Fluctuating asymmetry in *Apis mellifera* (Hymenoptera: Apidae) as bioindicator of anthropogenic environments

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Abstract: The successful distribution of *A. mellifera* is due to their ability to adjust to seasonal variations, considerable control over their internal physical environment and exploration of different resources. However, their populations have experienced different forms and levels of environmental pressure. This research aimed to verify the phenotypic plasticity in both size and shape of wings in *A. mellifera* using fluctuating asymmetry, based on geometric morphometrics from apiaries located in sites with high and low levels of anthropization. We sampled 16 locations throughout all five geographic regions of Brazil. At each site, samples were collected from 20 beehives installed in apiaries: 10 installed near high anthropogenic environments (Cassilândia - MS, Fortaleza - CE, Maringá - PR, Aquidauana - MS, Rolim de Moura - RO, Riachuelo - SE, Ubiratã - PR and Piracicaba - SP), and 10 in sites with low levels of human disturbance (Cassilândia - MS, Itapiúna CE, União da Vitória - PR, Aquidauana - MS, Rolim de Moura - RO, Pacatuba - SE, Erval Seco - RS, Rio Claro - SP). A sample of 10 individuals was taken in each hive, totaling 200 per location, for a total of 1 600 individuals. We used fluctuating asymmetry (FA) in size and shape of the forewing through geometric morphometrics. The FA analysis was conducted in order to check bilateral differences. The indexes of size and shape were submitted to analysis of variance (ANOVA), where the characters evaluated were used as factors to verify the size and shape differences. The results indicated an asymmetry on the shape of the wing ($P < 0.001$) but no asymmetry was observed on wing size. Considering FA as an environmental response and high and low impacted areas as a fixed factor, we observed significant differences ($P < 0.05$). The results for the wing shape in *A. mellifera* demonstrated that this feature undergoes more variation during ontogeny compared to the variation in size. We concluded that bee samples collected from colonies with higher levels of human disturbance had higher wing-shape asymmetry; the variation of fluctuating asymmetry in the wing shape of honeybees can be used as an indicator of the degree of environmental anthropization. Rev. Biol. Trop. 63 (3): 673-682. Epub 2015 September 01.

Key words: centroid size, forewings, geometric morphometrics, honeybees, shape, anthropization.

During embryonic development, the organism may face disruption from a genetic or environmental nature that needs to surmount for the appropriate phenotypic expression. This instability can be assessed by random disturbances suffered during development, enabling verification of increased phenotypic variance within one species (Knierim et al., 2007). The intense environmental changes resulting from human activities, such as deforestation and pollution, create increasing concerns about the ecological consequences of these human activities on natural populations (Polak, Opoka, & Cartwright, 2002). Moreover, genetic changes such as mutation, selection, inbreeding, and chromosomal disorder can increase asymmetry (Parsons, 1992).

Changes in the individuals' development, in natural populations, show up as promising bioindicators of environmental quality. Most part of the environment effects on the phenotypic responses is due to phenotypic plasticity (Scheiner, 1993). Plasticity is essential in adapting the organism to heterogeneous and unstable environments, since it allows the organism dynamic maintenance, although not all plastic responses are adaptive (Gotthard & Nylin, 1995; Via et al., 1995).

The fluctuating asymmetry (FA) is commonly used to evaluate subtle random and non-directional changes between the symmetry planes of the individuals during their ontogenetic development. FA has been studied because they reflect both genetic and environmental stresses (Palmer & Strobeck, 1986; Parsons, 1990).

The occurrence of asymmetric fluctuations is more related to organisms that develop under stress, because they generally have greater difficulty for regulating their development. Therefore, the fluctuating asymmetry can be used as a tool to estimate stress, since it represents a sensitive indicator of organism development, being helpful to elucidate how organisms respond to environmental and genetic changes (Lempa et al., 2000; Silva, Lomonaco, Augusto, & Kerr, 2009).The animals that exhibit bilateral symmetry, as the insects, usually have similar morphological structures on both sides of the body. Since the development of these structures is under genetic control, it is expected that they exhibit morphological symmetry on both sides, as the products of expression of the same genome (Leary & Allendorf, 1989). Bees have a great plasticity to occupy different niches and, on that basis, are able to establish themselves as wild populations in different regions. Since the beginning of the Africanization process of *Apis mellifera* Linnaeus in the Americas, this species has been the subject of many studies, making it possible to observe its great adaptability and wide distribution, and enabling the maintenance of a diverse gene pool (Nunes, Araújo, Marchini, & Moreti, 2012). The successful distribution of *A. mellifera* is due to their ability to adjust to seasonal variations and apply considerable control over their internal physical environment and by the resource exploration (Oliveira & Cunha, 2005). However, their populations experience different forms and levels of environmental pressure.

