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Original Article

Is leaf fluctuating asymmetry related to plant and leaf size in *Miconia albicans*, a common Melastomataceae species?

A assimetria flutuante de folhas está relacionada ao tamanho da planta e da folha em um arbusto de savana neotropical?

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Abstract

Fluctuating asymmetry, defined as random differences between the two sides of a symmetrical structure, has been often related to development stress in both plants and animals. In plants, leaf fluctuating asymmetry has been related to stresses such as pollution and fire and may also be related to leaf growth and herbivory rates. We assessed whether leaf fluctuating asymmetry is related to plant and leaf size in *Miconia albicans* (Sw.) Triana (Melastomataceae), a common multi-stem Neotropical shrub, in a Brazilian savanna area. We collected 15 leaves from each of 70 individuals, and measured fluctuating asymmetry as the difference in area between the right and left sides of the leaves using the central vein as reference. To avoid spurious results due to measurement error, the division along the central vein was performed independently by three researchers. We also measured the basal area and height of each stem of the plant individuals. We used linear models to assess the relations between leaf fluctuating asymmetry, plant size and leaf size. No consistent relations were observed between leaf fluctuating asymmetry and plant size, as the analyses performed on the fluctuating asymmetry values obtained by the different researchers showed different results. However, relative fluctuating asymmetry values, obtained by dividing the fluctuating asymmetry by the total leaf area, tended to be smaller in larger leaves. It thus appears that, in the study species, fluctuating asymmetry is related to the developmental conditions faced by the individual leaves and not by the plant as a whole.

Keywords: Cerrado, leaf asymmetry, leaf size, measurement error, *Miconia albicans*.

Resumo

A assimetria flutuante, definida como diferenças aleatórias entre os dois lados de uma estrutura simétrica, tem sido frequentemente relacionada ao estresse de desenvolvimento em plantas e animais. Nas plantas, a assimetria flutuante foliar tem sido relacionada a estresses como poluição e fogo e também pode estar relacionada ao crescimento foliar e taxas de herbivoria. Nós avaliamos se a assimetria flutuante foliar está relacionada ao tamanho da planta e da folha em *Miconia albicans* (Sw.) Triana (Melastomataceae), um arbusto neotropical multicaule comum, em uma área de Cerrado. Coletamos 15 folhas de cada um dos 70 indivíduos e medimos a assimetria flutuante como a diferença de área entre os lados direito e esquerdo das folhas usando a nervura central como referência. Para evitar resultados espúrios devido ao erro de medição, a separação ao longo da nervura centra foi feita independentemente por três pesquisadoras/es. Também medimos a área basal e a altura de cada caule de cada planta. Usamos modelos lineares para avaliar as relações entre assimetria flutuante foliar, tamanho da planta e tamanho da folha. Não foram observadas relações consistentes entre a assimetria flutuante da folha e o tamanho da planta, pois as análises realizadas nos valores da assimetria flutuante obtidos pelas/os diferentes pesquisadoras/es mostraram resultados diferentes. No entanto, os valores de assimetria flutuante relativa, obtidos pela divisão da assimetria flutuante pela área foliar total, tenderam a ser menores nas folhas maiores. Assim, verifica-se que, na espécie estudada, a assimetria flutuante está relacionada às condições de desenvolvimento enfrentadas pelas folhas individuais e não pela planta como um todo.

Palavras-chave: Cerrado, assimetria da folha, tamanho da folha, erro de medição, *Miconia albicans*.

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1. Introduction

Fluctuating asymmetry, understood as small and non-directional differences in size between the two sides of a bilaterally symmetric structure, may be related to developmental instability and developmental stress, although this relation is not certain (Palmer, 1996). In this way, it makes sense to expect fluctuating asymmetry to be non-adaptive, as it deviates the traits from their ideal symmetry (Palmer, 1994). This is because the development of individuals or structures subjected to stress may be hampered, precluding the formation of perfectly symmetrical features (Palmer, 1996). As a consequence, individual fitness should be affected and possibly reduced, as has been demonstrated for birds that had greater flight difficulties when they had fluctuating asymmetry in their traits (Tomkins and Kotiaho, 2001) and for houseflies, which were more preyed upon when they had high levels of asymmetry in the wings (Tomkins and Kotiaho, 2001). Still, even though a large part of the studies on fluctuating asymmetry have been performed with animals, plants can be an excellent model for such studies, given that they can have both bilateral and radial symmetry in inflorescences, leaves, fruits, and flowers (Freeman et al., 1993). Plants may also be good models due to their modular growth, which permits to assess asymmetry in different modules within the same individual (Graham et al., 1993). Studies on developmental instatbility in plants, although relatively few, have a long history - for example, Sakai and Shimamoto (1965) studied the leaves and central veins of 11 different tobacco (*Nicotiana tabacum*) varieties and detected significant differences between the veins of different plants of the same variety and between leaves and veins of plants belonging to different varieties.

