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Drone production, semen viability and spermatozoa longevity of Africanized *Apis mellifera*

André Luiz Halak[1], Pedro da Rosa Santos[2], Robson Marcelo Rossi[3], Priscila Wielewski[2], Gentil Vanini de Moraes[4] and Vagner de Alencar Arnaut de Toledo[2*]

[1]Cooperativa Agricola e Apícola das Beiras, Mangualde, Viseu, Portugal. [2]Programa de Pós-Graduação em Zootecnia, Departamento de Zootecnia, Centro de Ciências Agrárias, Universidade Estadual de Maringá, Avenida Colombo, 5790, 87020-900, Maringá, Paraná, Brasil. [3]Programa de Pós-Graduação em Bioestatística, Departamento de Estatística, Centro de Ciências Exatas, Universidade Estadual de Maringá, Maringá, Paraná, Brasil. [4]Departamento de Zootecnia, Centro de Ciências Agrárias, Universidade Estadual de Maringá, Maringá, Paraná, Brasil. *Author for correspondence. E-mail: vagner_abelha@yahoo.co.uk

ABSTRACT. Characteristics correlated with beekeeping production, less influenced by the environment and that can be controlled by management techniques, can help in the selection of colonies with higher production capacity, aiming to improve breeding programs. This research was carried out to evaluate the production of Africanized *Apis mellifera* drones and the longevity of spermatozoa from different genetic groups when supplemented with protein or not. Two genetic groups were used: one selected for royal jelly production from the Africanized honeybee breeding program and another without genetic selection. In both groups, the number of drone brood and the quality of semen were evaluated every 30 days, for five months. Statistical analysis was performed using Bayesian Inference. Statistical difference was found for the drone production among the treatments, and colonies without genetic selection produced more males (187.80 ± 11.15) than the selected colonies (93.07 ± 8.88). The selected colonies for royal jelly production presented greater efficiency in the reproductive cycle of males, because they produced fewer drones than colonies without genetic selection, however, with 31% greater semen viability.

Keywords: honeybee genetics; honeybee nutrition; soybean protein; Africanized honeybee reproduction.

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Introduction

Africanized honeybees have high genetic diversity and high selection potential (Baitala et al., 2010). The number of spermatozoa can be considered as the basis for the understanding of various aspects in honeybee biology, such as mating, drone fitness, polyandry and spermatozoa competition (Koeniger, Koeniger, Tingek, & Phiancharoen, 2005).

Characteristics correlated with beekeeping production, which are less influenced by the environment and that can be controlled by management techniques, can help in the selection of colonies with higher production capacity, aiming to improve breeding programs. Knowledge of male origin is one of the tools of animal breeding programs to select breeders in the next generations (Kahya, Gençer, & Woyke, 2008).

The time required for drones to reach sexual maturity depends on their genetics, as the earlier males reach their maturity; the more competitive they become (Rhodes, Hardem, Spooner-Hart, Andersen, & Wheen, 2011). Drones mate with queens when they reach 15-23 days of age, with an average of 21 days (Couvillon et al., 2010). The reproductive success of the drones depends on the size of the colony and the conditions during the development and maturity of the individuals (Abdelkader et al., 2014).

The knowledge about the qualitative characteristics of semen may allow the development of techniques to store semen for longer periods, maintaining the quality to be used in the instrumental insemination of queens, thus facilitating the insemination schedule. Czekónska, Chuda-Mickiewicz, and Samborski (2015) found that the number of drones required to collect a semen dose is quite varied. Moreover, even when a large amount of semen from a given male is transferred to a queen spermatheca, this does not necessarily imply that many worker honeybees will be produced, because the sperm entry into the spermatheca depends on the age of the queen and the care taken before and after fertilization (Cobey, Tarpy, & Woyke, 2013).

Naturally, much of the semen deposited by males is unused, only about 10% is transferred to the queen's oviduct, while 3% to 5% is stored into the spermatheca for use in egg fertilization (Seitz et al., 2015). The nutrition of these spermatozoa occurs by the presence of sugars, amino acids, vitamins, and lipids contained in seminal fluid (Baer, Heazlewood, Taylor, Eubel, & Millar, 2009).

