased the amount of royal jelly protein in hypo pharyngeal glands of nurse honeybees, which is not developed in forager honeybees [49].

Baitala et al. [33] using microsatellite markers identified seven alleles mrjp3 in the Africanized honeybees producing royal jelly and also confirmed the increased frequency of alleles C, D, and E of Mrjp3 in selected colonies. Production results from the genetic evaluations indicated that the analyzed queens had similar royal jelly production, suggesting that there was no difference to the alleles under selection for this feature, but the genotypes chosen for the matrix colonies with queens were being held in daughter queens and drones. Parpinelli et al. [50] verified the genetic variability of locus Mrjp3, Mrjp5, and Mrjp8 from colonies *Apis mellifera* L. Africanized, selected for royal jelly production since 2006 to identify molecular markers associated with the royal jelly production, and observed the fixing of these alleles during the reproduction process selection. Three sites were polymorphic and produced a total of 16 alleles and have been identified four new alleles for the locus Mrjp5 [50]. The effective number of alleles for the locus Mrjp3 was 3.81. The average observed heterozygosity was 0.4905, indicating a high degree of genetic variability for the locus analyzed. High values of the inbreeding coefficient (Fis) for locus Mrjp3, Mrjp5, and Mrjp8 indicated excess of homozygotes, i.e. the

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selection of *Apis mellifera* Africanized queens for royal jelly production is keeping alleles mrjp3 C, D, and E, despite the C allele has occurred with low frequency. However, the genetic variability of the queens is decreasing for the analyzed locus, with excess of homozygotes, but the large number of drones to fertilize the queens hampers the production of homozygous genotypes for the locus Mrjp3 [50].

Researches conducted at Maringa State University to get the best yields in the royal jelly production are based on assessments of honeybees over several years in colonies fertilized naturally [10], quantitative and molecular analysis, with selection based on genetic markers for protein MRJP3 [33] that relate to the colonies that have the most significant quantitative production of royal jelly [41, 42, 50].

Researchers, such as Mouro and Toledo [40], Toledo and Mouro [35], Baitala et al. [33], Toledo et al. [34], Faquinello et al. [10], Toledo et al. [43], and Parpinelli et al. [50], with our research contribute to the continuity of this research line. The selection of honeybees must occurs using controlled crossings with instrumental insemination techniques to achieve the homozygous individuals to define the allele that contributes most to the largest increase in production. However, it is very important to include sometimes a different queen with different genetic for keeping the heterosis.

Based on the above, this research was carried out to predict the genetic value of Africanized *Apis mellifera* L. honeybee colonies producing royal jelly, based on genetic information through production characteristics (larvae acceptance and royal jelly production per colony and per cup) with compilation of data collected from 2006 to 2011.

2. Material and methods

The experiment was conducted at the Experimental Farm of Iguatemi at Maringa State University, Brazil, from January to April 2011, and the data that contributed to the Africanized honeybees assessment system for prediction of breeding values in the parameters evaluated for royal jelly production were collected from 2006 to 2011, concurrent with the genomic DNA extractions of the same selected colonies with molecular genetic markers.

2.1. Identification of genomic DNA

Genetic information of selected and evaluated colonies was obtained from the Laboratory of Genetics and Cellular Biology at the University by Baitala et al. [33] and Parpinelli et al. [50]. All these authors followed the method for extracting the total DNA from nurse honeybee thorax, described by Bardakci and Skibiński [51] and adapted to be used in *Apis mellifera* L.

Polymerase chain reaction (PCR) was performed using specific primers synthesized to amplify the repetitive regions of the locus Mrjp3 [28], amplification reactions being carried out in a Techne thermal cycler TC-512, and amplification conditions for primer MRJP3 based on the method described by Albert and Schmitz [52].

2.2. Queen rearing and royal jelly production

Queen rearing was carried out in specialized laboratory to develop such activity in air-conditioned environment for larvae grafting, with an average temperature of 33 ± 2°C and relative humidity of 60 ± 10%. Colonies were provided by beekeepers from different regions of States of Parana, São Paulo, Mato Grosso, Mato Grosso do Sul, and Sergipe, as well as Paraguay and Colombia. Whenever a colony or swarm died, it was replaced by another one. All colonies were identified, and queens were marked with numbered plates on the thorax, located in a five frame hive or in a Langstroth hive, depending on the colony size and the season of the year. From this, we started to rear the first generation of daughters from those queens. The method used for queen production was adapted from Doolittle [47]. The grafting was simple with larvae aged between 0 and 24 h and controlled genealogies. Periodically, all colonies were genotyped to know their genealogy.

