



Bee's morphometrics and behavior in response to seasonal effects from ecoregions

A.R.S. Sousa¹, E.D. Araújo², K.P. Gramacho³ and L.A. Nunes⁴

¹Programa de Pós-Graduação em Biotecnologia e Recursos Naturais, Laboratório de Genética e Conservação de Recursos Naturais, Departamento de Biologia, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil

²Programa de Pós-Graduação em Ecologia e Conservação, Laboratório de Genética e Conservação de Recursos Naturais, Departamento de Biologia, Universidade Federal de Sergipe, São Cristóvão, SE, Brasil

³Programa de Pós-Graduação em Saúde e Ambiente, Laboratório de Estudos Biológicos e Produtos Naturais, Instituto de Tecnologia e Pesquisa, Universidade Tiradentes, Aracaju, SE, Brasil

⁴Programa de Pós-Graduação em Genética, Biodiversidade e Conservação, Universidade Estadual do Sudoeste da Bahia, Jequié, BA, Brasil

Corresponding author: A.R.S. Sousa
E-mail: arlikrafael@hotmail.com

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ABSTRACT. In the present study, we used morphological and behavioral analyses to assess the effects of seasonality and morphoclimatic patterns on the morphology, behavior, and distribution of 71 colonies of Africanized honey bees in 3 distinct ecoregions (Zona da Mata, Agreste, and Sertão) within the State of Sergipe, north-eastern Brazil. We found a high rate of gene flow among the studied colonies. However, there were pronounced morphological differences among localities and ecoregions, and body shape ($r = 0.06239$; $P = 0.05$) and size ($P < 0.001$) varied with altitude. Regional groups were separated by phenotypic plasticity, rather than

genetic divergence. We also found a significant difference in the hygienic behavior of these populations between the dry and rainy seasons ($P = 0.022$; $\alpha = 0.05$) and between ecoregions ($P = 0.001$; $\alpha = 0.05$). The main modulator of hygienic behavior was the influence of temperature ($\rho = 0.065$; $P = 0.471$; $\alpha = 0.05$) and altitude ($\rho = -0.294$; $P = 0.001$; $\alpha = 0.05$) on rainfall ($\rho = 0.274$; $P = 0.002$; $\alpha = 0.05$). This supports the hypothesis that environmental factors influence the expression of hygienic behavior trait. The influence of environmental factors on the morphology, behavior, and distribution of Africanized honey bees, together with the identified polyphenisms, indicate high genetic variability within these populations that can be exploited in future bee handling and breeding programs.

Key words: Africanized honey bees; Gene flow; Hygienic behavior; Geometric morphometrics; Phenotypic plasticity; Polyphenism

INTRODUCTION

Bees are essential to the balance of ecosystems and play a fundamental role as pollinators that contribute to the reproduction and dispersal of the majority of angiosperm species, many of which are economically important. The role of bees as pollinators developed through a complex coevolutionary process over the past 100 million years (Cardinal and Danforth, 2013). Although quantification of pollination services is difficult, several studies have demonstrated their value for agribusiness and related ecosystems (Gallai et al., 2009; Lautenbach et al., 2012). The loss of this natural capital could adversely affect the maintenance of wild plant diversity, ecosystem stability, agricultural production, food security, and human welfare (Thomann et al., 2013).

The increasing decline in bee species worldwide, as a result of climate change (González-Varo et al., 2013), proliferation of parasites and pathogens (Wagoner et al., 2013), indiscriminate use of pesticides (Van der Sluijs et al., 2013), and the synergistic effects of these factors, has attracted attention and resulted in studies aimed at the characterization, technological exploitation, and sustainable use of these species of pollinators.

Apis mellifera is widely distributed throughout tropical regions and occurs on the American continent as polyhybrids produced by cross-breeding between European subspecies and the African subspecies *A. m. scutellata*. The great genetic plasticity and adaptability shown by these honey bee groups has contributed to their spread throughout several morphoclimatic patterns in tropical regions. These groups have acquired their own characteristics and are known as Africanized honey bees (Gonçalves, 1974).