Based on this, the present study aimed to evaluate the production of Africanized *Apis mellifera* honeybee drones, spermatozoa longevity and viability in a group of genetically selected drones for royal jelly production, and in another group without genetic selection. In addition, the study evaluated if protein supplementation influences these evaluated parameters.

Material and methods

Two groups of colonies were established, the first consisting of six daughter colonies of a queen with genetic selection for royal jelly production, and the second consisting of six colonies without genetic selection. The colonies were established in the apiary of the Experimental Farm of Iguatemi, from the State University of Maringá in the Apiculture and Meliponiculture Laboratory.

From each of the 12 queens, 30 daughters were produced by the larvae grafting method. Subsequently, six queens from each genetic group were randomly selected with weight at emergence from 180 mg to 200 mg. After that, they were marked and introduced into the colonies to evaluate drone production.

The colonies received energy supplementation based on flaxseed and palm oils and protein based on soybean and brewer's yeast (Sereia et al., 2010), with 24.7% of crude protein. After 70 days of the queen's introduction, the colonies began to produce drones and were managed for swarm control by removing brood combs and queen cells.

For both genetic groups, the number of male broods over time was evaluated by the introduction of combs into a wooden support with 4 cm^2 (Al-Tikrity, Hillmann, Benton, & Clark, 1971). The brood was counted every 30 days, in a total period of five months. Both male production and semen collection were evaluated from August 2013 to May 2014 and from October 2013 to February 2014. For emergence, age control and subsequent semen collection, the drone combs were identified by the colony number and placed in a proper incubator with mean temperature of 34ºC (± 2) and humidity of 60% (± 10%). After emergence, in order to differentiate the colonies, the individuals were marked on the dorsal thorax with a proper ink of different colors, and then, they were returned to their original colony until they reached sexual maturity. When the colonies were receiveing only the energy supplementation, the first three semen collections were performed and evaluated over time. After these collections, the colonies were supplemented with 24.7% crude protein for 45 days, and the other three collections were performed.

About 30 drones were handled from each mother colonies for each semen mixture. For this, traps were used on the hive entrance of each colony to capture the drones, considering only the identified ones. Semen was collected individually and bottled in glass capillaries. For dilution, saline solution (0.9%) was employed to prevent dryness of semen in the capillary, being used 60% of solution for each semen part.

After semen collection, capillaries were stored at room temperature with mean temperature of 30ºC (± 2), and their viability was verified every 24 hours by evaluating motility and vigor. An aliquot of semen was deposited on a microscopy slide, covered by a coverslip and observed under phase contrast microscope in 100-fold magnification, allowing the morphological observation of spermatozoa. Motility is characterized by the presence of movement and expressed as a percentage that can vary from 0 to 100%. Vigor can be characterized within a score from 1 to 5, where 5 is the best vigor.

Statistical analysis was performed using Bayesian Inference. Such investigation becomes very interesting when the data volume is small, because unlike the frequentist models, there are no restrictions in the Bayesian model.

For the number of male brood, it was considered that the response (number of male brood, Y_i) follows the Poisson distribution with parameter λ, this is, $Y_{ij} \sim$ Poisson (λ_j), i = 1, 2, ..., n (hive) and j = 0, 1 (0: unselected e 1: selected). *A priori,* non-informative distributions were considered, this is $\lambda_j \sim$ Gama (10^{-3}, 10^{+3}) according to OpenBugs parameterization, a software (Markov Chain Monte Carlo Methods) that allows to simulate *a posteriori* distribution using MCMC algorithms.

The procedure was performed independently for each of the five sample occasions and for the total. For a comparison between the general mean of the groups (selected and unselected) and between periods (within each group), it was considered that the response follows the normal distribution. The parameters μ and τ,

respectively, mean and precision were simulated by non-informative *a priori* distributions, accordingly, $\mu \sim N(0, 10^{-6})$ and $\tau \sim Gama(10^{-3}, 10^{+3})$, such that the standard deviation is given by $= 1/\sqrt{\tau}$, according to the OpenBugs parameterization.

For drone spermatozoa viability, it was analyzed the viability time (in days), considering the model:

$$\gamma_{ij} = \mu + T_j + \varepsilon_{ij}$$

in which:
y_{ij} = observed time i, in treatment j;
the following effects:
μ = constant inherent in all observation;
T_j = treatment effect j, j = 1, 2, 3, 4 (1: group without selection and without supplement, 2: group without selection and with supplement, 3: group with genetic selection without supplement, 4: group with genetic selection with supplement);
ε_{ij} = random error associated with each observation.

