Sociobiology 66(3): 448-456 (September, 2019) DOI: 10.13102/sociobiology.v66i3.3444

Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Infestation and Reproduction of *Varroa destructor* Anderson and Trueman and Hygienic Behavior in Colonies of *Apis mellifera* L. (Africanized Honeybee) with Queens of Different Genetic Origins

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Article History

Edited by
Cândida Aguiar, UEFS, Brazil
Received 10 May 2018
Initial acceptance 01 June 2018
Final acceptance 15 April 2019
Publication date 14 November 2019

Keywords
Honeybee selection; parasite resistance; honey production

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Abstract

The variables related to the varroa mite and the hygienic behavior in Africanized honeybee colonies were evaluated from October 2013 to June 2014. Three groups of different genetic origins were evaluated. One group consisted of queens from honeybee selection program of the Maringá State University (Maringá/PR). These queens were selected for royal jelly production by molecular markers for the expression of the MRJP3 protein. Another group consisted of queens from the queen producer Gian Bejger (Santa Terezinha/SC). These queens were selected for hygienic behavior and monitored for *Varroa destructor* Anderson and Trueman infestation. The last group was composed of queens from captured swarms/colonies that did not undergo any selection process and were chosen randomly in the apiary where the experiment was installed (Irineópolis/SC). Colonies with queens of Maringá presented an increase in the invasion rate and total reproduction of varroa as there was reduction of hygienic behavior over the evaluation period. Colonies with queens of Santa Terezinha presented the highest ($p<0.05$) hygienic behavior with an average of 92.0%. This group presented the lowest ($p<0.05$) total and effective reproduction of the mite (1.7 and 0.9 of total and fertile offspring, respectively). Colonies with queens of Irineópolis presented the lowest hygienic behavior (78.0%) ($p<0.05$) and the highest total (12.6) ($p<0.05$) and effective (5.3) ($p<0.05$) reproduction of the mite. The use of selected queens, with hygienic behavior, interferes with the varroa population dynamics, contributing to the reduction of the invasion and total and effective reproduction rates of the mite.

Introduction

The hygienic behavior of honeybees is a natural resistance mechanism to the brood diseases and parasites (Wilson-Rich et al., 2009), characterized by uncapping and removal of dead and diseased brood, being an inherited characteristic (Rothenbühler, 1964). The *Varroa destructor* Anderson and Trueman mite (Anderson & Trueman, 2000) is an ectoparasite of brood and adult individuals of *Apis* bees. Since the *V. destructor* introduction in Brazil for more than 30 years, infestation levels remain low and do not cause potential damage to colonies (Junkes et al., 2007; Wielewski et al., 2013; Schafaschek et al., 2016).

Several factors contribute to this condition, among them the presence of the Africanized honeybee, which are more resistant than the European ones, mainly due to the reduced female fecundity of the *V. destructor* in the brood cells of Africanized *Apis mellifera* L. workers (Rosenkranz et al., 2010). Other important characteristics that limit the mite population are grooming behavior (Moretto et al., 1993; Moretto et al., 1995; Nganso et al., 2017), mite mortality in adult honeybees (Evans & Spivak, 2010), removal of varroa-infested brood (Guerra Jr et al., 2000), descendant mites mortality (Mondragón et al., 2006; Rivera-Marchand et al., 2012). The climatic conditions and the time of year

also influence the reproductive capacity of varroa (Moretto et al. 1991; Currie & Tahmasbi, 2008), with mite females producing more offspring during pollen production than in other year seasons (Mondragón et al., 2005).

It is also found that there is a genetic association between the hygienic behavior and the infestation level in adult honeybees by *V. destructor* mite (Arechavaleta-Velasco et al., 2012; Tsuruda et al., 2012). The Africanized honeybees selection for the hygienic behavior characteristic decreases the total reproduction rate of the mite and, consequently, the infestation rate in adult honeybees (Wielewski et al., 2012). Some strains of honeybees have the ability to keep the mite population at low levels, apparently by reducing the reproductive success of mites in worker brood (Harris et al., 2012). This behavior related to the removal of varroa-infested brood was described by Harbo and Harris (2009) as "*Varroa Sensitive Hygiene"* (VSH). The two main characteristics of this behavior are the removal of infected brood and the high frequency of non-reproductive mites.