The starting–finishing colonies were mini-hives [53]. For queen rearing, 10 queens from each selected colonies were produced in each generation. Each colony was settled from two overlapped nucs with a queen excluder between them. In this, mini-hive colony was a cup bar frame with 30 acrylic cups, 15 in the upper bar, and 15 in the lower with different genealogies, identified and randomly distributed (**Figure 2**). After 10 days, the queens' cells were removed from the starting–finishing colonies and were placed in incubators until the queens emerged in glass vial of 20 mL with a piece of paper, identifying the genealogy and hive number. Newly emerged queens were anesthetized with carbonic gas (CO_2), identified with a numbered label in the upper thorax, placed in a plastic cage, and brought stored until their introduction in the colonies, for royal jelly production.

Figure 2. Withdrawn of frame with queen cells for queen production.

After emergency, the queens were anesthetized with CO_2 for the measurement of body weight (mg) in a precision digital scale 0.001 g and length and abdomen width (mm) through digital precision caliper 0.01 mm–0.0005". Queens with body weight above 180 mg were allocated in JZsBZs™ type cages and kept in an incubator with nurse honeybees and introduced in five-frame hives to be mated naturally. This introduction occurred after at least 24 h after the supersedure to avoid risk of plunder and being a period of greater acceptance [47]. As the queens were inseminated naturally, the information regarding paternal genealogy was considered as unknown.

The beginning of oviposition was monitored to start royal jelly production evaluations after 50 days as this ensured that all the worker honeybees were daughters from the new queen. Terada et al. [54] verified that the average longevity of an Africanized worker is 26.3 days.

For royal jelly production, in each colony was introduced a frame containing three bars and 100 artificial cups in total. After 66–72 h frames were removed (**Figure 3**), larvae were discarded and royal jelly collected with suction device. Larvae grafting were scheduled, based on the schedule followed by Wielewski et al. [11].

Figure 3. Bar with cups showing the larvae acceptance and royal jelly produced.

2.3. Statistical analysis

Data of weight, length, and width of the abdomen in all generations of selection of the newly emerged queens were subjected to analysis of variance (ANOVA) using SAS software [55] and the averages in every generation for each evaluated trait were compared by Tukey's test at 5%.

After obtaining the data of body weight, length, and width of the abdomen of the newly emerged queens, proceeded to the genetic evaluation using the software *Multiple Trait Gibbs Sampling in Animal Models* (MTGSAM), developed by Van Tassel & Van Vleck [56], making the Bayesian estimation using the Gibbs sampling method.

The animal model used was in the following:

$$y = X\beta + Za + e$$

where y is the vector of observations; X is the incidence matrix of fixed effects, contained in the vector β; β is the vector of fixed effects; Z is an incidence matrix of additive genetic effects; a is the vector of additive genetic effects; and e is the vector of random errors associated to each observation.

where y, a, and e have normal multivariate joint distribution, as follows:

$$\begin{bmatrix} y \\ a \\ e \end{bmatrix} \sim NMV \left\{ \begin{bmatrix} X\beta \\ 0 \\ 0 \end{bmatrix}; \begin{bmatrix} ZGZ' + R & ZG & R \\ GZ' & G & 0 \\ R & 0 & R \end{bmatrix} \right\}$$

In unicaracter analysis, G is the genetic variance and covariance matrix as $A\sigma_a^2$, A being relationship matrix, and σ_a^2 is the additive genetic variance; R is the residual variance matrix given by $I\sigma_e^2$, I being identity matrix, and σ_e^2 is the residual variance of the trait.