It was considered that the response (Y_{ij}) follows normal distribution, that is, $Y_{ij} \sim N(\mu_j, \sigma_j^2)$, $i = 1, 2, ..., n_j$ for j^{-th} treatment levels. For each μ_j and σ_j^2 were considered a priori, respectively, $\mu_j \sim N(0, 10^{-6})$ and $\sigma_j^2 \sim Gamma(10^{-3}, 10^{+3})$, according to the OpenBUGS parameterization.

Comparisons were made between the posterior distributions of the considered groups mean values. Means were considered different at 5% significance level, the groups whose credibility intervals for the average differences did not include the value 0.

The *a posteriori* marginal distributions for all parameters were obtained through the BRugs package of the R program (R Development Core Team, 2019). Therefore, 11,000 values were generated in a MCMC process, and considering a sample disposal period of 1,000 initial values; thus, the final sample contains 10,000 generated values.

Results and discussion

Differences were found for drone production between treatments, and the unselected colonies produced more males (187.80 ± 11.15) than the selected (93.07 ± 8.88). Drone brood counts, means and their standard deviations over the five months are shown in Table 1.

Table 1. Mean number of drone brood and standard deviation for the two genetic groups.

Group		Months					Total	Mean
		October	November	December	January	February		
Selected	Mean	b 61.00 C	b 104.50 A	b 106.20 A	b 111.80 A	b 81.80 B	b 465.30	b 93.10
	Standard deviation	± 3.17	± 4.17	± 4.18	± 4.33	± 3.69	± 8.82	± 8.88
Unselected	Mean	a 135.00 D	a 228.30 B	a 267.80 A	a 206.30 C	a 101.50 E	a 938.70	a 187.80
	Standard deviation	± 4.79	± 6.20	± 6.75	± 5.88	± 4.15	± 12.56	± 11.15

[a, b] Means followed by different lowercase letters in the columns are statistically different between groups; A, B, C Means followed by different capital letters in the lines are statistically different between periods by Bayesian comparisons.

There was a difference ($p < 0.05$) in male brood production over the months, ranging from 61 offspring in October to 111.80 offspring in January in the group with genetic selection (Table 1). For the unselected group, there was also a difference over the months, with the lowest number of broods in February (101.50) and the largest in December (267.80). The group without selection obtained, on average, 153% more of produced drones than the selected colonies. Possibly the largest drone brood production in December and January was due to favorable weather conditions for the reproduction of Africanized honeybees, with high temperatures and low air humidity. In addition, summer succeeds spring, when there is a greater abundance of natural resources for honeybees to develop and start the reproductive cycle, having as one of the main characteristics the increase in male production (Smith, Ostwald, Loftus, & Seeley, 2014).

The data indicated that the protein supplementation does not increase nor influences the semen viability. For Africanized honeybees, male production represents a high investment due to the high energy and protein demand, therefore, there is a need in worker honeybees to regulate the number of males (Boes, 2010), because compels space in the comb that could be used for production of workers or food storage. For this reason, males are only produced when environmental conditions are favorable to colony growth (Amiri, Micheline, Rueppell, & Tarpy, 2017).

Bratkowski, Pirk, Neumann, and Wilde (2012) reported that the drone production has a great influence on the transmission of genetic material. Selected colonies for royal jelly production require large numbers of nursing honeybees, which means that these colonies were more efficient and produced fewer males brood (almost 50% less – Table 1). However, these colonies generated individuals with higher spermatozoa viability (more than 30%, Table 2). Baitala et al. (2010) and Parpinelli, Ruvolo-Takasusuki, and Toledo (2014), studying molecular markers, reported that there was an increase in the percentage of homozygous throughout the generations, which indicates that genetic groups tended to homozygosis for royal jelly production. Table 2 shows the time (in days) of drone spermatozoa viability by treatment and the means and deviations over five months.

Table 2. Bayesian estimates for drone spermatozoa viability time (in days) by treatment, considering the mean of three collections.