Recently has been found changes in the *V. destructor* reproduction rates in several regions of Brazil that have been attributed to the change from the Japanese haplotype (J) to the Korean haplotype (K) of varroa (Strapazzon et al., 2009). Carneiro et al. (2007) found an increase in the reproductive capacity of *V. destructor* in State of Santa Catarina, where the mite fertile female percentage increased from 56% in the 1980s to 86% in 2005/2006. The difference in the female percentage that produced deutonymphs, that is, female offspring that can reach the adult stage was of 72% in 2005/2006, against 35% in 1986-1987.

The recent arrival of the K haplotype in Brazil (Garrido et al., 2003; Guerra et al., 2010) requires further investigation about the dynamics of this parasite-host interaction, as it is still little known whether the effects intensity caused by *V. destructor* depends on honeybee genotype, mite genotype or of the both (Calderón et al., 2010).

The objective of this research was to evaluate the relationship between the hygienic behavior traits, infestation and invasion rates, as well as the reproductive success of the *V. destructor* mite, in colonies of Africanized honeybees with queens of different origins and selected for different traits. The hypotheses tested were: i. queens originated from a selection process present higher hygienic behavior; ii. The higher hygienic behavior interferes in the reproductive dynamics of *V. destructor*, reducing reproduction rates and consequently the infestation levels in adult honeybees.

Material and methods

The experiment was carried out in an apiary located in the Irineópolis municipality, in the State of Santa Catarina, Brazil, at latitude 26°17'29" South and longitude 50°51'18" West, with an altitude of 762 m. The predominant climate in the region is the humid mesotherm, with mild summers (temperate or Cfb of Köppen). The predominant vegetation is mixed ombrophilous forest, with secondary vegetation and areas of agricultural activities.

Three groups of queens were evaluated, coming from different regions and selected by different processes for different traits. One group consisted of queens from honeybee selection program of the Maringá State University, from Maringá city, located in the Northwest region of the Paraná State, located at latitude 23°25'30" South and longitude 51°56'20" West, with altitude of 550 m and humid mesothermic climate, with hot summers (subtropical or Cfa of Köppen). These queens were selected for royal jelly production by molecular markers for the expression of the MRJP3 protein (Baitala et al., 2010), showing a tendency of homozygosis for this locus (Parpinelli et al., 2014) after years of selection.

Another group consisted of queens from the queen producer Gian Bejger of the Santa Terezinha municipality, in the State of Santa Catarina, located between the limits of the northern plateau of Santa Catarina and the upper Itajaí valley, at latitude 26°46'43" South, longitude 50°00'29" West and altitude of 610 m, with predominant moist mesothermal climate with mild summers (temperate or Cfb of Köppen). These queens were selected for hygienic behavior and monitored for *V. destructor* infestation. The last group was composed of queens from captured swarms/colonies that did not undergo any selection process and were chosen randomly in the apiary where the experiment was installed (Irineópolis / SC) described previously. For all groups evaluated, the queens were produced using the Doolittle adapted method (1889). Queens were mated in the air in local from origin of the groups using individual mating nucs.

The queens were introduced in the experimental apiary from December 2012 to April 2013 in 40 standard Langstroth hives with ten frames each, being 15 colonies from Maringá/PR, nine colonies from Santa Terezinha/SC and 16 colonies from Irineópolis/SC. From these 40 colonies, those that survived the winter of 2013 were evaluated, being four from Maringá, four from Santa Terezinha and 12 from Irineópolis groups. In the region of the experimental apiary, the winter is from June 21 to September 21. The loss of many colonies in the winter of 2013, can be explained by a severe winter occurred, including the occurrence of snow, that is uncommon in the region. The establishment of the colonies with queens from other regions may have been difficult because an influence of climatic variables compared of the colonies with local queens, as verified by Schafaschek et al. (2016). The introduction of the queen so late December to April, may have been difficult the initial development of this colonies because a shortage of flowering in this period in the region. Therefore, many colonies from this group were lost in the winter.