Africanized honey bees have, as one of their main features, high resilience to diseases due to the innate ability of some of their colonies to detect, uncap and remove dead or diseased brood, thereby preventing the spread of diseases in their nests. This feature is called hygienic behavior (Rothenbuhler, 1964) and is a form of social immunity (Cotter and Kilner, 2010; Locke et al., 2012). Hygienic behavior is economically important due to its influence on colony resistance to parasites and pathogen infestations (Rosenkranz et al., 2010) and its relationship with other characteristics of interest, such as honey and propolis production (Wielewski et al., 2012; Güler and Toy, 2013; Padilha et al., 2013).

Hygienic behavior is an attribute that has been widely explored (Büchler et al., 2010; Rinderer et al., 2010; Pernal et al., 2012) as an index trait for honey bee-breeding selection. Although hygienic behavior is correlated with the expression of groups of genes (Lapidge et al., 2002; Oxley et

al., 2010), its manifestation is believed to be determined by a complex interaction among environmental (Panasiuk et al., 2009; Güler and Toy, 2013), social (Panasiuk et al., 2010), and epigenetic factors (Herb et al., 2012).

Apis populations are distributed throughout diverse climates and regions of the American continent and selective pressures differ between these sites. This has promoted morphophysiological and behavioral variability among these groups and resulted in the development of typical ecotypes that fit various climatic domains (Meixner et al., 2010). In this context, geometric morphometrics is an accurate and inexpensive tool (Francoy et al., 2008) for the study of the differences among populations of bees. Geometric morphometrics can be used to determine the relationships between the causes and consequences of variations in the adjustment of individuals to their natural habitat (Lawing and Polly, 2010).

In this study, we characterized the divergences among colonies of Africanized honey bees and assessed the influence of environmental factors in three distinct ecoregions, such as temperature, precipitation, and seasonality, on the morphology and behavior of these groups of bees.

MATERIAL AND METHODS

Study area and sample collection

The experiments were performed in three distinct geomorphological regions of the State of Sergipe, northeastern Brazil. The morphoclimatic patterns were homogenous within the study sites and heterogeneous between them. The studied regions were therefore 3 ecologically well-defined areas called ecoregions (Table 1 and Figures 1 and 2).

Table 1. Origin, geographic location, elevation and number of samples of *Apis mellifera* colonies from the State of Sergipe.

Location	Latitude	Longitude	Elevation	Climate	Number of samples
São Cristóvão	11°0'54"	37°12'21"	31 m	Tropical humid	7
Estância	11°16'4"	37°26'16"	10 m	Tropical humid	8
Japarutuba	10°35'34"	36°56'24"	109 m	Tropical humid	8
Brejo Grande	10°25'28"	36°27'44"	17 m	Tropical humid	8
Frei Paulo	10°32'56"	37°32'2"	272 m	Dry tropical	8
Aquidabã	10°16'52"	37°29'40"	226 m	Dry tropical	8
N.S. da Glória	10°13'06"	37°25'13"	234 m	Semiárid	8
Canindé do S.F	9°38'31"	37°47'16"	214 m	Semiárid	8
Poço Verde	10°42'28'	38°10'58"	273 m	Semiárid	8

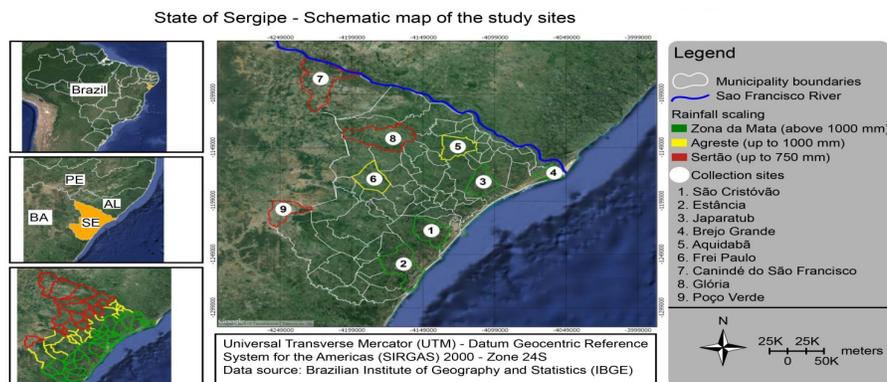


Figure 1. Ecoregions of Sergipe and *Apis mellifera* collection sites.