Treatment[1]	Mean	Standard deviation	Standard error	Median	$P_{2.5\%}$	$P_{97.5\%}$	σ
NS_SS	9.54 b	±0.74	0.19	9.54	8.08	11.00	2.77
NS_CS	9.86 b	±0.73	0.19	9.86	8.34	11.31	2.74
S_SS	12.21 a	±0.71	0.18	12.20	10.79	13.62	2.69
S_CS	13.27 a	±0.61	0.16	13.26	12.04	14.49	2.31

[1]NS_SS: Not selected without supplement; NS_CS: Not selected with supplement; S_SS: Selected without supplement; S_CS: Selected with supplement; [a, b]Means followed by different letters, in the same column, are statistically different by Bayesian comparisons.

The mean time viability of spermatozoa of the non-selected colonies that received protein supplementation was 9.86 days, and for the unselected that did not receive protein supplementation was 9.54 days, with no difference ($p > 0.05$). Drones from colonies with genetic selection that received supplementation obtained viability of 13.27 days, and without supplementation 12.21 days, with no difference between supplementated and non-supplementated colonies (Figure 1). Protein supplementation also did not influence spermatozoa longevity, even though the presence of proteins in seminal fluid is necessary for spermatozoa viability (Avila, Sirot, Laflamme, Rubinstein, & Wolfner, 2011).

Therefore, the results obtained for both genetic groups with food supplementation indicated that food does not influence on semen quality. However, Baer et al. (2009) considered that drone semen is rich in proteins, with about 57 proteins detected in seminal fluid, and thus, supplementation could help in the sperm quality.

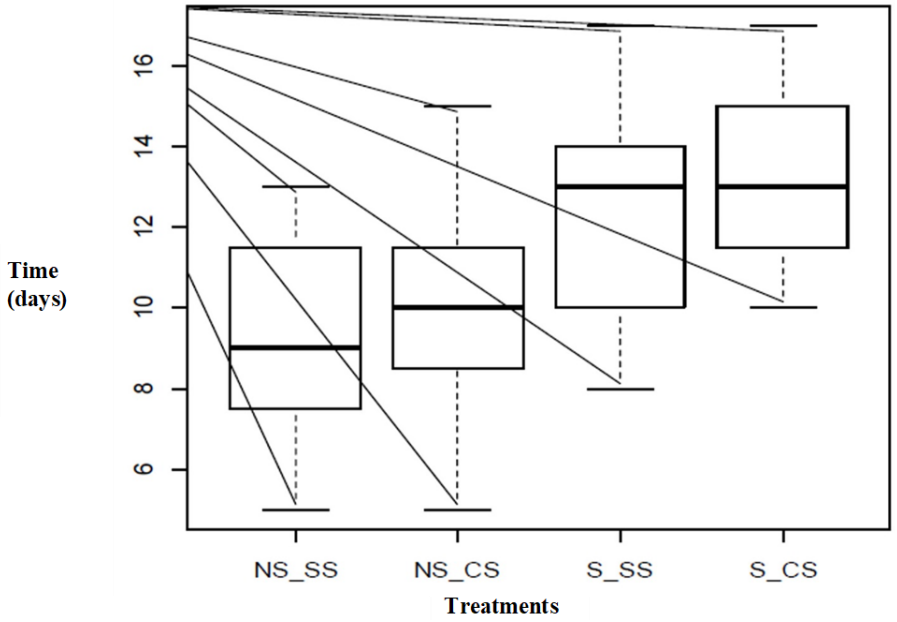

Figure 1. BoxPlot of time (days) of drone spermatozoa viability by treatment, mean result of three collections. NS_SS: Not selected without supplement; NS_CS: Not selected with supplement; S_SS: Selected without supplement; S_CS: Selected with supplement. In X axis – Treatments; In Y axis – Time (in days).

However, there was a difference ($p < 0.05$) when analyzing the means of spermatozoa viability in different groups. The unselected group obtained mean of 9.70 days, being approximately 31% lower than selected group, which presented a mean of 12.74 days. Since the cost of producing and maintaining males

alive is very high for the colony, selected colonies were more efficient than unselected colonies by producing fewer males brood (Table 1), however, these individuals presented higher spermatozoa viability (Table 2).

A mean sperm motility of 60% was observed. Billard and Cosson (1992) reported that spermatozoa motility is 1-2 min in most species, and spermatozoa movement varies during the motility phase. The same authors observed that dilution is an important factor in determining activation dynamics, which was confirmed by the optimal dilution of 60% saline solution (0.9%) for each semen sample.