Avoiding swarming and to provide space for colonies development, it was adopted in management the replacement of two to four combs with brood and feed by frames with new wax foundation, as the colonies presented total occupation

of the nest combs. Thus, the provision of space for the performance of the queen laying was provided according to the need of each evaluated genetic group, allowing the expression of its real development potential. The queen laying on the supers was avoided using queen-excluder screens. The supers were added according to the development of each colony and the nectar flow in the region.

The evaluations were carried out monthly, from October 2013 to June 2014. The infestation percentage by *V. destructor* in adult honeybees, pupae invasion rate, mite total and effective reproduction, hygienic behavior of honeybees, total colony weight and honey productivity were evaluated.

The *V. destructor* infestation in adult worker honeybees was evaluated using the method described by Stort et al. (1981), in which approximately one hundred adult worker honeybees were collected from a comb and placed in a bottle with alcohol 70%. Subsequently, stirred the contents and performed the separation of honeybees from the mites to evaluate the colony infestation, in percentage. The invasion rate in pupae was evaluated according to the method proposed by De Jong and Gonçalves (1981). This method included the retrieval of a comb of worker-capped brood from each colony and the removal of 100 worker pupae (50 from one side of the comb and 50 from another side) with brown eye and body at the beginning of pigmentation.

Adult female mites and their offspring (eggs, protonymphs and deutonymphs) in pupae and alveoli were analyzed by attached light and counted to obtain the invasion rate and the total and effective reproduction. The invasion rate in worker pupae was obtained by the formula:

Invasion rate (%) = (number of invaded pupae/number of analyzed pupae) x 100.

Total reproduction (RT), represented by the total number of offsprings produced by mite, was determined by the formula:

RT = total number of offsprings/number of original adult female mites.

The mite effective reproduction (RE), that is, the number of viable progenies for reproduction, was determined by the formula:

RE = (number of deutonymphs + young adults)/nº of original adult mite females.

The hygienic behavior, represented by the percentage of uncapped brood cells (%), was evaluated using the Rothenbühler adapted method (1964). For this purpose, a comb containing pupae of workers corresponding to the pupal stage with pink eyes (17 to 18 days) was collected from each colony. A 5 x 6 cm comb section (approximately 100 capped brood-pupae of the comb) was cut and then frozen for 24 h. This section was photographed for later counting of the number of capped cells. After 24 h of freezing, the sections were returned to the respective colonies and, again, photographed 24 h after the return, for analysis of the uncapped wells.

The colonies were weighed at night, when all worker honeybees were inside the hives. The hive weight was considered with the combs and the weight of the empty supers, feeder and queen excluder screen was discounted to calculate the weight of the colonies. The honey yield was evaluated after two harvests, carried out in November and December of 2013, being calculated by the weight difference between full and empty supers after extracting the honey.

Statistical analyzes of the differences in hygiene behavior traits, varroa invasion rate in worker pupae, total reproduction, effective reproduction and varroa infestation in adult honeybees according to the origin of the queen and the evaluation period as well as the interaction between these two factors were performed using the generalized linear models procedure, by PROC GENMOD of SAS version 9.3 (SAS, 2012). The binomial distribution with logit binding function was admitted for the hygienic behavior and varroa invasion rate in worker pupae and the Poisson distribution with log binding function was used for the total reproduction, effective reproduction and varroa infestation in adult honeybee traits. The means for these traits were compared by the t-test at the 5% level of significance. For this test the means were compared two by two.

The colony weight data were submitted to variance analysis, implemented in PROC GLM of SAS version 9.3 (SAS, 2012), and linear regression curves were estimated as function of the evaluated months. The means for different groups of queens were compared by Tukey test at the 5% level of significance.

