MORPHOLOGICAL DIVERSITY AND HYGIENIC BEHAVIOR OF *APIS MELLIFERA UNICOLOR*

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ABSTRACT

The characterization of bees is important for optimized and sustainable management of beekeeping systems. To this end, morphometric and hygienic behavior studies of the Apis mellifera unicolor bee from three different regions (Centre, South-East and West) were carried out. From a morphological point of view, two bee strains differing in coloration were identified in these regions: black bee strains and yellow bee strains. In some areas (western region), a cohabitation of yellow and black bees was observed in the same hive. Morphometric measurements revealed a biogeographical variation in the regions studied, which can be divided into three groups: central bees with long front and rear wings and a large pollen sac, western and south-eastern bees with a long proboscis and a well-developed mentum, and finally central-eastern bees with long legs and a well-developed tibia. The pin test was used to study hygienic behavior. The results showed that there were no significant differences between intact, uncapped, partially cleaned and cleaned cells from the regions studied, with the exception of cleaned cells from western regions. A 13.07% decrease in hygienic capacity was observed in the central regions when comparing our results with previous studies.

Key words: *Morphometry, Hygienic behavior, Varroa destructor, Apis mellifera unicolor*

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1. INTRODUCTION

Honey bees play an important role in the pollination of various cultivated and wild plant species, and in agricultural production (honey, pollen, wax, royal jelly, propolis). Therefore, they actively contribute to the development and safeguarding of the biodiversity of ecosystems by promoting the sustainability of flowering plants. They are also considered as a true bio-indicator of the health of the environment [1].

Madagascar has an endemic bee species described as *Apis mellifera unicolor* [2; 3]. It is characterized by its uniform black color and small size [3]. According to Ralalaharisoa Ramamonjisoa in 1992, the Malagasy bee subspecies presents two ecotypes: an ecotype of altitude characteristic of the Highlands, which is not very aggressive, and an ecotype of the coastal regions of low altitude, more aggressive [4]. When nectar and pollen resources are in sufficient quantities, the first type forms relatively large sedentary swarms while the second type forms smaller, very easily migratory colonies. Moreover, the latter accumulates reserves in lesser quantity [5].

Genetically, *Apis mellifera unicolor* belongs to the African evolutionary lineage [6]. Genetic studies conducted in 2012-2014 showed that bee populations in Madagascar were organized into two genetic subclusters that would correspond to their geographic distribution [7].

In February 2010, *Varroa destructor*, the main threat to beekeeping in the world, was officially declared to be present in Madagascar [8; 7]. Since then, it has spread to many regions of the island.

Despite all the measures (destruction of the first outbreaks, ban on transhumance and import of acaricide products) taken to control its spread, *V. destructor* continues to spread and impact beekeeping production.

Since its arrival in Madagascar, *V. destructor* has been considered the main threat to beekeeping. Co-evolution between this parasite and *A. m. unicolor* is still in its infancy, and a balance between the two species has yet to be achieved. As a result, many colonies that have not received acaricide treatment have been decimated [7].

Like all social insects, bees use a number of mechanisms to defend themselves. One of these defense mechanisms is hygienic behavior (HB). HB is the main mechanism by which worker bees detect diseased brood, uncap the cells and remove infested larvae or pupae [9]. This behavior is of major interest in the control of major brood diseases such as American foulbrood [10].

In this study, we propose to study the morphometric diversity of *A. m. unicolor* bees to identify the different ecotypes or races of bees found in different regions of the island, and the hygienic behavior of bees to assess the different levels of tolerance or resistance to varroa mites in different regions.