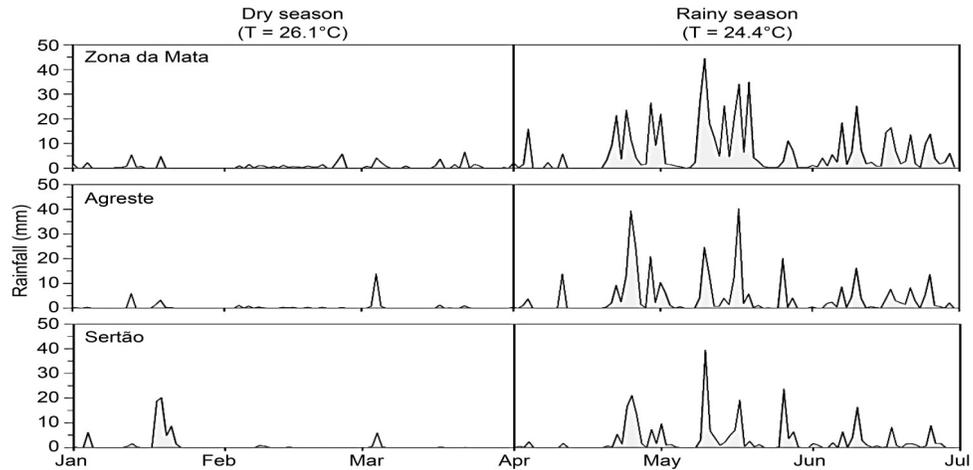


Figure 2. Rainfall in each ecoregion during dry and rainy periods.

Morphometrics

The right forewings of 710 individuals from 71 commercial colonies, 10 specimens each, were placed between microscope slides and photographed with a digital camera attached to a stereomicroscope using the image capture Leica Application Suite version 2.0 software. A total of 17 homologous landmarks (Figure 3) were plotted at the wing-vein intersections, as described by Franco et al. (2008), using the tpsDig software version 2.4 (Rohlf, 2005). The images were then aligned, and centroid coordinates were obtained with the aid of the MorphoJ software (Klingenberg, 2011).

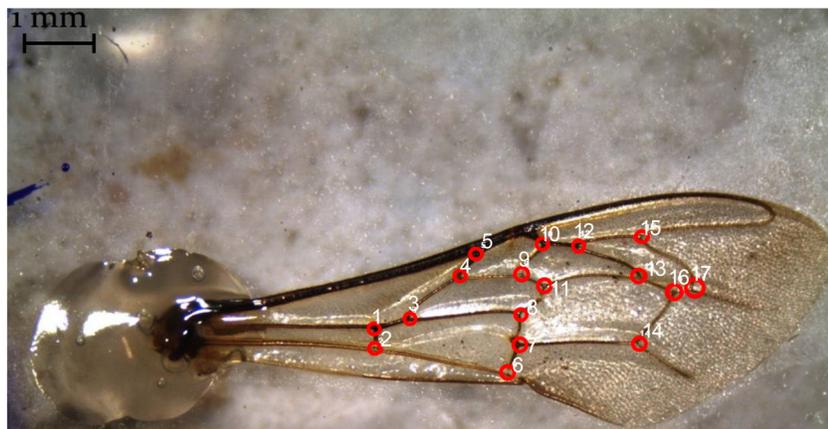


Figure 3. Seventeen anatomical landmarks plotted at the wing vein intersections of the Africanized honey bee, *Apis mellifera* L.

The canonical variable technique was used to maximize the explanatory value of each canonical variable with respect to morphological differences between the studied populations and to test whether the group means were significantly different. To facilitate geometric interpretation of the data similarities between groups were identified in two-dimensional scatterplots in which each axis represented a canonical variable. The Mantel test with 5000 permutations, as implemented in the NTSYSpc program, was used to assess correlations among the matrices' altitude, shape, and size. To verify the reliability of the results, a cross-validation test was performed.