This study aimed to verify the phenotypic plasticity in both size and shape of wings in *A. mellifera* using fluctuating asymmetry based on geometric morphometrics. Furthermore, we evaluated whether adverse conditions related to human actions affect the bilateral symmetry of wings or not.

MATERIALS AND METHODS

The study was conducted in a two years period, from April 2009 to April 2011. We sampled 16 locations in Brazil, distributed in 13 municipalities (Table 1), covering five geographic regions of Brazil. At each site, samples were collected from the 20 beehives installed in apiaries: 10 installed near highly anthropogenic environments (Cassilândia - MS, Fortaleza - CE, Maringá - PR, Aquidauana - MS, Rolim de Moura - RO, Riachuelo - SE, Ubiratã - PR and Piracicaba - SP), and 10 in places considered of low levels of human disturbance (Cassilândia - MS, Itapiúna - CE, União da Vitória - PR, Aquidauana - MS, Rolim de Moura - RO, Pacatuba - SE, ErvalSeco - RS, Rio Claro - SP). A total of 10 individuals were sampled in each hive using falcon tubes, totaling 200 individuals per location, and 1 600 when both high and low impacted areas were considered. According to Palmer (1994), a total of 30 individuals are enough to verify the presence of FA.

The environment was chosen in a way to maximize the degree of conservation and anthropization. Regarding the classification of places with high and low degree of human disturbance, environments considered highly anthropogenic were chosen for major urban, farming areas with insecticide applications, and industries with large areas (regions with

TABLE 1
Locations, geographic coordinates, characterization of the sample collection area and kind of impact of *Apis mellifera* population

Sites	Geographic Coordinates	Area	Anthropization level	Kind of impact
Cassilândia-MS	19°6′46″S, 51°44′2″W	Urban area, garbage dump	High	Pollution, deforestation
Fortaleza-CE	3°43′1″S, 38°32′34″W	Urbanarea	High	Pollution, deforestation
Maringá-PR	23°25′30″S, 51°56′20″W	Sugarcane crop, fruits, corn, soybean and insecticide application	High	Deforestation, pollution (intoxication)
Aquidauana-MS	20°28′15″S, 55°47′13″W	Urban area	High	Pollution, deforestation
Rolim de Moura-RO	11°43′31″S, 61°46′40″W	Urban area and garbage dump	High	Pollution, deforestation
Riachuelo-SE	10°43′40″S, 37°11′13″W	Plant of sugar cane and insecticide application	High	Deforestation, pollution (intoxication)
Ubiratã-PR	24°32′42″S, 52°59′16″W	Sweetcorn crop and insecticide application	High	Deforestation, pollution (intoxication)
Piracicaba-SP	22°43′30″S, 47°38′56″W	Urban area	High	Pollution, deforestation
Cassilândia-MS	19°6′46″S, 51°44′2″W	Savana	Low	Undetected
Itapiúna_CE	4°33′50″S, 38°55′19″W	Forest	Low	Undetected
União da Vitória-PR	26°13′48″S, 51°5′9″W	Forest	Low	Undetected
Aquidauana-MS	20°28′15″S, 55°47′13″W	Forest	Low	Undetected
Rolim de Moura-RO	11°43′31″S, 61°46′40″W	Forest	Low	Undetected
Pacatuba-SE	10°27′10″S, 36°39′3″W	Forest	Low	Undetected
Erval Seco-RS	27°32′56″S, 53°30′14″W	Forest	Low	Undetected
Rio Claro -SP	22°24′39″S, 47°33′39″W	Forest	Low	Undetected

high pollution rates). On the other hand, environments considered with low levels of human disturbance were chosen because they are areas of permanent preservation of remaining forests (Atlantic Forest, Planalto, Cerrado), riparian areas or ecological reserves (Table 1).

All sampled specimens were stored in ethanol at -20 °C. Later, the forewings of both right and left sides of each individual were removed, with the help of entomological forceps. Subsequently, these structures were placed between two microscopic slides, to keep them flat and prevent distortion, during the image capture process. The images were acquired and digitized with a camera attached to a stereomicroscope using the Leica Application Suite version 3.4.1. From the images of the wings, coordinates of the landmarks were obtained on both sides using software TpsDIG2 version 1.40 (Rohlf, 1998), as shown in figure 1.