There is growing evidence that fluctuating asymmetry, individual fitness, and developmental stability are related to environmental stress (Lens et al., 2002; Cuevas-Reyes et al., 2013). Developmental instability measures, including fluctuating asymmetry, serve as a proxy to indicate that the organism's physiological processes are failing to prevent environmental stress (Freeman et al., 1993). Thus, it can be expected that phenotype formation is not just a function of mRNA regulation, but also of metabolic pathways and physiological processes that are subject to environmental external changes (Freeman et al., 1993).

There are two main ways in which environmental stress affects the traits symmetry. The first is through developmental noise, which tends to hamper the symmetrical development of bilateral traits. The second way is through increased developmental instability, which can be defined as the variation in the expression of a given phenotype when a genotype is under stressful conditions (Møller and Swaddle, 1997) and tends to lead to a decrease in the individual's capacity to buffer environmental stress (Klingenberg and Nijhout, 1999; Klingenberg, 2003; Lens et al., 2002). On the other hand, individuals who develop in environments with low levels of stress may have a greater ability to prevent random errors in development (Freeman et al., 2005).

In plants, leaf fluctuating asymmetry has been observed to be related to a variety of stresses, such as fire (Alves-Silva and Del-Claro, 2013), pollution (Ivanov et al., 2015), and urbanization (Cuevas-Reyes et al., 2013). Leaf fluctuating asymmetry may also be affected by environmental stress faced by individual leaves (Cowart and Graham, 1999) and has been observed to correlate with factors such as leaf growth rate and chemical composition (Lempa et al., 2000) and herbivory (Santos et al., 2013). Environmental stress may also affect plant development in other ways, and factors such as inappropriate soil conditions (Haridasan, 1988), high herbivory pressure (Vranjic and Ash, 1997) and impacts of invasive species (Pires et al., 2012) may all hamper plant development, decreasing plant size and possibly increasing fluctuating asymmetry. Similarly, it is possible that the development of leaves subjected to a greater stress will be hampered, resulting in smaller and more asymmetrical leaves. However, such patterns are not always observed - for example, Ishino et al. (2012) did not detect differences in leaf fluctuating asymmetry between plants growing at the edge and in the interior of a Cerrado fragmento. However, the fluctuating asymmetry in the leaves of another Cerrado plant species, *Miconia falax* (Melastomataceae), was greater in the shaded interior than on the exposed edge of a Cerrado site, but was not affected by galls (Alves-Silva, 2012).

Size and growth rate are often used as indirect measures to quantify stress in organisms (Roff, 1986; Kozlov and Niemelä, 1999). However, plants can develop compensatory physiological strategies to deal with stressful environments, which in turn makes it difficult to identify the relationship between stress and developmental instability (Kozlov et al., 2002; Freeman et al., 2005). However, the relation between leaf fluctuating asymmetry and either plant size or leaf size has been little explored except from a methodological and experimental perspective (Graham et al., 2015; Maldonado-López et al., 2019).

We tested whether leaf fluctuating asymmetry is related to plant size and leaf size in a resprouting Neotropical shrub in a Brazilian Cerrado area. We hypothesized that fluctuating aysmmetry would be negatively related to plant performance, as indicated by plant and leaf size. Thus, we predicted that there would be a a negative relation between leaf fluctuating asymmetry and plant size, with more asymmetrical leaves on smaller individuals. We also expected leaf fluctuating asymmetry to be negatively related to leaf size, with greater asymmetry values in smaller leaves.

2. Material and Methods

2.1. Study area

We performed this study in a *cerrado sensu stricto* (Brazilian savanna) area (~ 875 m.a.s.l.) in the *campus* of the Federal University of São Carlos, São Carlos, SP, Brazil. The Cerrado occupies a total of 180 hectares at this site and the sampled area has approximately 12 hectares. Part of the study area has been preserved since at least 1970 whereas part was used a eucalypt plantation until the 1990s and is currently in an advanced stage of regeneration. The climate in the region is characterized by a dry winter and a rainy summer, with an annual precipitation of 1339mm and average temperature of 22.1°C (Oliveira and Batalha, 2005). The study area was patchily burnt by an anthropogenic fire in August 2006, five years before the study (Dodonov et al., 2014).