The following linear equation was used to verify the relationship between honey bee wing centroid size (the independent variable) and altitude at which the colony was found (the explanatory variable):

$$y = a + bx \quad (\text{Equation 1})$$

where y is the wing centroid size (mm), x is the colony altitude (m), a is the intercept, and b is the slope coefficient; a and b are unknown parameters to be estimated.

Hygienic behavior

Hygienic behavior was evaluated by the pin-killing method (Figure 4) according to the methodology described by Newton and Ostasiewski Jr. (1986) and modified by Gramacho and Gonçalves (2009). Relationships between environmental variables and hygienic behavior were evaluated using Spearman correlations. Variations in removal rates among ecoregions and between dry and rainy periods were evaluated by the Kruskal-Wallis test. Path analysis (Wright, 1921) was used to evaluate the correlations among temperature, rainfall, and altitude, and then estimate the direct and indirect effects of these explanatory variables on the hygienic behavior of the colonies. Statistical analyses were performed with the aid of the R Development Core Team software (2008).



Figure 4. Comb of an Africanized honey bee colony that was subjected to the hygienic behavior test by the pin-killing method. Area (A) includes the treatment cells, and area (B) includes the control cells.

RESULTS

The first 14 canonical variables accounted for 80% of the total variation in wing shape among the studied *A. mellifera* colonies (Table 2). No groups were formed, which suggests a high rate of gene flow between colonies (Figure 5). This result was further supported by cross-validation analysis in which only 33% specimens were correctly classified according to region, indicating that *A. mellifera* individuals from the studied regions were similar to one another.

Table 2. Canonical variables, eigenvalues, variance percentage, and percentage of cumulative variance obtained from analysis of *Apis mellifera* colonies in the State of Sergipe.

VCA	λ_i	σ^2 (%)	σ^2 cumulative (%)
1.	2.998	23.966	23.966
2.	0.971	7.763	31.729
3.	0.824	6.592	38.322
4.	0.666	5.33	43.652
5.	0.629	5.033	48.684
6.	0.613	4.905	53.59
7.	0.542	4.34	57.93
8.	0.519	4.151	62.081
9.	0.466	3.731	65.812
10.	0.447	3.58	69.392
11.	0.432	3.456	72.848
12.	0.369	2.95	75.798
13.	0.339	2.711	78.509
14.	0.302	2.417	80.926
15.	0.295	2.365	83.291
16.	0.263	2.107	85.398
17.	0.238	1.904	87.301
18.	0.230	1.841	89.142
19.	0.193	1.546	90.689
20.	0.185	1.481	92.17
21.	0.159	1.276	93.446
22.	0.146	1.172	94.618
23.	0.133	1.066	95.685
24.	0.103	0.829	96.514
25.	0.102	0.817	97.331
26.	0.094	0.754	98.085
27.	0.076	0.615	98.701
28.	0.064	0.515	99.216
29.	0.052	0.417	99.633
30.	0.045	0.367	100.000

VCA = canonical variable; λ_i = eigenvalues; σ^2 = variance.

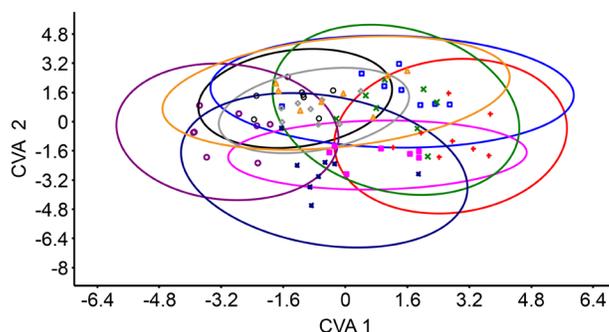


Figure 5. Scatterplot of *Apis mellifera* colonies from each of 9 locations in relation to the Cartesian axis established by the canonical variables (CVA 1, CVA 2). Light blue: Canindé do São Francisco; Red: Brejo Grande; Dark green: Estância; Pink: São Cristóvão; Yellow: Poço verde; Black: Aquidabã; Light green: Glória; Purple: Frei Paulo; Dark blue: Japarutuba.