Four hundred individuals from four locations were selected for the asymmetric preliminary analysis, in order to verify the measurement error. Hence, each individual was measured twice. According to Palmer (1994), tests such as this should be used, to verify the measurement error, using at least 30 individuals.

The indexes of size were submitted to analysis of variance (ANOVA), where the characters evaluated were used as factors, to verify the size differences. The fluctuating asymmetry (FA) analysis was conducted in order to check bilateral differences. To calculate FA, both sides of the wing were measured, as well, the mean difference between the right and left sides, i.e., FA = $[(\Sigma|(R-L)|/n]$, and compared using the paired T-test, to see whether the distributions have means equal to zero (Palmer & Strobeck, 1986; Swaddle, Witter, & Cuthill, 1994). FA was measured in all individuals for the determination of size and shape.

The univariate analysis of variance, ANOVA, was performed, to verify the asymmetry of the wing centroid size, where the centroid size was treated as an independent variable, the side of the body as a fixed effect, and the individuals as a random effect (Klingenberg & McIntyre, 1998). To obtain the F value for the side effect and individuals, we used the interaction between individual and side as the denominator. To obtain the F value for the interaction between individual and side, the measurement

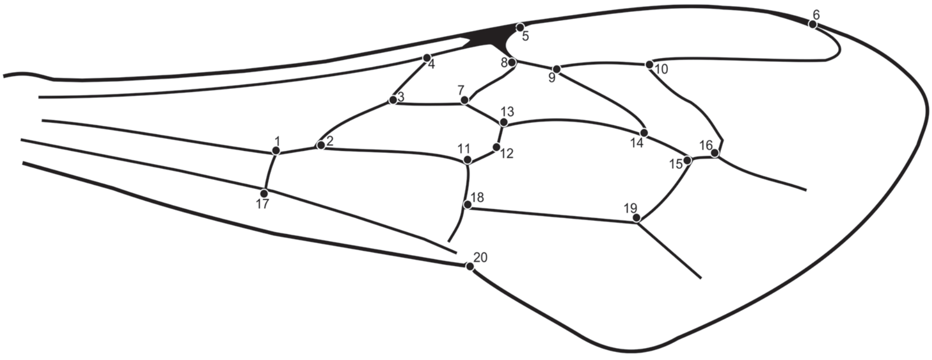

Fig. 1. Forewing of *Apis mellifera* with anatomical landmarks used for analyzes.

error as denominator was used (Palmer, 1994; Klingenberg & McIntyre, 1998).

According to the genetic model of Via and Lande (1985), which evaluates quantitatively the phenotypic plasticity, the total phenotypic variation of a character is the sum of its genetic and environmental components, as well the interaction between these factors. Therefore, the occurrence of phenotypic plasticity in size has been estimated by the environmental component of variation (Falconer, 1989).

Procrustes ANOVA analysis was performed to analyze the asymmetry of the wing shape of the populations and the configurations of the species aligned (Klingenberg & McIntyre, 1998; Palmer & Strobeck, 2003). In a similar way, we analyzed the asymmetry to the centroid size, where the side of the wings was treated as a fixed effect, and the individuals as random effect. Thus, the effect of individual consists in individual variation of the shape, and the side and individual effect corresponding to FA. The term of the interaction between side and individual is the measure of fluctuating asymmetry (small random differences between left and right sides, between individuals) and the residue, which is the variability between replicates, are treated as the measure of error (Klingenberg & McIntyre, 1998).

Procrustes distance analysis was performed using the data obtained from the wing to assess the differences and the asymmetry level between the various localities sampled. With the Procrustes distances values, we calculated the heritability of wing-shape variation in *A. mellifera* using the multiple variance analysis model by Goodall (1991) and intraclass correlation coefficient (Sokal & Rohlf, 1995) as reported by Monteiro et al. (2002). The causes for fluctuating asymmetry have been studied, and it was believed that it was completely related to the environment (Palmer, 1994); however, it has a genetic basis that remains unknown with the heritability close to zero (Palmer & Strobeck, 1986; Parsons, 1990; Fuller & Houle, 2002; Leamy & Klingemberg, 2005).

RESULTS

The effect of the ANOVA for the size of the wing, using the centroid size, was not significant for populations from União da Vitória-PR, Rolim de Moura-RO, (environments with low levels of human disturbance, P = 0.515), Maringá-PR and Piracicaba-SP (highly anthropogenic environments, P = 0.32). The other locations showed significant differences in wing size, in the differences between left and right sides (L-R) and in the effect of interaction between side and individual. In the ANOVA, the centroid size made it possible to verify that some populations, of *A. mellifera*, showed the presence of directional asymmetry.