2.2. Study species

Miconia albicans (Sw.) Triana (Melastomataceae) is a multiple-stem Neotropical woody species common in open vegetation (Miyanishi and Kellman, 1988) and very abundant in the study area (Dodonov et al., 2014), where it seldom reaches a height of over 3 m (Dodonov et al., 2011). It has simple coriaceous leaves, 4-16 × 1.6-6.5 cm in size, with the abaxial surface covered by trichomes and a glabrescent adaxial surface; its branches, petioles and inflorescences are also covered by trichomes (Goldenberg, 2004). It has small flowers distributed in panicles and berries with 25 to 35 seeds (Goldenberg, 2004).This species is apomyctic, producing sterile pollen and being little visited by potential pollinators (Goldenberg and Shepherd, 1998). The fruits, in turn, are consumed by at least 19 bird species (Allenspach and Dias, 2012). This is an aluminum-accumulating species (Haridasan, 1988) that responds to fire by intense resprouting (Dodonov et al., 2014; Hoffmann and Solbrig, 2003) and usually suffers low herbivory levels, in part due to the leaf pilosity (Paleari and Santos, 1998); however, it may be strongly affected by the galling nematode *Ditylenchus* sp (Viana et al., 2013).

2.3. Sampling

We sampled a total of 70 *M. albicans* individuals located along 7 transects in the study site, with 30 to 50 m between adjacent transect, in January 2011 (Figure 1). In each transect, we placed 10 sampling points distant 15 m from one another and sampled the *M. albicans* individual closest to each sampling point. Due to extremely high abundance of this species in the study area, the closest individual was seldom farther than 5 m from each sampling point. We haphazardly selected and collected 15 fully expanded leaves on each individual, transported them in plastic bags into the laboratory, and digitized them with a digital scanner, with the adaxial side downwards (Figure 1). When possible, and considering that *M. albicans* is highly attacked by the galling nematode *Ditylenchus* sp. (Viana et al., 2013), we selected leaves with little or no herbivory damage and none or few galls. We then measured fluctuating asymmetry by digitally dividing each leaf along its central nervation (Figure 2) and calculating the area of each half in the Image J software (Schneider et al., 2012). To control for measurement error, the division along the central nervation was performed by three separate researchers (ALB, LHA, PD), thus obtaining three fluctuating asymmetry values for each leaf. Subsequently, we calculated an average of the measurements performed by the three researchers and used it as a response variable in the analyses alongside the individual measurements.

We also measured the basal diameter and total height of each stem of all the stems belonging to each individual, with a Vernier caliper and a foldable 2-m ruler, between January and July 2011. These data could not be obtained for one individual, which was only included in the analysis of the relation between leaf fluctuating asymmetry and total leaf area (see below). The number of stems per individual varied between 1 and 14, with a total basal area between 0.88 and 160 cm^2 and a maximum height between 87 and 492 cm, which is representative of the variation in size