2. MATERIAL AND METHODS

2.1 Study sites

The investigation was done from October to December 2019 in three different localities of Madagascar. The first site was the rural commune of Antsenavolo, district of Mananjary, Vatovavy region (South-East Region), which benefits from a humid tropical climate. It extends between longitude 48°02'56.29"E and latitude 21°23'32.1 "S. The average temperature for the commune of Antsenavolo was 28°C with a maximum of 30°C and a minimum of 25°C (from October 2019 to December 2019). Fort Duchesne (Central Region) is located in the city of Antananarivo between longitudes 47°32'46.9 "E and latitude 18°54'33.5 "S. The site has a subtropical climate with dominant summer rains characterized by a hot and humid summer. The average temperature was 23°C with a maximum of 29°C and a minimum of 18°C (from October to December 2019. The relative humidity was 84% in November and 86% in December2019. The last site was Antanimasaja, it is located between longitude 46°20'43.1 "E and latitude 15°43'01.8 "S in the city of Mahajanga (Western Region). It has a hot and dry tropical climate. The average temperature was 30°C with a maximum of 33°C and a minimum of 26°C (from November 2019 to December 2019).

2.2 Morphological study

The study took place from October to November 2019. The bee samples were collected in the eco-climatic zones of the West (Maintirano, Mahajanga), the Center (Antananarivo), the Central East (Fandrina, Ambositra) and the Southeast (Mananjary). A total of 11 apiaries and 37 hives of Langstroth type (Central and Southeast regions) and Dadant (Western region) were visited for bee sampling . In each apiary, three to four hives were observed and bees were collected. Depending on the availability of biological material and the voluntary participation of beekeepers in the study, 30 workers were collected from each location with at least 10 bees per hive.

The collection was performed on bees inside the hive. They were put in a transparent glass bottle of 100ml and killed with ether. Then in the lab, they were counted and measured according to different morphometric characters. Measurements were taken under a microscope with a graduated ocular at the agricultural entomology laboratory of the University of Antananarivo. The magnification used was 10 times for the observation of legs, wings and proboscis and 20 times for the observation of pollen sacs and the cubital index [11;12;13]. During the collections, the behavior of the bees was noted (aggressiveness, frame holding).

In this study, two statistical analyses were performed. The analysis of variance (ANOVA) was used to compare the different sites and the 18 morphometric traits of bees (gloss, paragloss, Paragloss, proboscis, forewing length, front wing width, posterior wing length, posterior wing Width, cubital index, hip, trochanter, coxa, tibia, metatarsal, tarsal, hind leg length, pollen bag length, pollen bag width) . The Principal Component Analysis (PCA) was carried out to identify groups of variables that were highly correlated with each other and to make a grouping of individuals in relation to the variables studied and the axes carrying the information.

2.3 Hygienic behavior (HB)

HB tests were done from November to December 2019: November 28th for the South-East region (municipality of Antsenavolo), December 04th for the Central region (municipality of Manjakandrina) and December 11th for the West region (Antanimasaja). For this study, 45 hives were involved including 15 hives per region.

Bee broods were killed to measure the hygienic behavior of the bee colony [14;15]. The pin test method was chosen over the freezing method because it is easier to apply in the field, less expensive to implement, and requires less time. The tendency of honeybees to dispose of dead broods is faster [16].

One frame of brood cells was taken from each hive. Two sections of brood cells, each containing 25 capped cells, with 50 cells per colony, were selected. In each cell, the brood was killed with an entomological pin n°2. The pin was inserted in the middle of the cell and pushed into the brood body until it reached the base of the cell. The tested frame was quickly replaced in the hive after the puncture, noting the time (T0). At 6 h after perforation, the frame was removed again and photographed. Then, the number of cleaned and uncleaned cells was counted on the photos taken in the lab. For the reading of the results, the punctured cells were classified into four different categories: intact cells (I), uncapped cells (A), partially cleaned cells with brood still remaining (B), cleaned cells, that is to say completely uncapped and the brood removed (C).

Data analysis was performed using R software version 3.5.1 (R Core Team, 2018). The residual distribution of the data was visually checked for normality. The distribution of cleaned cells rate, measured 6 h post-puncture, was examined considering the entire data set (N = 45) and for each region separately (15 colonies from the central region, 15 colonies from the southeastern region, and 15 colonies from the western region). An analysis of variance (ANOVA) was performed using the general linear model procedure (glm function, quasipoisson family, identity link) and mean separations using the chi-square test (Chisq function). A Tuckey test was then used.