For bicaracter analysis, the G matrix is $G_0 \otimes A$, A being the relationship matrix, and G_0 is the additive genetic covariance matrix as follows:

$$G_0 = \begin{bmatrix} \sigma_{a_1}^2 & \sigma_{a_1 a_2} \\ \sigma_{a_2 a_1} & \sigma_{a_1}^2 \end{bmatrix}$$

The matrix R is given by $R_0 \otimes I$, I being identity matrix by equal order to drone number, and R_0 is the residual covariance matrix, as below:

$$R_0 = \begin{bmatrix} \sigma_{e_1}^2 & \sigma_{e_1 e_2} \\ \sigma_{e_2 e_1} & \sigma_{e_1}^2 \end{bmatrix}$$

In analysis strategy, it was used Gibbs chains of 58,500 cycles resulting from 650,000 cycles were generated, respectively, with initial disposal of 65,000 iterations and sampling intervals for every 10 iterations. The convergence of chains was tested by Heidelberger and Welch [57]

test, implemented in Convergence Diagnosis and Output Analysis (CODA)—R software—Version 2.12.1. [58].

Colonies were classified into upper and lower, with the predicted values, considering the average genetic values of the parameter used as classification criteria. Each genotype was estimated probability rating in the higher and lower classes, from the PROC GENMOD routine from Statistical Analysis System [55] in which it was considered the data binomial distribution with logarithmic linkage. Classification probabilities for each genotype were tested by *T* test, using PROC GENMOD routine with 5% of significance.

Comparing the averages of genetic values in function of genotypes, proceeded averages multiple compilation test implemented in PROC GENMOD routine. To reduce interference of environmental effects in royal jelly production, the model used for prediction of breeding values, the environmental effects of year were considered—2006–2011, time—the four seasons of the year and type—colony model, as having flat distribution, and collection and distribution of chi square.

Bayesian inference was used by MTGSAM software as a tool to define the genealogy and predict breeding values for each colony in the traits, as larvae acceptance per grafting (%); royal jelly production per colony (g); and royal jelly production per cup (mg). The use of this type of analysis is appropriate to raise the accuracy of the dataset that follows this kind of beekeeping analysis protocol.

SAS software was used to determine the differences between production parameters in relation to the predicted values for the evaluated colonies. Considering that each colony is a superorganism [59], it was worked for analyzes concerned with different individuals—78 units of repetitions, a corresponding period to 6 years from 2006 to 2011. The genotypes identified that appear more frequently over the years of selection in the colonies submitted for royal jelly production were as follows: DE, DC, CE, EF, and FG [50].

3. Results

3.1. Performance rating

Table 1 represents the statistical differences found for the selected colonies evaluated by the production parameters, among the high and low classes of genetic value, considering the genotypes. It was observed that there were no statistical differences for the total larvae acceptance per grafting (%) among the categories of DE and EF genotypes, being FG the genotype ranked as the one that had the worst performance for this parameter with total chances of appearing below the average obtained. In this case, the DC genotype stands out for having 71% chance of being ranked above the average of the values found for acceptance. For total royal jelly production per colony (g), it repeated practically the same performance conditions between genotypes, and although there was no statistical difference, the highlight was DE genotype with 48% of chance to be classified as being high genetic value for this parameter. When performed royal jelly production per cup, DE genotype achieved the highest

rating, presenting 60% of probability to be above the average, significantly differentiating from EF genotype and not differing from the others. The EF genotype had the worst rating, there was 78% chance of this genotype be classified as low genetic value for this parameter.

Genotype	Class	Evaluated parameters		
		Total larvae acceptance per grafting (%)	Royal jelly per colony (g)	Royal jelly per cup (mg)
DE	High-genetic value	0.50a	0.48a	0.60a
	Low-genetic value	0.50	0.52	0.40
DC	High-genetic value	0.71a	0.43a	0.43ab
	Low-genetic value	0.29	0.57	0.57
CE	High-genetic value	0.50a	0.25a	0.25ab
	Low-genetic value	0.50	0.75	0.75
EF	High-genetic value	0.44a	0.33a	0.22b
	Low-genetic value	0.56	0.67	0.78
FG	High-genetic value	0b	0b	0.33ab
	Low-genetic value	1	1	0.67

Means followed by the same letters within the classes, in the same column, are not statistically different from each other by *T* test ($P > 0.05$).

Table 1. Probability of classification of high and low genetic value of different genotypes for total larvae acceptance per grafting, royal jelly production per colony, and royal jelly production per cup.