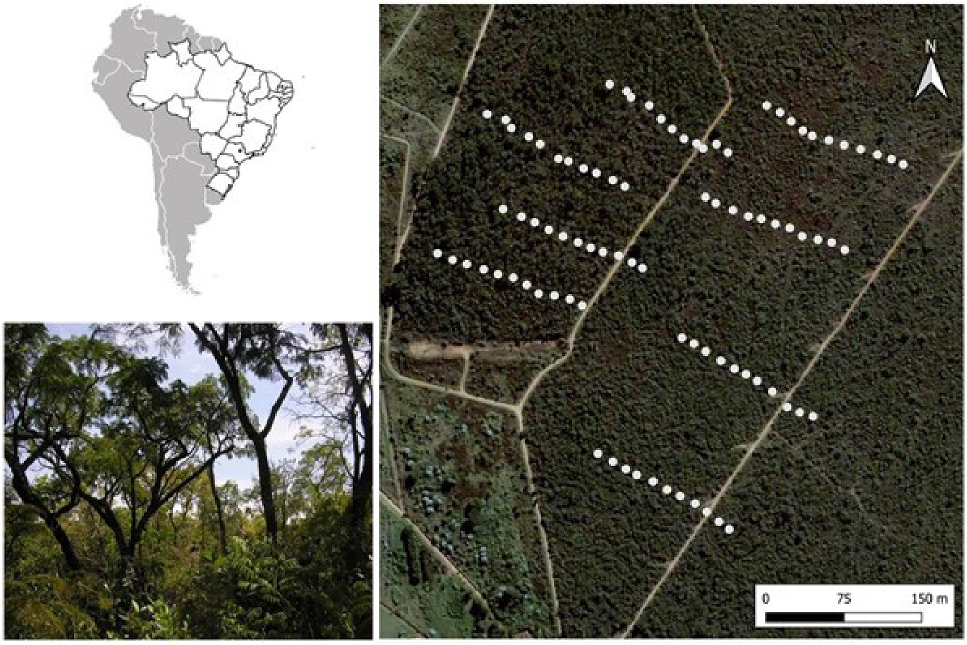

Figure 1. Location of the study site in Brazil, location of the sampling points in the study site and a representative photo of the study site.

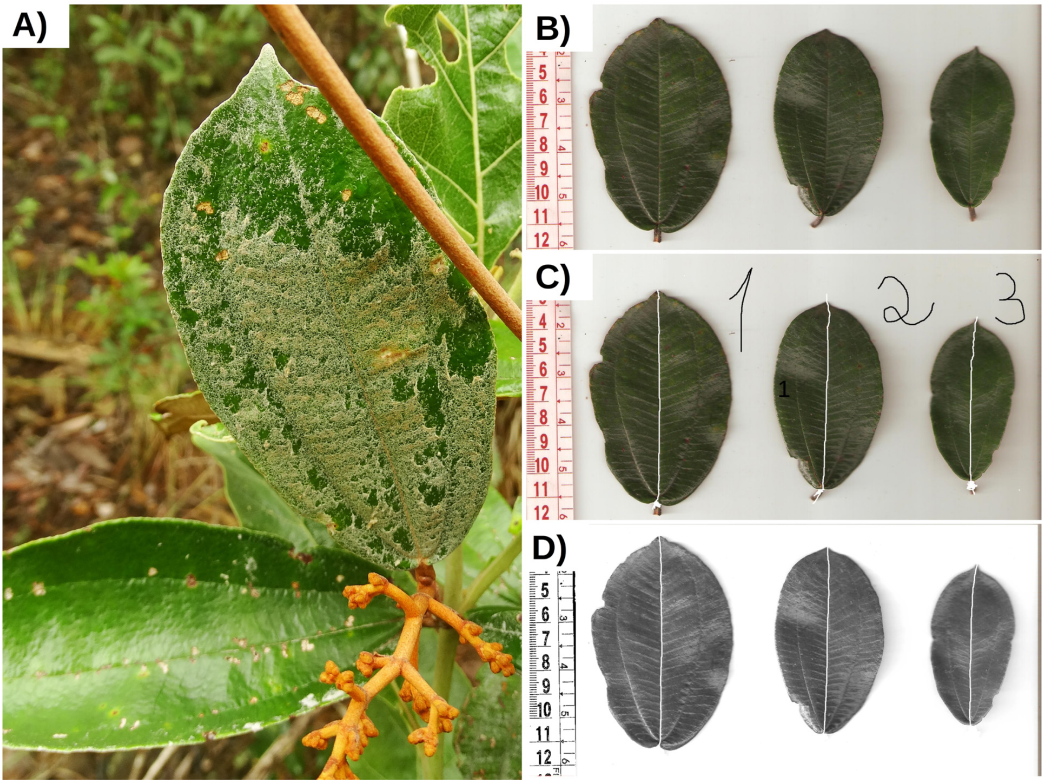

Figure 2. Leaves of *Miconia albicans* (A) and an example of how leaf fluctuaing asymmetry was calculated: three leaves with their adaxial side scanned (B), these same leaves divided along their central vein and numbered (C), and these same leaves in grayscale and with their petioles removed for the calculation of area in Image J (D). After calculating the area of the right and left sides, fluctuating asymmetry was calcualted as the absolute difference between the right area and the left area of each leaf. Scale in cm.

presented by *M. albicans* in our study (Dodonov et al., 2011; Dodonov et al., 2014).