On the other hand, the effects of ANOVA, on the wing-shape asymmetry, showed significant results in all populations, regardless of where the samples were obtained and whether they presented a high or low level of disturbance. This result demonstrated the existence of fluctuating asymmetry in all populations of *A. mellifera*, when wing shape was evaluated. From the interaction analysis performed, to verify the presence of FA and to compare the populations analyzed, we observed that colonies collected close to where insecticide application occurs, such as Maringá-PR, Riachuelo-SE and Ubiratã-PR, presented a greater asymmetry value, when compared to localities with low levels of human disturbance (Fig. 2). Furthermore, we found that samples collected from locations, classified with a high degree of human disturbance, such as Aquidauna-MS and Rolim de Moura-RO, also showed a higher degree of asymmetry, compared to the colonies sampled in environments with a low degree of human disturbance. Thus, from all 16 locations sampled, 63 % of the colonies collected in contaminated environments, showed greater asymmetry when compared to others. Significant differences ($P < 0.05$) were observed when FA was regarded as the environmental response and high and low impacted areas were considered a fixed factor. The only samples that observed a higher value of FA, were those populations located in areas considered with low levels of human disturbance, such as Cassilândia-MS, Itapiúna-CE and Rio Claro-SP. With regards to wing shape heritability, a value of $h^2 = 0.33$ with EPM standard error = 0.0008 was found.

The presence of varying degrees of FA, in the regions studied by evaluating the wing shape of *A. mellifera,* enables to see that these differences may also be related to the geographic distance between colonies ($P = 0.0143$ and $r = 0.29$). This is expected since geographical proximity is associated with greater genetic and climate similarity between colonies.

DISCUSSION

The differences between individuals are often represented using size variation between

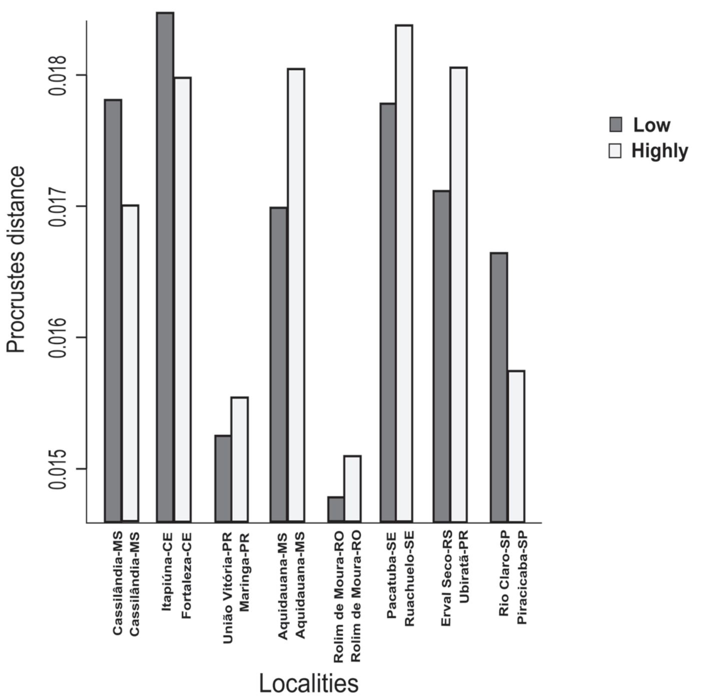

Fig. 2. Interaction of the presence of fluctuating asymmetry in wing of *Apis mellifera* in different collection sites, where L are areas that have a low degree of human disturbance, and H are highly anthropogenic environments.

them. However, according to Schneider, Leamy, Lewis and DeGrandi-Hoffman (2003), size and some structures analysis may have some restrictions for assessing the asymmetry, and may not be good indicators for the analysis of asymmetry in bees. It is consistent with the results found for the size asymmetry in some populations of *A. mellifera* (União da Vitória-PR, Rolim de Moura-RO, ($P > 0.05$), Maringá-PR and Piracicaba-SP). Clarke & Oldroyd (1996), studied males and workers of *A. mellifera* and observed that some characters do not show significant size correlation, usually because the wing size in insects is correlated with the overall size of the body. Therefore, it is possible that identical genotypes exhibit differences associated with the use of nutritional resources and with different environmental conditions. We observed the presence of directional asymmetry for wing size in some populations, a result that corroborates Smith, Crespi and Bookstein (1997) and Schneider et al. (2003), that evaluated hybrid and pure populations of *A. mellifera* from Procrustes superimposition and symmetry analysis.