When all the *A. mellifera* colonies were compared as a whole, no significant differences in wing morphology were found between them. However, when the colonies were compared by ecoregions, distinct groups were formed, suggesting that location affected the wing-shape variation of these honey bees. Moreover, when ecoregions were compared, the second canonical variable accounted for 100% of specimen variation (Table 3). To facilitate the visualization of colony similarities, the scores obtained for the first two canonical variables were used to construct a two-dimensional plot defined by two canonical vectors (Figure 6). In this plot, colonies were clearly clustered by ecoregion.

Table 3. Canonical variables, eigenvalues, variance percentage, and percentage of cumulative variance obtained from analysis of *Apis mellifera* in three distinct ecoregions of the State of Sergipe.

VCA	λ_i	σ^2 (%)	σ^2 cumulative (%)
1	0.320	57.729	57.729
2	0.234	42.271	100.000

VCA = canonical variable; λ_i = eigenvalues; σ^2 = variance.

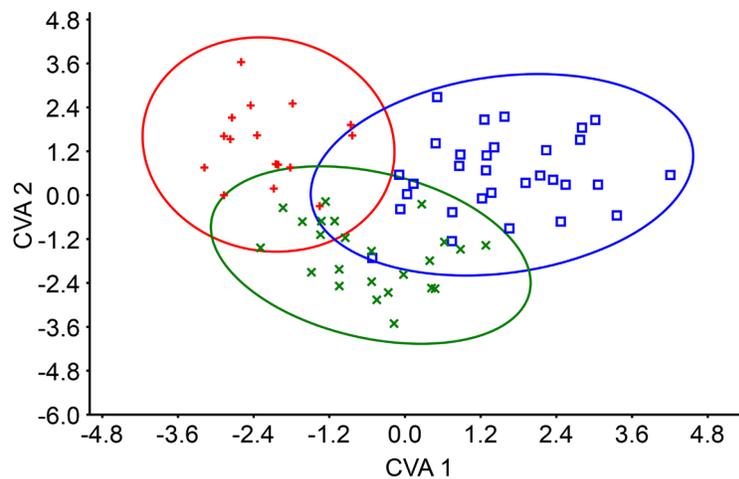


Figure 6. Scatterplot of the *Apis mellifera* colonies from each of three ecoregions in relation to the Cartesian axis established by canonical variables obtained from wing morphometrics (CVA 1, CVA 2). Blue: Zona da Mata; Red: Agreste; Green: Semiárid.

Thus, when the colonies were evaluated according to the apiaries, only the first two canonical variables were necessary to account for 72.107% of the total individual variation (Table 4). This variation can also be observed in the scatterplot in Figure 7.

Table 4. Canonical variables, eigenvalues, variance percentage, and percentage of cumulative variance obtained from analysis of *Apis mellifera* among apiaries of the State of Sergipe.

VCA	λ_i	σ^2 (%)	σ^2 cumulative (%)
1	1.303	47.03	47.03
2	6.952	25.077	72.107
3	5.515	19.894	92.001
4	2.217	7.998	100

VCA = canonical variable; λ_i = eigenvalues; σ^2 = variance.

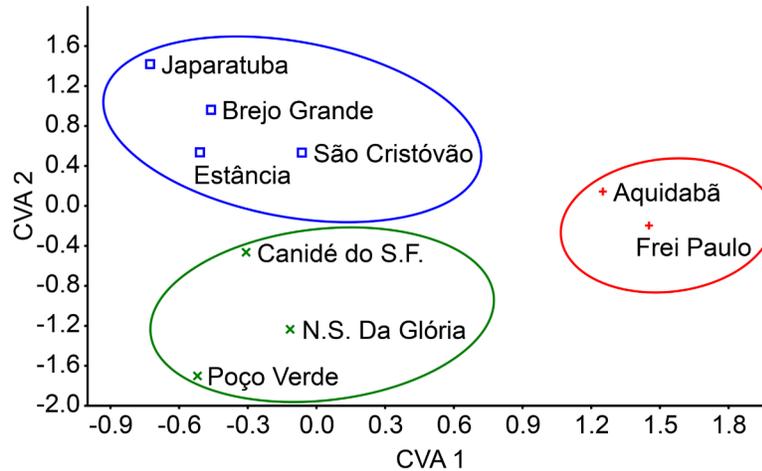


Figure 7. Scatterplot of the *Apis mellifera* apiaries in relation to the Cartesian axis established by the canonical variables (CVA 1, CVA 2) and the distance between apiaries according to the Mahalanobis' generalized distance.