The mean spermatozoa vigor value was 4 points, and this intensity of spermatozoa movements is characterized within a score from 1 to 5. To perform this evaluation, we used different semen dilutions for a 100-fold magnification, being 0% dilution, 50% and 60% diluted in 0.9% saline (Figure 2).

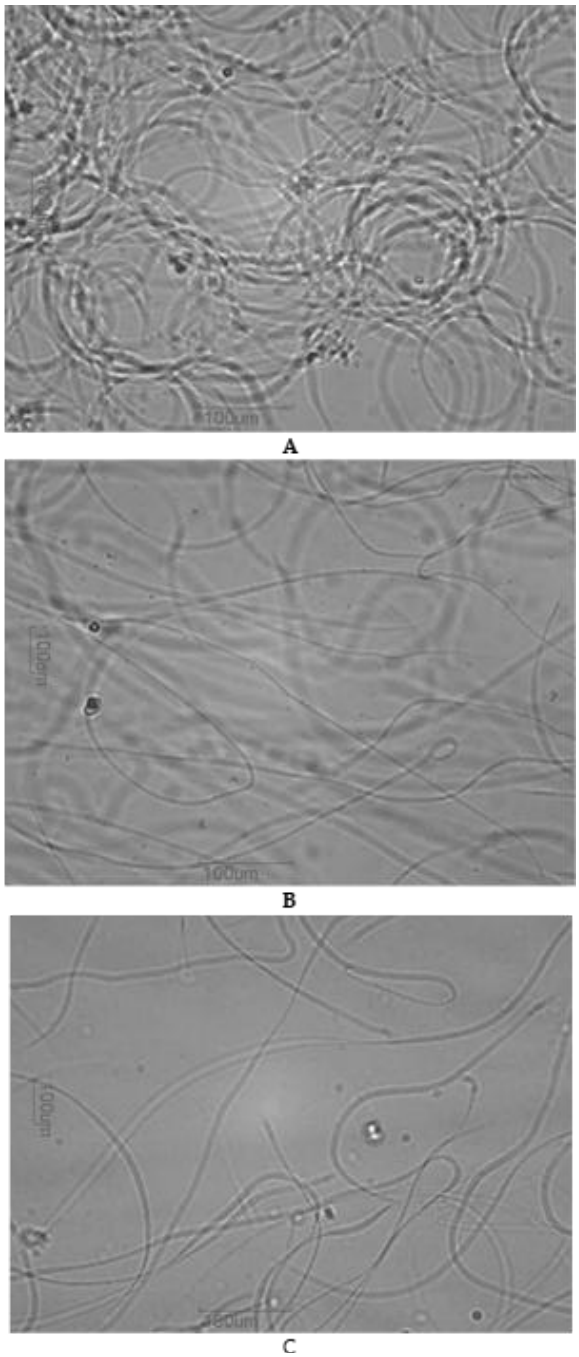

Figure 2. Drone spermatozoa in 100-fold magnification at different saline dilutions (0.9%). A: pure semen, B: 50% diluted semen, C: 60% diluted semen. The dilution was done with saline solution (0.9%).

Gençer and Kahya (2011) reported that the mean spermatozoa viability in queen oviducts after insemination is 88.10%, demonstrating the importance and care with semen collection and storage, as there

is loss throughout the process, until the arrival at the spermatheca. The same authors reported that spermatozoa collected correctly and not killed during the procedure have viability similar to that of spermatozoa in the seminal vesicles, which is 98.10%.

Maintaining spermatozoa viability with physiological vigor is critical to increase the chances of males transmitting their genes and leaving offspring. A better responsive organism can explain the longer storage life of semen from genetically selected drones, in order to protect semen by synthesizing antioxidant defense proteins and binding proteins (King, Eubel, Millar, & Baer, 2011).

Conclusion

The selected colonies for royal jelly production presented greater efficiency in the reproductive cycle of males, because despite producing fewer drones than colonies without genetic selection during the evaluated period, they produced males with 31% greater semen viability. This difference in semen quality can benefit and facilitate genetic propagation through offspring. The protein supplementation had no effect on semen quality on both kinds of the studied colonies.

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