3. RESULTS

3.1 Morphological differences between strains

Two different morphotypes of bees in terms of coloration were observed in the surveyed areas. In the western part, hives with black bees, yellow bees, and composites of both morphotypes (black and yellow bees) were observed. In the other sites (Central and Southeast), there was only one morphotype of bee, black bees.

To complete this study, measurements on 18 morphometric characters were performed on these different populations: Forewing length, Pollen bag length, Posterior wing length, mentum, trochanter, proboscis, gloss. tarsal, pollen bag width, coxa, metatarsal, posterior wing Width, cubital index, tibia, Hind leg length, Front wing width, paragloss and hip. The results showing significant values are recorded in the following table – 1.

Table – 1: Morphometric characteristics of bees

Groups	N	Central East		West		Southeast	Center
Sites		*Fandriana*	*Ambositra*	*Mahajanga*	*Maintirano*	*Mananjary*	*Fort Duchesne*
Glosse	30	3.01a ± 0.06	2.96b ± 0.11	2.79c ± 0.14	2.74c ± 0.35	2.78c ± 0.32	2.45d ± 0.08
Mentum	30	1.53d ± 0.06	1.52d ± 0.07	1.81a ± 0.03	1.81a ± 0.05	1.77b ± 0.04	1.66c ± 0.07
Proboscis	30	5.8b ± 0.012	4.97a ± 0.14	5.15b ±0.01	5.2a ± 0.36	5.09 a ± 0.03	4.71c ± 0.12
Forewing length	30	8.04c ± 0.06	8.09b ± 0.07	7.99d ± 0.03	8.02c ± 0.04	8.03c ± 0.05	8.24a ± 0.08
Hip	30	1a ± 0.05	1a ± 0.06	0.98a ± 0.06	0.95b ± 0	0.95b ± 0.02	0.70c ± 0.01
Trochanter	30	0.62c ± 0.06	0.67b ± 0.06	0.64c ± 0.04	0.72a ± 0.06	0.62c ± 0.05	0.57d ± 0.02
Tibia	30	2.81c ± 0.07	3a ± 0.19	2.98a ± 0.02	2.5d ± 0.06	3.03a ± 0.03	2.9b ± 0.10
Hind leg length	30	10.52a ± 0.16	10.51a ± 0.28	9.98b ± 0.16	10.38c ± 0.14	10.6a ± 0.33	10.25b ± 0.19
Pollen bag length	30	1.63a ± 0.06	1.56a ± 0.05	1.62a ± 0.04	1.53b± 0.05	1.54b ± 0.04	1.691b ± 0.06

a,b,c,d- differences significant at p ≤ 0,05

The correlation circle was determined by representing the 18 morphometric traits on the factorial plane, the first two axes represented a cumulative inertia rate of 44.44% and 32.70% of the total variation.