A Mantel test (Table 5) showed a highly significant positive correlation between shape and altitude matrices, with less than 5% error. This indicates collinearity between these effects. Therefore, wing shape was influenced by altitude.

Table 5. Mantel test for the comparison of shape and altitude matrices using measurements of *Apis mellifera* wings with 5000 permutations.

Structure	Compared matrices	R	R ²	P
	D ² of Procrustes vs altitude	0.06239	0.00389	0.0098*
Wing	D ² of Procrustes vs size	-0.07949	0.00631	0.8384 ^{NS}

NS = not significantly different; *significantly different.

The estimated coefficients of regression for wing centroid size on colony altitude indicate that altitude had a positive linear relationship with centroid size (slope = 0.001; Table 6 and Figure 8).

Table 6. Estimated coefficients for regression of wing centroid size on colony altitude with the respective standard deviations, *t* values and P values.

	Estimates	Standard deviation	<i>t</i> value	P value
Intercept	6.571	0.028	237.241	<2·10 ⁻¹⁶
Altitude	0.001	0.0001	8.475	<2·10 ⁻¹⁶

Of the 63 colonies subjected to pin-killing treatment, 28 had mean proportions of hygienic behavior that were greater than 80% (Figure 9). Kruskal-Wallis tests (Figure 10) revealed the hygienic behavior of the Africanized honey bees between seasons ($P = 0.022$; $\alpha = 0.05$) and ecoregions ($P = 0.001$; $\alpha = 0.05$). The mean proportions of hygienic behavior for each region were: Zona da Mata ($81.85 \pm 11.81\%$), Agreste ($68.59 \pm 17.85\%$), and Sertão ($57.27 \pm 31.85\%$).

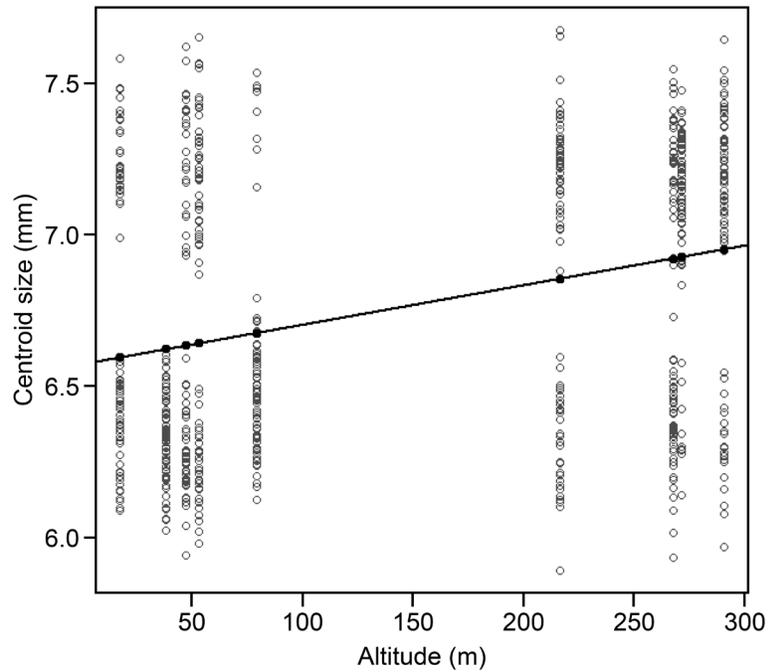


Figure 8. Linear relationship between observed and estimated *Apis mellifera* centroid size (mm) and altitude (m). Open circles represent observed centroid sizes; closed circles represent centroid sizes predicted by the linear equation. The straight line represents the linear relationship between centroid size and altitude.



Figure 9. A representative non-hygienic *Apis mellifera* colony (A) in which dead brood were not removed after 24 h, and a representative hygienic colony (B) in which dead brood had been removed by the bees.