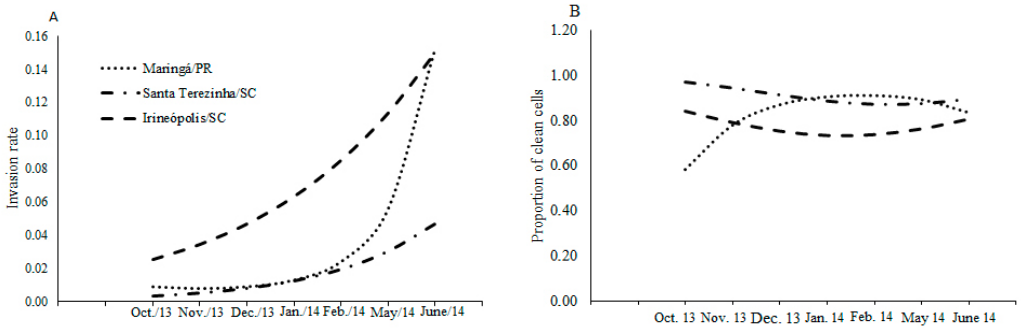

Fig 1. Invasion rate by *Varroa destructor* in worker pupae (A) and hygienic behavior of *Apis mellifera* bees (B) in colonies with queens from Maringá/PR, from Santa Terezinha/SC and from Irineópolis/SC.

Results

There was an interaction effect between the origin of the queen and the evaluation period for the varroa invasion rate in worker pupae (Fig 1 A). The hygienic behavior showed effect of the origin of the queen and interaction between the origin of the queen and the time of evaluation (Fig 1 B).

There was also interaction between the origin of the queen and the evaluation time for the number of adult female mites in worker pupae cells. Thus, this variable was not included in the evaluation model of total offspring number and the fertile offspring number. The number of adult mites had quadratic interaction with the total offspring number and linear interaction with the fertile offspring number. The total and effective reproduction had an effect of the origin of the queen for the Maringá and Santa Terezinha groups and interaction effect between the origin of the queen and the evaluation period for the three groups evaluated. This interaction was quadratic for Maringá (Fig 2 A) and Irineópolis (Fig 2 C) groups and linear for Santa Terezinha (Fig 2 B) group.