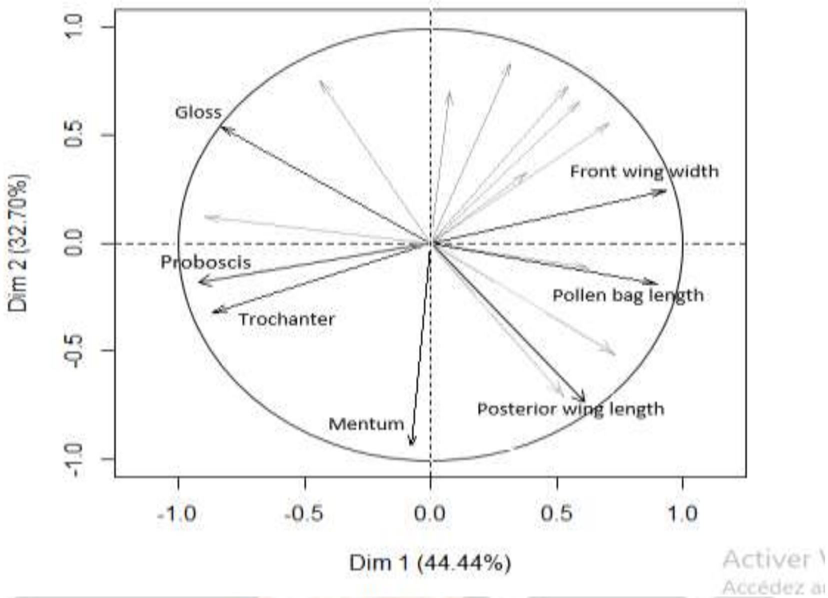


Figure -1: Graphical representation of the 18 morphometric variables within the correlation circle of the factorial design.

The variables were organized into two groups (Figure 1):

The first group was mainly composed of 7 significant variables with high contribution (higher or equal to 5%). Within this group the Forewing length, Pollen bag length, Posterior wing length, mentum , trochanter, proboscis, and gloss.

The second group corresponded to 11 morphometric characters. They were tarsal, pollen bag width , coxa, metatarsal, posterior wing Width, cubital index, tibia, Hind leg length, Front wing width, paragloss and hip.

Figure -2 shows the analysis of point clouds on the factorial plane.

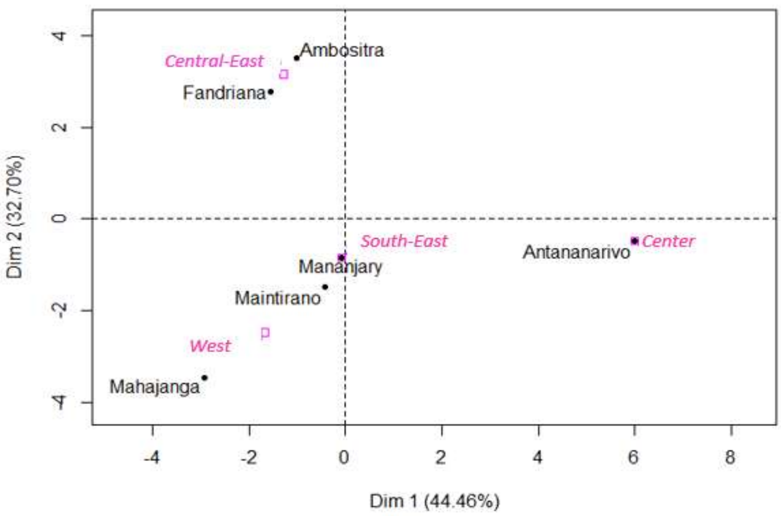
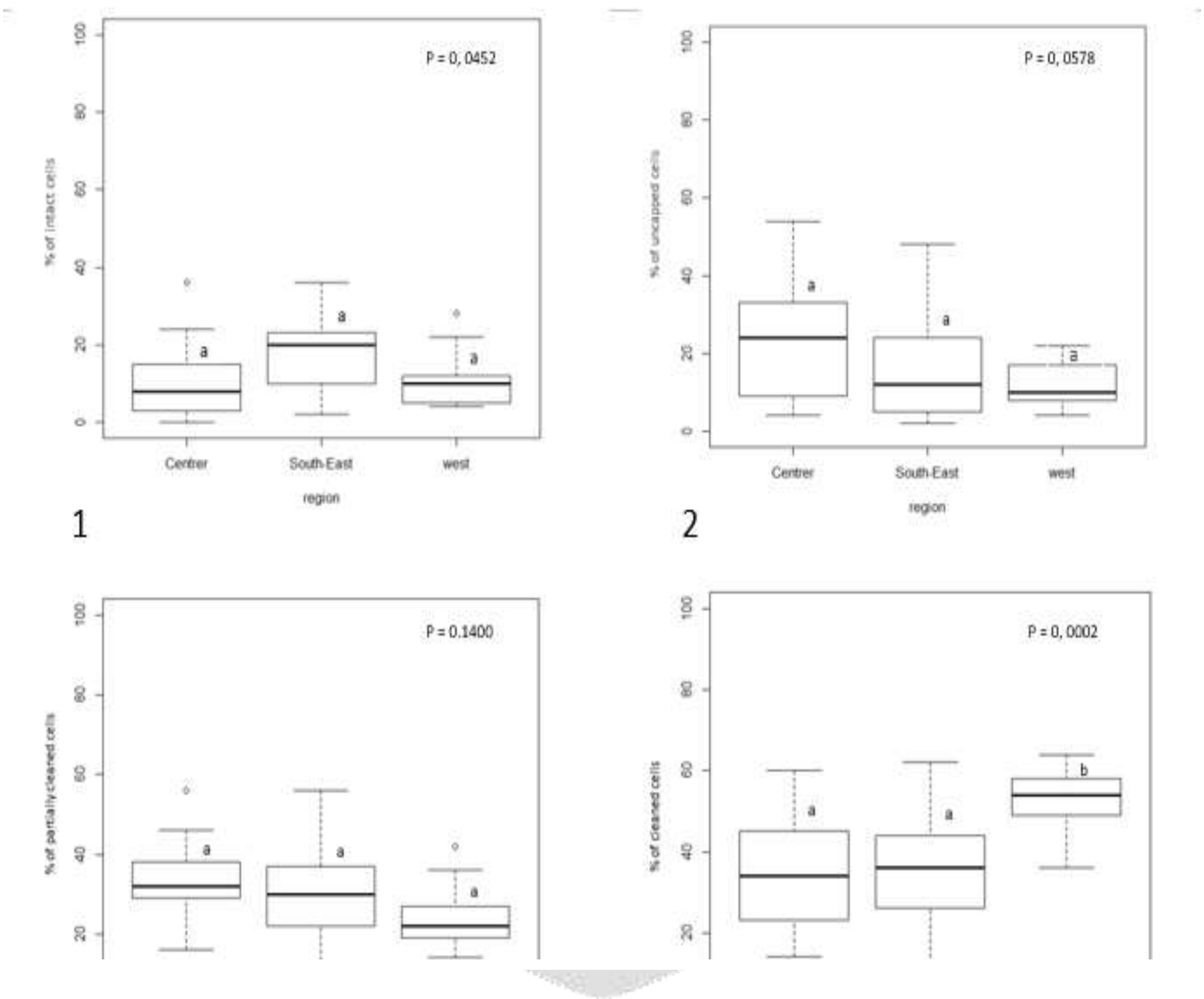