3.2. Prediction of genetic values

Averages of predicted genetic values for each genotype are presented in **Table 2**. To larvae acceptance, the genetic values of DE and DC genotypes were higher in relation to the EC, EF, and FG genotypes. There was no difference between genotype DE and all others. The DC genotype differed significantly from FG genotype, which presented the worst performance in this parameter. For total royal jelly production per colony, DE genotype was superior not only differentiating from DC and CE genotypes and did not differ significantly from EF and FG genotypes.

	Genetic values		
Genotype	**Total larvae acceptance per grafting (%)**	**Royal jelly per colony (g)**	**Royal jelly per cup (mg)**
DE	0.8043ab	0.4505a	0.0234a
DC	2.3118a	−0.0955ab	−0.007b
CE	−6.7991ab	−1.1013b	−0.0388b
EF	−4.2494ab	−0.612b	−0.0109ab
FG	−9.2538b	−1.2472b	−0.0283ab

Means followed by the same letter, in the same column, do not differ statistically from each other by *T* test ($P > 0.05$).

Table 2. Average genetic values for the characteristics evaluated in terms of different genotypes.

4. Discussion

For more accurate estimates, taking into account data distribution and the possibility of working with a small sample size, Bayesian inference is being increasingly used in honeybee husbandry trials because it produces accurate estimates of the variance components, genetic values [10], and credibility intervals, contributing to an efficient selection program [60]. However, studies using these methods have not been conducted for the royal jelly production in honeybees until not long ago [10, 11]. Metorima et al. [61] recommend this method for data without restriction of this nature, and it should be used as a tool in obtaining more accurate estimates for research in honeybees.

Genetic value is part of the genotypic value transmitted from parents to offspring. The prediction of genetic values helps evaluations between statistical analyzes on quantitative genetics with molecular genetics. Costa-Maia et al. [9] reported that accurate genetic parameter estimation allows prediction of the genetic value of the animal and, therefore, identification of genetically superior individuals.

All laboratory studies indicated that certain alleles are disappearing while others are settling, which can be observed by increase in the frequency of the relevant alleles in the royal jelly production. After DNA extraction from nurse honeybees, Baitala et al. [33] and Parpinelli et al. [50] noted an increase in the frequency of alleles C, D, and E on bees forming part of phenotypic and molecular genetic evaluation system for royal jelly production.

The reduction of heterozygosity contributes to the fact that the alleles C, D, and E have appeared more frequently and reducing the frequency for the F and G alleles. Observing the parameter royal jelly production per colony, we can see that the D allele has an important contribution, as were the DE and DC genotypes that stood out. During the time that genetic evaluations were conducted for the royal jelly production, alleles that most closely related to the increased production were maintained throughout the selection process, agreeing to Parpinelli et al. [50]. Parpinelli et al. [50] reported that the selection based on royal jelly production for MRJPs is leading to homozygosity of these loci, especially MRJP3, which presented the lowest value of observed heterozygosity.

To total larvae acceptance after the grafting, only the FG genotype was classified as inferior, while other were all high, highlighting the DC genotype with 71% chance of being so classified as superior. In a survey that compared the Doolittle and the "Starter" methods for royal jelly production, Baumgratz et al. [62] found larvae acceptance percentages of 63% for the first method and 50% for the second, values greater than 29.20%, observed by Toledo et al. [34]. Faquinello et al. [10] reported average larvae acceptance of 52.13%, which agrees with the performance of almost all genotypes in this study. Sereia et al. [39] observed differences for the grafted larvae acceptance with a mixture of linseed oil + palm oil and isolated soy protein + brewer's yeast that had, respectively, 63.45% and 63.75% when compared with palm, linseed, isolated soy protein, yeasts and controls I and II (45.80%, 49.71%, 50.32%, 50.95%, 49.60%, and 52.17%, respectively).

The same classification was repeated for the parameters royal jelly production per colony and royal jelly production per cup, whose average for Faquinello et al. [10] was 6.26 g and 190.07 mg, respectively. This was due to the fact the DE genotype has differed statistically only from EF genotype, with no difference between the other. Baumgratz et al. [62] observed productions per cup of 268 and 269 mg. Faquinello et al. [10] found positive correlations between royal jelly production per colony and royal jelly production per cup, total larvae acceptance rate after the grafting with royal jelly production per colony, and low correlation between total larvae acceptance after the grafting rate and royal jelly per cup. Muli et al. [63] when evaluating the royal jelly production potential between two subspecies of African *Apis mellifera* L., they found that the collection after 3 days had higher yields of royal jelly per cup—349.5 mg than gathering after 2 days—236.3 mg.