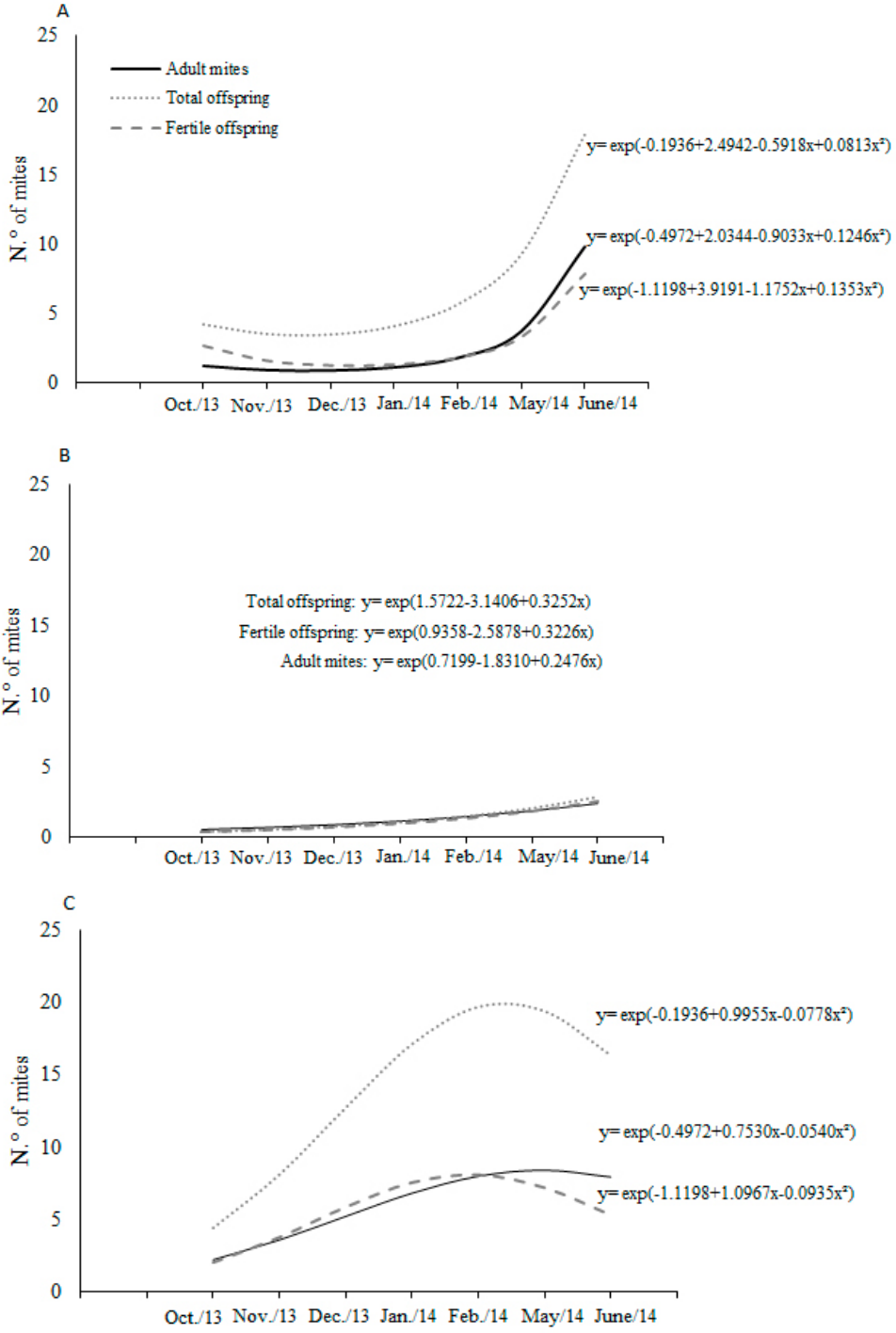

Fig 2. Number of adult mites in worker cells. number of total offspring and number of fertile offspring of *V. destructor* in *A. mellifera* bee colonies with queens from Maringá/PR (A), Santa Terezinha/SC (B) and Irineópolis/SC (C).

Varroa infestation in adult honeybees had an isolated effect of the origin of queen and the evaluation period (Fig 3). As expected, the number of mites detected in the samples was in function of the number of collected honeybees. So to control the sampling, the number of collected honeybees was considered as covariate in the model in order to improve accuracy and reduce model error. It was found that the lowest infestation rates occurred in November and December (Fig 3). The mean infestation rates are presented in Table 1.

The hygienic behavior was related to the varroa invasion rate in pupae and mite total reproduction. However, this relationship presented different behavior for each queen group. It was found, by means of the equation derivation, that for the queens from Maringá, when a decrease of the hygienic behavior began, near February of 2014, there was a marked increase of the varroa invasion rate in pupae and in the its total reproduction. Effective reproduction also increased, but in a later period, as of May 2014. The mean for this variable over the period was 2.3 fertile offspring and the hygienic behavior of 84.0% (Table 1). Differently from the other groups of queens, the Maringá group had a reduction of the mite effective reproduction until February of 2014 (Fig 2 A).

The Santa Terezinha group also presented an increase in the varroa invasion rate in pupae from the reduction of hygienic behavior (Fig 1). However, they had high rates of hygienic behavior and remained stable throughout the period, with an average of 92.0%. They also presented the lowest ($p<0.05$) values of total reproduction (1.7), and effective reproduction (0.9) of the mite (Table 1).

The Irineópolis group presented the lowest mean ($p<0.05$) of hygienic behavior (78.0%), occurring an increase in the invasion rate, with the decrease of this behavior. They presented maximum value of total and effective reproduction in February 2014 (Fig 2 C), when the lowest values of hygienic behavior were observed (Fig 1 C). This group presented the highest mean ($p<0.05$) of total reproduction and the effective reproduction (Table 1).