It is possible to verify the presence of FA in the wing-size in some populations of *A. mellifera*, similar to that found by Clarke, Oldroyd and Hunt (1992), which evaluated asymmetry size between males and females of *A. mellifera,* and confirmed that inbreeding has no effect on the stability in the development of this species. They also found that in the haplodiploid system, the level of heterozygosity does not seem to be an important factor in the stability of the development.

In the analyses performed for this study, we observed that the shape represents better symmetry differences when compared to size, which are similar to results found by Del Lama, Gruber and Godóy (2002). Those authors also found that the shape of the wing contain better structures to asymmetry study, because it apparently is more affected by the environment when compared with the size. According to Leary and Allendorf (1989), a single feature can provide a good index of stress suffered during the individuals' development. All populations showed significant differences between left and right sides, as well as in the interaction between side and individual, suggesting a directional asymmetry and fluctuating asymmetry.

The results for the shape of the wing of *A. mellifera*, demonstrate that this feature undergoes more variation during ontogeny compared to the variation in size. These results are consistent with those found by Smith et al. (1997) that noted the presence of fluctuating and directional asymmetry on the shape of the wing of *A. mellifera*, and affirmed that directional asymmetry is more common than expected, and suggested that this asymmetry is related to the position of the larvae, prepupae and pupae in the brood cell. In addition, studies performed by Abou-Shaara and Al-Ghamdi (2012) showed asymmetric patterns on wings of Carniolan (*A. mellifera carnica*) and Yemeni honeybees (*A. mellifera jementica*), using conventional and geometric morphometric analysis, suggesting that temperature, seasons and viruses may have contributed to the presence of asymmetry.

The reason why the locations with low degree of human disturbance (Cassilândia-MS, Itapiúna-CE, and Rio Claro-SP) have shown greater asymmetry may be associated with various environmental factors that generate noise during development. In view of the fact that individuals were collected in forest fragments, could be the factor that is influencing this asymmetry. Studies by Lens, Dongen, Wilder, Brooks and Matthysen (1999) evaluating species of birds found in forest fragments, and comparing them with the birds deposited in museums (collected more than 50 years ago, before the fragmentation of the forest), found an increase in FA. That is, fragmentation can cause stress on these organisms, which may affect their development. A similar cause should be occurring in bee populations collected in environments recognized as preserved.

The greater asymmetry observed in places with insecticide application, corroborates studies by Abaga et al. (2011): a high level of developmental instability, in *A. mellifera,* was significantly associated with the use of insecticides in cotton farms; it was verified that the FA index, in the tarsus length, was increased, implying that FA can be considered as a biomarker that reflects the stress induced by insecticide treatments. Another factor that may contribute to the existence of FA is that all individuals have been collected in apiaries and the handling could cause changes in behavior, and be a stress generator.

The presence of different degrees in asymmetry, in the regions studied, is similar to that found by Mazeed (2011), evaluating wing patterns in Egyptian (*A. mellifera lamarckii*) and Carniolan honeybees (*A. mellifera carnica*). They verified the presence of fluctuating and directional asymmetry, mainly in Carniolan honeybees (as they are not found naturally in Egypt), and therefore, should not be adapted to the region. This suggests that the geographical location and adaptation to different environmental conditions, to which each organism is subjected, affect the degree of asymmetry. Furthermore, this same study draws the attention to the genetic differences between lineages and inbreeding as the possible causes of asymmetry.

The asymmetry exists technically in different scales and all structures will be asymmetrical to some extent (Palmer, 1996). The low heritability observed in the wings of *A. mellifera* was expected, since FA is a random variation that is related to the environment (Palmer, 1994) to which the genetic basis remains unknown with heritability close to zero (Leamy & Klingenberg, 2005). The heritability of a trait is a parameter that varies in time and space, and the low heritability can be indicative of a strong constraint on development and evolution (Monteiro, Diniz-Filho, dos Reis & Araújo, 2002). These authors also, found significant heritability values for the shape of the forewing in *A. mellifera* ($h^2 = 0.2935$; $P < 0.001$), a similar result was found in the present study ($h^2 = 0.33$).