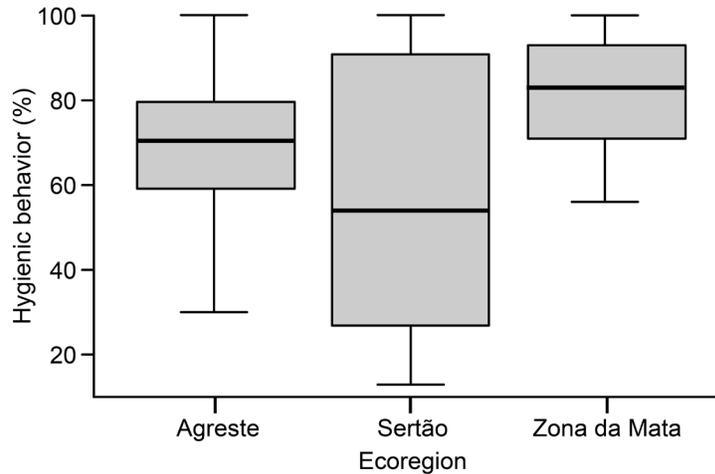


Figure 10. Box plot of *Apis mellifera* hygienic behavior (%) in the three studied ecoregions: Agreste, Sertão, and Zona da Mata.

As shown in Figure 2, precipitation rates fluctuated between the dry and rainy seasons, influencing the percentage of brood removal (Figure 11). In addition, the Spearman's correlation coefficients between hygienic behavior and the explanatory variables temperature ($\rho = 0.065$; $P = 0.471$; $\alpha = 0.05$), rainfall ($\rho = 0.274$; $P = 0.002$; $\alpha = 0.05$) and altitude ($\rho = -0.294$; $P = 0.001$; $\alpha = 0.05$) indicate significant associations between the hygienic behavior of honey bees, rainfall, and altitude. Increases in rainfall were directly associated with an increase in hygienic behavior, whereas increases in altitude were associated with decreases in hygienic behavior. The relationships between environmental conditions and the hygienic behavior of Africanized honey bees are shown in Table 7 and Figure 12.

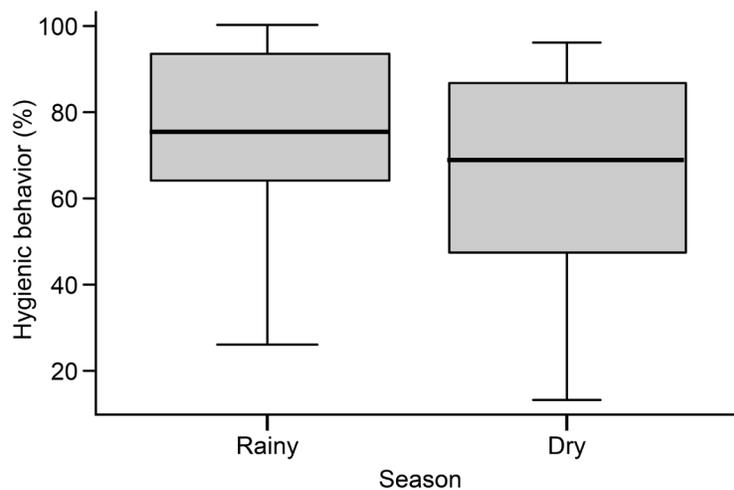
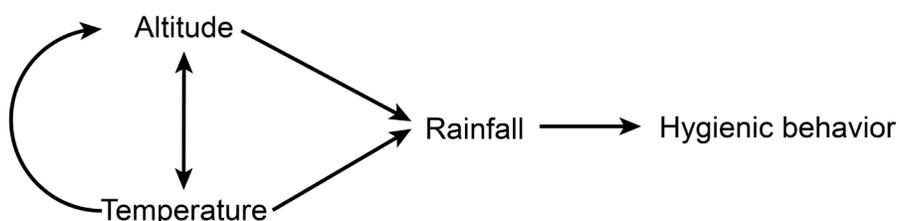


Figure 11. Box plot of *Apis mellifera* hygienic behavior (%) during the rainy and dry seasons.

Table 7. Estimated direct (diagonal) and indirect effects of temperature, rainfall, and altitude on honey bee hygienic behavior.