Figure - 2: Principal component analysis of individuals by region.

The collected bees were split into 3 groups (figure -2). The first group was formed by the central bees (Antananarivo) with long fore and hind wings and a large pollen sac. The second group was represented by the bees of the West (Mahajanga and Maintirano) and the Southeast (Mananjary) which have long proboscis and a well-developed mentum. The third group was formed by the bees of the Center-East (Ambositra and Fandriana) having long legs and a well-developed tibia.

3.2 Hygienic behavior

Differences in hygienic behavior were observed in the bee populations studied. They were evaluated at the level of intact (1), uncapped (2), partially cleaned (3) and cleaned cells (4). The following figure-3 shows the results obtained in the different regions after 6h of testing.



cleaned cells (4) recorded after 6h of testing. The same letters (a) indicate that there is no significant difference and different letters (a,b) indicate that there is a significant difference.

According to the figure-3, significant differences were observed on the cleaned cells. (ANOVA, P=0.0002). The major significant difference was observed between in the western regions where bees were able to clean more efficiently. The Tukey test confirms that the West region shows a significant difference with the South-East (Tukey HSD, P= 0.0017) and Center (Tukey HSD, P=0.0008) regions.

Table - 2 Hygienic behavior responses after 6h of testing. For each region are indicated, n: the number of colonies tested, range and mean ± SD of the percentage of intact, uncapped and cleaned cells.