Royal jelly production is greatly influenced by the environment. The genetic correlation indicated that the selection increased royal jelly production per colony, the larvae acceptance, and royal jelly production per cup [10]. Li et al. [64] concluded that the hypo pharyngeal glands of nurse honeybees selected for royal jelly production were significantly higher than in non-selected honeybees. By microscope images, it could be seen that the royal jelly secretion period on selected honeybees was higher than in non-selected honeybees [65]. This highlights the importance of selection, genetic evaluation of individuals, and the starting of a breeding program of honeybees, mainly Africanized.

The average obtained for genetic values, DE genotype obtained positive values for the three parameters, followed by DC genotype also presented that the average value of larvae acceptance in a positive way, i.e. classified as superior. All other genotypes presented negative values for the average genetic value, and that means were rated lower. For total larvae acceptance after the grafting, there were only differences between DC and FG genotypes, being the DC genotype higher than the others. When total larvae acceptance is small can increase the royal jelly production per colony by increasing in the amount of royal jelly deposited per cup [66]. Genetic values were predicted royal jelly production and the use of genetic evaluation techniques presented that the alleles D and E—referring to MRJP—is the most genetic value to produce royal jelly.

The environmental influences and genetic differences in mating level hampering honeybee breeding [67]. Royal jelly production is a controllable genetic trait [68] and for its high

commercial value, there is a need for tools for the establishment of breeding programs [4, 33, 34, 40, 42]. Harbo and Rinderer [69] reported that the selection of superior genotypes with honeybees involves the use of improved queen replacement techniques, instrumental insemination, and assisted selection with molecular marker. However, in the literature, there is little data available for molecular markers associating to royal jelly production [4, 10, 11, 33, 34, 40, 42, 70]. The selection of more productive queens benefits everyone interested in beekeeping and in breeding control and selection [71], for this is necessary evaluate thousands of colonies in several apiaries, accurate record keeping, and if possible, a insemination laboratory [72].

Studies, such as Mouro and Toledo [40], Toledo and Mouro [35], Baitala et al. [33], Toledo et al. 34, Faquinello et al. [10], Toledo et al. [43], Parpinelli et al. [50], and this present research with predicted values are important and should be taken into account for the implementation of breeding programs. These data allow the continuation of this research line with selection based on the prediction of genetic values of the selected colonies. However, with controlled crosses using instrumental insemination techniques, it is possible to obtain homozygous for the allele that contributes to the largest increase to the royal jelly production. Moreover, the high allelic polymorphism for MRJP3 protein is an indicator that this biomolecular marker can be used in studies of the genetic structure of Africanized honeybees and so, after the selection process, establishes a breeding program [33, 70] that should be adopted by the government so that this technology would be widespread in the country and among beekeepers.

As a result of the analyzes, it is concluded that alleles D, E, and C are most important when the production parameters evaluated are larvae acceptance after the grafting and royal jelly production per colony and per cup. Thus, DE, DC, and EC genotypes should be kept in the evaluation system for royal jelly production, while the others should be discarded or replaced as it had the worst performance for these important parameters in production.

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Author details

Katia Ostrovski-Tomporoski[1], Patrícia Faquinello[2], Fabiana Martins Costa-Maia[3], Maria Claudia Ruvolo-Takasusuki[4], Pedro da Rosa Santos[5] and Vagner Arnaut de Toledo[5*]

*Address all correspondence to: abelha.vagner@gmail.com

1 Federal University of Paraná, Curitiba, State of Paraná, Brazil

2 Federal Institute Goiano, Ceres, State of Goiás, Brazil

3 Paraná Federal Technology University, Campus Dois Vizinhos, State of Paraná, Brazil

4 Biotechnology, Genetics and Cell Biology Department, State University of Maringá, State of Paraná, Brazil

5 State University of Maringá, State of Paraná, Brazil

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