	Temperature	Rainfall	Altitude
Temperature	0.084	-0.093	0.069
Rainfall	-0.025	0.311	0.074
Altitude	-0.034	-0.138	-0.172

Indirect effects are effects that one explanatory variable exerts on the dependent variable as a result of its direct effect on another explanatory variable. The effect of temperature via precipitation (row 1, column 2) was -0.093 (Table 7), indicating that temperature had a negative indirect influence on hygienic behavior that resulted from its negative association with rainfall. The main indirect effect was the effect of altitude via rainfall: the altitude influenced rainfall, which in turn affected the hygienic behavior of honey bees. Overall, the main environmental variable modulating hygienic behavior, either directly or indirectly, was rainfall (Figure 12).

**Figure 12.** Chain diagram illustrating the effects of the primary (rainfall) and secondary (altitude and temperature) explanatory variables on the response variable (*Apis mellifera* hygienic behavior).

DISCUSSION

The results presented in this study show that the distinct morphoclimatic patterns of the studied ecoregions influenced the shape ($r = 0.06239$, $P = 0.05$) and size ($P < 0.001$) of *A. mellifera* wings, suggesting that altitude played an important role in the separation of the populations. Similar studies (Kekecoglu and Soysal, 2010; Parker et al., 2010) have also demonstrated how the distribution of bee populations over ecologically distinct regions is associated with differentiation between groups and the great dispersal ability and high phenotypic plasticity of these insects contributes to their adaptation to different geomorphological regions (Le Conte and Navajas, 2008). Topographic variation has been shown to interfere in the development of organisms, contributing to the formation of polyphenisms (Hepburn and Radloff, 2011). According to Klok and Harrison (2009), altitude influences the size and shape of organisms' structures because at high altitude, the rarefied air causes hypoxia that compromises the development of individuals due to low energy supply. Furthermore, air density decreases with altitude and provides less support for beating wings, thus changing the ideal ratio between the wing size and weight of these insects (Harrison et al., 2010). The present study demonstrated that, due to intense gene flow among the studied colonies, the high variability in wing size and the pronounced differences between apiaries and ecoregions are the result of phenotypic plasticity rather than genetic divergence among populations. This confirms the results reported by Gruber et al. (2013). In the present study, therefore, we were able to characterize the differences between honey bee populations using multivariate analysis

in combination with wing-venation morphometric data. Characterization of these divergences and understanding the causes and consequences of variations in the composition of bee ecotypes can be exploited in handling and breeding programs, as previously described by Rahimi and Mirmoayed (2013).

Moreover, it was evident that both season ($P = 0.022$; $\alpha = 0.05$) and ecoregion ($P = 0.001$, $\alpha = 0.05$) effects affected the hygienic behavior of Africanized honey bees. Hygienic behavior was directly influenced by rainfall (0.311) and indirectly influenced by altitude (-0.172) and temperature (0.084). This supports the hypothesis that environmental conditions affect the expression of hygienic behavior.

Panasiuk et al. (2009) and Güler and Toy (2013), also, reported differences in removal rates between periods of high and low nectar flow that corresponded to months with relatively high and low precipitation. During rainy periods, there is more food available than during dry periods. This results in greater honey bee vitality and serves as a stimulus for comb cleaning and food storage. Besides, the relatively large number of bees in the colony during rainy periods and the clustering that occurs among them may facilitate the detection of dead, diseased or parasitized brood. Thus, the present study corroborates the results of Costa-Maia et al. (2011) and Pernal et al. (2012), who suggested that environmental conditions are significantly involved in the manifestation of hygienic behavior. However, Stanimirović et al. (2008) and Padilha et al. (2013) found that hygienic behavior has a strong genetic component.

Our findings support the hypothesis that there are divergences in the populations of Africanized honey bees in distinct apiaries and among the ecoregions of the State of Sergipe and that environmental factors influence the morphology and behavior of these groups. In addition, the high gene flow observed in the studied populations denotes their high genetic variability, which implies that they have potential for future handling and breeding programs.

More studies related to the interaction between genetic and environmental factors are needed in order to understand the effects of geographic variation on the hygienic behavior and morphology of Africanized honey bees.

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