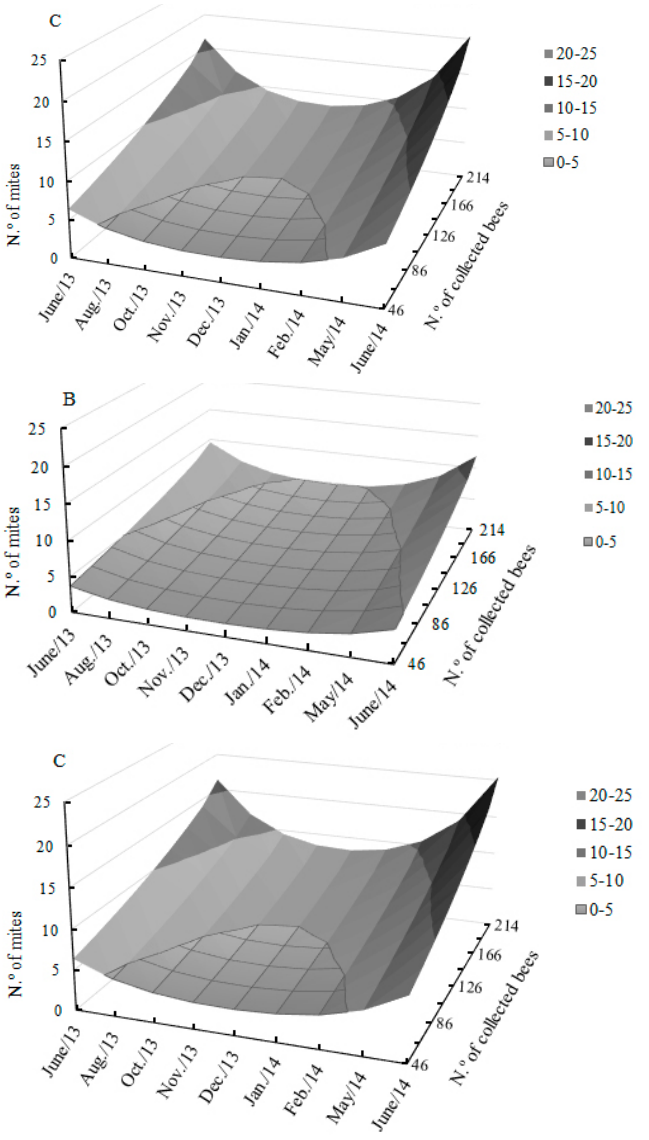

Fig 3. Infestation by *Varroa destructor* in *Apis mellifera* bee colonies with queens from Maringá/PR (A), Santa Terezinha/SC (B) and Irineópolis/SC (C).

Table 1. Mean ± standard error, for the analyzed variables, from October 2013 to June 2014, in colonies with queens from Maringá/ PR, Santa Terezinha/SC and Irineópolis/SC, in Irineópolis, Santa Catarina State, Brazil.

	Varroa invasion rate in worker pupae (%)	Hygienic behavior (%)	Total reproduction (number of total descendants)	Effective reproduction (number of fertile offspring)	Varroa infestation in adult bees (%)
Maringá/PR	2.0±0.10 b[1]	84.0±0.06 b	5.7±0.09 b	2.3±0.14 b	4.9±0.07 b
Santa Terezinha/SC	1.0±0.20 c	92.0±0.08 a	1.7±0.16 c	0.9±0.23 c	3.5±0.96 c
Irineópolis/SC	6.0±0..06 a	78.0±0.03 c	12.6±0.04 a	5.3±0.06 a	6.1±0.45 a

[1]Means followed by the same letter in the same column are not statistically different ($p> 0.05$). by the t test.

As expected, there was an effect of the evaluation period on the colonies weight (Fig 4), with a significant reduction in the weight from January. This was possibly due to the reduction in the bee population due to the decrease in the availability of food resources in the environment.

The honey productivity was evaluated only for the groups from Santa Terezinha and Irineópolis. The Maringá group delayed the honey storage in the supers. This group presented a rapid development of the colony, driven by the high rate of laying of the queens. However, the use of the

queen excluder screens, in this case, provided little space for the colony development, causing high swarming rates in the colonies of this group. This fact did not allow sufficient workers to carry out nectar storage, and consequently, honey production was impaired. So, this group presenting an insufficient amount of honey and only at the end of the apicultural crop, that is, at the end of December and beginning of January. Due to the shortage of flowering from this period, it was decided not to harvest the honey in the Maringá group not to harm the survival of the colonies. There was no significant difference in honey productivity between Santa Terezinha and Irineópolis groups. The mean honey production was 16.3 kg/colony for Santa Terezinha and 13.4 kg/colony for the Irineópolis groups.