Regions	% of intact sells	% of uncapped sells	% of partially cleaned sells	% of cleaned sells
Center n = 15	2 - 36 10,26 ± 10,16	4 - 54 22,40 ± 15,34	8 - 56 32,40 ± 15,34	14 - 60 34.93 ± 14,65
South-East n =15	2 - 36 17,73 ± 9,22	2 – 48 16,80 ± 13,72	8 - 56 29,46 ± 12,83	12 - 62 36 ± 13,77
West n= 15	4 - 28 10,53 ± 6,86	4 - 22 12,13 ± 5,82	14 - 42 24,66 ± 7,98	36 - 60 52,66 ± 7,27

4. DISCUSSION

In Madagascar, two populations of bees that differ in color have been observed. In the central part of the island and the southeastern region, the bees are uniformly black in color. On the other hand, in the western region, they appeared under black or yellow colors. The study identified for the first time a cohabitation of yellow and black bees in the same hive, especially in the western zone. B. Amssalu stated in 2004 in *Apis mellifera* colonies in the Ethiopian region, a mixture of black and yellow bees can be present in the same colony [17]. Bee colonies can even show variations in coloration between black and yellow in the same locality and under the same eco-climatic conditions.

According to Rasolofoarivao in 2015, no genetic differences have been identified between these bee populations [18]. The genetic clusters identified did not correspond to these morphological variations but rather to large geographical areas. The colorimetric criteria and color patterns of individuals are therefore not determinant for the differentiation of bee ecotypes in Madagascar.

Nevertheless, morphometry remains one of the criteria for differentiating subspecies and ecotypes of bees [19]. Indeed, the results obtained show a great morphometric variability of the populations of *A. m. unicolor* bees in Madagascar. These variabilities were mainly observed at the level of the hind legs, the fore wings and the proboscis. These results confirmed those of Louveaux (1977) who demonstrated the morphometric variability within the species *Apis mellifera* itself [20].

The wing measurements of bees can also vary according to the environment and other abiotic factors [18; 21; 13]. Wing size (length and width) is a parameter that influences,bee flight on the one hand, and the quantity of pollen that can be collected [22]. Measurements of the ulnar index were considered to be the most precise indicators, and are specific to each subspecies or ecotype of bee [11; 3]. For *A. m. unicolor* from Madagascar, the highest value of cubital indices was observed in the bees of Fandrina from the eastern region (Ic: 2.33a ± 0.016mm), while the lowest value was in the bees of Maintirano from the western region (2f ± 0.4mm). Between these two extremes, intermediaries were identified along the East-West axis of the Great Island, such as the bees of the highland region in Antananarivo (2.23b ± 0.15mm), those of Ambositra (2.21c ± 0.20mm), Mananjary (2.16d ± 0.08mm) and finally Mahajanga (2.04 e ± 0mm). The results obtained thus demonstrated a diversity between the bee populations present in the studied regions. All these places have very different environments and resources might be more difficult to gather in high density areas like Antananarivo, the capital than in forested areas with natural parks and high diversity of endemic flora [23] and less populated areas.

Furthermore, the proboscis also plays an important role in the adaptive success of bees in terms of nectar collection. The variations in proboscis lengths obtained ranged from 4.71 ± 0.12mm to 5.8 ± 0.012mm from East to West. Compared to other subspecies of *A. mellifera*, (*A. m. liguistica* (6.50 mm), *A. m. carnica* (6.60 mm) and *A. m. caucasia* (7.00 mm) [24; 19;13], the lengths of the proboscis of the populations of bees of Madagascar were clearly lower. The workers of *A. m. unicolor* are among the smallest of the genus [25], and there is a strong allometric relationship between the size of the bee and its tongue. Mattu and Verma (1984) suggest that the small size in the length of the bees' tongue at the sites presented in this study is probably an adaptation to the particular flora on which these bees feed [26]. Thus, the disparity observed in the bees of Madagascar is probably the result of this adaptation to the variations of the melliferous plants and their environment in particular the Malagasy flora is very diversified. About 12 000 to 14 000 species of plants [27] have been recorded on the island, 90% of which are endemic [28].