It seems to us that the degree of human disturbance can increase the intensity of fluctuating asymmetry, however, different types of human disturbance may result in different pressures of asymmetry. Environmental factors, location of the apiary and management, can directly influence the results. Studies by Silva, Lomonaco, Augusto and Kerr (2009) found that environmental factors and anthropogenic interference influence the developmental stability in the size of *Euglossa pleosticta*.

The adjustment to the habitat is the response that causes asymmetry. Populations located in urban areas tend to be more negatively affected and present a greater FA than others, as noted by Weller and Ganzhorn (2004) in populations of carabid beetles, which showed that specimens collected closer to the cities showed greater FA levels. Weller and Ganzhorn (2004) concluded that the urban effect makes the communities move out, causing a predicted loss of species, a reduction in body size, and to the FA, an increase in species that are susceptible to urbanization. Those results are similar to the ones found in the present work, where populations of *A. mellifera,* collected in urban areas (Aquidauana-MS and Rolim de Moura-RO), showed greater asymmetry effects when compared with populations of the same localities, inferring the contribution of urbanization in the presence of asymmetry in these organisms. Therefore, understanding the influences of human impacts on urban landscapes is important to reduce the negative effects on biodiversity remaining (Pickett et al., 2001).

In this study it was observed that the shape of the wing is more suitable for the study of asymmetry when compared with the size of the wing. We observed that the colonies collected in highly anthropogenic areas exhibit greater asymmetry compared with those collected in environments with low levels of human disturbance. Indeed, morphological characters may also indicate habitat quality. However, since it is a pioneering and still prospective study, the use of genetically closely related colonies and a better description of the environmental variables, can significantly improve the accuracy of the fluctuating asymmetry tests in environmental quality assessment, using the shape of the wings of *Apis mellifera*.

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RESUMEN

Asimetría fluctuante en *Apis mellifera* (Hymenoptera: Apidae) como bioindicador de ambientes antropogénicos. La distribución exitosa de *A. mellifera* se debe a su capacidad para adaptarse a las variaciones estacionales, controlar considerablemente su ambiente físico interno y por la exploración de recursos. Sin embargo, sus poblaciones experimentan diferentes formas y niveles de presión ambiental. Esta investigación evaluó colmenares, ubicadas en entornos con distintos niveles de antropización. Ambientes considerados altamente antropogénicos fueron escogidos: áreas urbanas, agrícolas con aplicaciones de insecticidas, y las industrias con grandes áreas (regiones con altos índices de contaminación). Por otra parte, los entornos considerados con bajos niveles de perturbación humana fueron elegidos: áreas de preservación permanente de los bosques restantes (Mata Atlántica, Planalto, Cerrado), áreas ribereñas o reservas ecológicas. Se muestrearon 16 localidades que abarcan las cinco regiones del Brasil. Estamos utilizando la asimetría fluctuante desde el tamaño y la forma del ala anterior por técnicas de morfometría geométrica. En cada sitio, las muestras se obtuvieron de las 20 colmenas instaladas en los colmenares: 10 instalados cerca de entornos altamente antropogénicos (Cassilândia - MS, Fortaleza - CE, Maringá - PR, Aquidauana - MS, Rolim de Moura - RO, Riachuelo - SE, Ubiratã - PR y Piracicaba - SP) y 10 en sitios de bajo nivel de perturbación humana (Cassilândia - MS, Itapiúna_CE, União da Vitória - PR, Aquidauana - MS, Rolim de Moura - RO, Pacatuba - SE, Erval Seco - RS, Rio Claro - SP). Los índices de tamaño y forma fueron sometidos a análisis de varianza (ANOVA), donde se utilizaron los caracteres evaluados como factores, para verificar las diferencias de tamaño y forma. La asimetría de análisis fluctuante (AF) se llevó a cabo con el fin de comprobar las diferencias bilaterales. Los resultados indican la existencia de la asimetría de la forma del ala ($P < 0.001$), pero no se observó asimetría del tamaño del ala. Considerando AF como respuesta ambiental y áreas de alto y bajo grado de alteración humana como factor fijo, observamos diferencias significativas ($P < 0.05$). Los resultados, para la forma de ala de la *A. mellifera*, muestran que esta característica se somete a más variación durante la ontogenia en comparación con la variación en el tamaño. Llegamos a la conclusión de que las colonias de abejas recogidas en ambientes con niveles más altos de la perturbación humana tienen una mayor asimetría en forma de ala, por lo que la asimetría fluctuante en forma de alas de las abejas puede ser utilizada como un indicador del grado de antropización del medio ambiente.

Palabras clave: tamaño de centroide, alas anteriores, morfometría geométrica, abejas, forma, antropización.

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