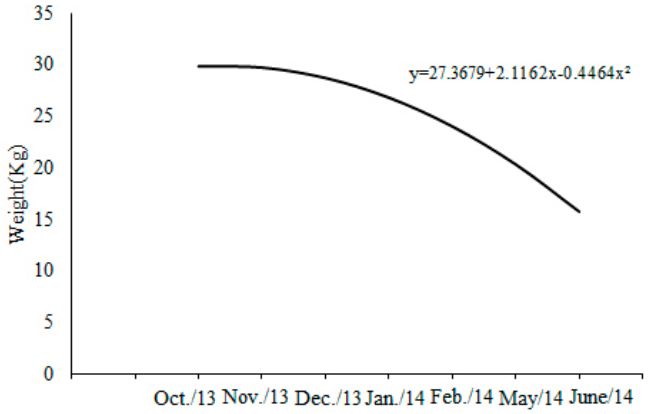

Fig 4. Weight of *Apis mellifera* bee colonies regardless of the queens' origin from October 2013 to June 2014.

Discussion

The results found in this research are in agreement with Wielewski et al. (2012), who found that the selection of Africanized honeybees for hygienic behavior traits reduces the infestation rate in adult honeybees by varroa, by reducing the mite total reproduction. These authors obtained values of total reproduction of 1.02 individuals for the same lineage of the Maringá group, evaluated in Maringá / PR as well as infestation rates of 8.3% and invasion rates of 9.5%. However, in this experiment the mean value of total reproduction for Maringá group was higher (5.7), infestation rates (4.9%), and invasion rates (2.0%) were lower than those found by Wielewski et al. (2012).

The total reproduction values obtained for the Santa Terezinha group (1.7) are similar to those detected by Moretto et al. (1996), which found a total reproduction of 1.7 descendants evaluating colonies of Africanized honey bee in the Rio do Sul municipality/SC, in the period of 1986/1987. Carneiro et al. (2007), however, found changes in the *V. destructor* reproductive capacity in the State of Santa Catarina, after 20 years, where there was a 60% increase, with females leaving around 2.6 descendants in the region of Blumenau/SC.

More recently, Carneiro et al. (2014) evidenced an increase of 22% in relation to Carneiro et al. (2007), with a mean number of 3.2 descendants per reproductive cycle. Besides, the beekeepers of the States of Santa Catarina and Rio Grande do Sul are bringing thousands of European queen honeybees from Argentina in last years, what can explain this arise in mite reproduction. The selection process of the Santa Terezinha group, based on high hygienic behavior and low varroa infestation, was probably the one that input the trait of reduced total and effective reproduction, associated to the high hygienic behavior of this group.

The introduction of genetically selected queens in this experiment, contributed to the reduction of total and effective reproduction of mite, since the group of Irineópolis queens, which did not undergo a rigorous selection process, presented a total and effective reproduction significantly high (12.6 and 5.3, respectively). This group also had the highest rates of infestation in adult honeybees (6.1%) (Table 1). The results agree with Wielewski et al. (2012), where the low total reproduction represented low invasion and infestation rates by varroa for the groups with selected Africanized honey bee queens. Harbo and Harris (2001) also found that queens selected for decreased varroa mite reproduction maintain this trait even when free-mated with unselected drones, working with European honeybee.

The finding that the Maringá group presented reduction of the effective reproduction until the month of February of 2014, is possibly due to some traits of this group acquired by the selection process. Despite the increase in hygienic behavior until the month of February, there was no reduction in total reproduction, except for effective reproduction. Some important traits that limit the mite population in Africanized *A. mellifera* are the grooming behavior, adult bees mite mortality (Junkes et al., 2007) and descendants mite mortality (Mondragón et al., 2006).