Bees use their legs to collect pollen and other resources. The average length of the hind legs of Malagasy bees in the samples collected was between 10.6 ± 0.33mm and 9.98 ± 0.16mm and was greater than that observed by [29] in *Apis m. intermissa* in Algeria (6.32 ± 0.172 mm), but much less than that observed by Ajao et al. (2014) in Nigeria in *Apis m. adansoni* (12.1 ± 0.03mm) [30] both subspecies belonging to the A lineage, such as *A. m. unicolor* [3]. Regarding the differentiation between the studied sites, bees from Fandrina (10.52a ± 0.16mm), bees from Ambositra (10.51a ± 0.28mm) and bees from Mananjary (10.6a ± 0.33mm) have well developed legs. The dimension of the hind leg of the bee is an important parameter because it is essential and determines the pollen harvesting capacity [26]. Similarly, honey production is positively correlated with the length of the hind leg and especially with the length of the basket at the tibia [31]. In general, the difference in size is most noticeable on the tibia, where the pollen basket is located. As the hind leg is essential for pollen transport, its size is probably related to the floristic richness of the locality where the species lives. Thus, bees from the Southeast and Central East have long legs compared to bees from the central and western regions, so bees in these areas can collect more.

The hygienic behavior of bees can be variable within subspecies and their populations [10]. Given that colonies that can only clean 0-16% of punctured cells in 6 h are considered unhygienic [32;33;34], thus highly susceptible to varroasis. The results of this study showed that the Western colonies managed to clean at least 52.66 ± 7.27% of the perforated cells in 6h. Statistical analysis by Tukey's test confirmed that there were significant differences between the fully cleaned cells in the western region.

Our results (central region with 34.93 ± 11.92% of cells cleaned at 6h) are different with those obtained by Rasolofoarivao et al., in 2015(central region with 48 ± 18% of cells cleaned at 6h) in the central region [35] because a 13.07% decrease in the hygienic capacity of the bees was noticed 4 years later. According to Panasiuc et al. in 2009[36], the hygienic behavior varies between years and periods of the season. This variation may depend on various factors. Air temperature and relative humidity, precipitation and natural nectar production have been considered as the major environmental factors influencing the performance of hygienic behavior [37;38; 39].

Since its accidental introduction, *V. destructor* has been detected in all three study regions (Southeast, Central, West). As soon as it was identified in the southeastern and central regions, beekeepers have conducted their colonies differently from those in the eastern region. Indeed, the highly hygienic colonies of the bee populations in the western region offer them an ability to adapt to limit the damage caused by *V. destructor* (tolerance). Indeed, these colonies have developed a capacity to live without varroa control measures, although the traits responsible for this tolerance are not yet clear.

5. CONCLUSION

In order to develop beekeeping, the first step is the mastery of the subspecies and ecotypes used. The results of this study allowed the detection of two ecotypes of bees in Madagascar with a different distribution. It appears that the bees of the West present significant differences compared to the bees of the other regions from the point of view of the coloration, the morphometry. The bee colonies in the West are hygienic colonies that detect and extract from the cells, whether capped or not, the larvae and pupae of bees that are sick or infested by varroa mites. This behavior could provide a framework for sustainable control of brood parasites including *V. destructor*.

However, further studies on the selection of varroa resistant bees (surviving several years without treatment) should be considered to strengthen the fight against *V. destructor*. The interest is to reduce the number of treatments, which would save time for the beekeeper, and decrease the number of potentially stressful interventions for the colonies. Varroa resistance seems to be an interesting tool in the long term to complement the current recommendations. New research is currently underway to try to understand more precisely these mechanisms and to be able to select these resistant strains by simple criteria.Highly hygienic colonies could be used as part of a *V. destructor* control program. Hygienic colonies could also have great economic interests, as they have been shown to produce more honey and pollen [40].

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