The selection of honeybees for hygienic behavior possibly also selected for these other limiting traits for mite population in the colony. Calderón et al. (2010) reported that the reduction of the varroa mite reproductive capacity is the most important factor in the tolerance of honeybees to this parasite. The limitation of mite growth population due to the reduction of its reproductive capacity was also found in colonies of European honey bee by Locke and Fries (2011). Pinto et al. (2012) found a negative correlation between invasion rate and hygienic behavior in Africanized honeybees from Taubaté and Viçosa, in the southeastern region of Brazil, suggesting that colonies with higher hygienic behavior have the lowest rates of varroa invasion.

Another factor explaining these results is that the Maringá group, from a honeybee selection program, has been working since 2007 with molecular markers for the expression of the MRJP3 protein involved in the royal jelly production (Baitala et al., 2010). Parpinelli et al. (2014) indicated that these honeybees tended to homozygosis for this locus. Li et al.

(2008), analyzing the proteins of the hypopharyngeal glands of winter honeybees, selected for high royal jelly production, detected a great variety of proteins, similar to those found in royal jelly, especially MRJP3. MRJPs are the main proteins present in the royal jelly secreted by the hypopharyngeal glands of the workers. Studies on the influence of brood attractiveness on varroa invasion rate have shown that queen larvae and queen larvae extracts were significantly less attractive than worker and drone larvae and that the royal jelly presented a repellent effect (Calderone et al. 2002).

In general, the levels of varroa infestation in adult honeybees found in this study were low. However, it was found that the groups with selected queens, Maringá group, selected by molecular markers for the MRJP protein expression and the Santa Terezinha group, selected for low *V. destructor* infestation and high hygienic behavior had the lowest rates of varroa infestation in adult honeybees, while the Irineópolis group, with unselected queens, had the highest varroa infestation. The values found for Maringá and Santa Terezinha groups confer with the infestation indexes found by Strapazzon et al. (2009) and Carneiro et al. (2014), in the region of Blumenau/SC, which remained around 3.0% and 4.1%, respectively.

The infestation levels observed in this research presented a reduction in the spring and summer months, increasing from the end of summer and reaching the highest levels in the autumn months for the three groups of evaluated queens (Fig 3). The results are similar to those obtained by Toledo and Nogueira-Couto (1996), who found that the infestation rate in adult Africanized honeybees decreased from winter to spring decreased in the summer and increased again in the fall. This behavior was possibly due to the reduction of the honeybee population in the colonies in the following period at the end of the summer and during the winter, leading to a higher concentration of the mites on the remaining honeybees.

The delay in the honey storage in colonies with queens from Maringá may have been influenced by the management adopted with the use of queen excluder. The limited space in the nest due to the use of these screens may have caused the swarming of the colonies with higher posture capacity. This provided less population of foraging honeybees during the period of greater nectar flow, which occur from September to November, delaying the storage for the harvest.

The results corroborate with Faquinello et al. (2011) who found that superior genotypes can express themselves differently depending on the production system to which they are submitted. Schafaschek et al. (2016) also found that queens from different regions had their development influenced by the adopted management and local climatic variables, with advantage to the adapted local queens. Therefore, it is recommended the adoption of different management for colonies with queen from other regions. As well as, selected queens for high production and with high laying ability need be provided with ideal conditions for production, as space for laying and abundant food. This may contribute to the higher expression of productive potential of the selected strains.

The results confirm the first tested hypothesis that the queens originated from a selection process present higher hygienic behavior. In the experiment the queens originated from selection processes (Maringá/PR and Santa Terezinha/SC) had the highest mean of hygienic behavior. The second hypothesis tested, that the higher hygienic behavior interferes in the reproductive dynamics of *V. destructor*, reducing reproduction rates and consequently the infestation levels in adult honeybees, was also confirmed. It was found that the high hygienic behavior promoted the reduction of the invasion, total and effective reproduction rates of the *V. destructor* mite and in the infestation levels in adult honeybees.

Acknowledgment

To the beekeepers Gian Bejger and Domingos Bejger, for the donation of queens of one evaluated group. To the Bee Research Group - GPBee, of the Maringá State University, for aid in queen production. To CNPq, processes numbers 479329/2009-5, 308283/2011 and 311663/2014-1, and Fundação Araucária protocol 15095 - agreement 422, for the financial support.

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