2.4. Data analysis

We calculated total fluctuating asymmetry (FAtot) as the absolute difference between the areas of the right and the left sides of the leaf. As these values were correlated with total leaf area, we also calculated a relative fluctuating asymmetry (FArel) by dividing FAtot by the total leaf area. Fluctuating asymmetry is defined as random deviations from a perfect symmetry, and is therefore expected to have a mean of zero and a normal distribution (Palmer and Strobeck, 1992). We thus calculating confidence intervals for the difference between the right and left areas of the leaf by means of a bootstrap-T analysis, which is a more robust form than a simple bootstrap and does not assume a normal distribution (Manly, 2007). We also performed a maximum-likelihood mixture analysis (Dempster et al., 1977; Hammer et al., 2001), which adjusts one or more normal distributions to the data in order to assess whether the data may be described by a combination of two or more normal distributions. In addition to the curve distribution parameters, this analysis provides a probability value indicating the proportion of data points explained by each curve. We adjusted between one and four normal curves and selected the model with the lowest Akaike's Information Criterion (AIC); when the diffence in AIC between two models was smaller than two, we selected the model with the smallest number of curves.

To assess the relation between plant size and leaf fluctuating asymmetry, we performed a stepwise multiple regression between mean fluctuating asymmetry (FAtot and FArel) per individual and four different plant size parameters. For this, we started with the full model and gradually removed or added variables until AIC no longer decreased. For plant size, we used the number of stems, total basal area, mean basal area per stem, and maximum height (the height of the tallest stem) of each individual. We did not use mean height per stem because it was correlated to maximum height (R=0.77). To assess the relation between fluctuating asymmetry and leaf area, we adjusted a mixed linear model between FArel and leaf area, including the individual plant as a random factor. As an exploratory analysis showed that the greatest effect of leaf size appears to be on the maximum, and not the average, FArel values, we also performed a quantilic regression betweel FArel and leaf size, with the quantiles 0.1, 0.25, 0.5, 0.75, and 0.9. To account for the non-independence of leaves collected from the same plant, we used restricted randomizations to assess significance by randomizing the FArel values among the leaves from each individual, but not among plants. We used 4999 randomizations and calculated the slope for each randomization. We then assessed significance as the proportion of randomized slopes (including the observed slope, as it is considered to be one of the values that could be observed under the null hypothesis) which were equal to or greater than the observed slope. These restricted randomizations were based on the general guidelines given by Manly (2007).

Considering that measures of fluctuating asymmetry may be greatly affected by measurement error, we performed separate analyses for the values obtained by the three researchers and one additional analysis using the means of the asymmetry values obtained by the three researchers, and considered only the relations shown to be significant in all analyses as representative. We used the softwares Past 2.17c (Hammer et al., 2001) for the analysis and R 4.1.0 (R Development Core Team, 2021) for the other analyses, with the nlme package (Pinheiro et al., 2019) for the mixed models and the quantreg package (Koenker, 2021) for the quantilic regression. The data and codes used for this study are available at <https://github.com/pdodonov/publications>.

3. Results

The difference between the right and left sides of the leaves varied from -11.89 cm^2 to 9.56 cm^2, representing a maximum of 33% of the leaf area. The bootstrapped confidence intervals for the mean difference did not include zero for any of the researchers, with the lowest 2.5% confidence limit being of 0.035 cm^2 and the highest 97.5% confidence limit being of 0.39 cm^2. The results of the mixture analysis were less consistent, and models with two, three and four curves were selected for the different sets of difference values. However, regardless of the number of curves selected, two of them always accounted for at least 88% of the data points, showing that a large part of the data may be represented by a combination of two normal curves (Figure 3).

We did not find a clear relation between plant size and leaf fluctuating asymmetry, as the analyses performed on the values obtained by different researches and on mean values showed different results (Table 1). Thus, FAtot increased with maximum plant height for one researcher but was not related to any variables for the other two researchers nor for the mean values; conversely, FArel increased with total basal area and decreased with mean plant size for two researchers and for the mean values, but was not related to any variables for the third researcher (Table 1).

On the other hand, all models calculated for individual leaves showed a negative relation between FArel and total leaf area, with greater mean FArel values in smaller leaves ($p < 0.03$; Table 1; Figure 4).

The R^2 of the corresponding linear model, however, was very small, below 0.01. The quantile regression showed that the 0.9 quantile decreased significantly with leaf area ($p < 0.04$ in all regressions; Figure 4)) whereas the 0.1, 0.25, 0.5 and 0.75 quantiles had no regression with leaf area ($p > 0.12$ in all regressions) (Table 1).

4. Discussion

We observed a slight indication of directional asymmetry in the study species, as the right area was on average slightly larger than the left area. However, this directional asymmetry was quite small relative to the range of asymmetry values. In addition, the data may be described by a combination of two normal curves both of which are centered close to zero. Thus, leaf asymmetry of *M. albicans* may not be explained by simple random

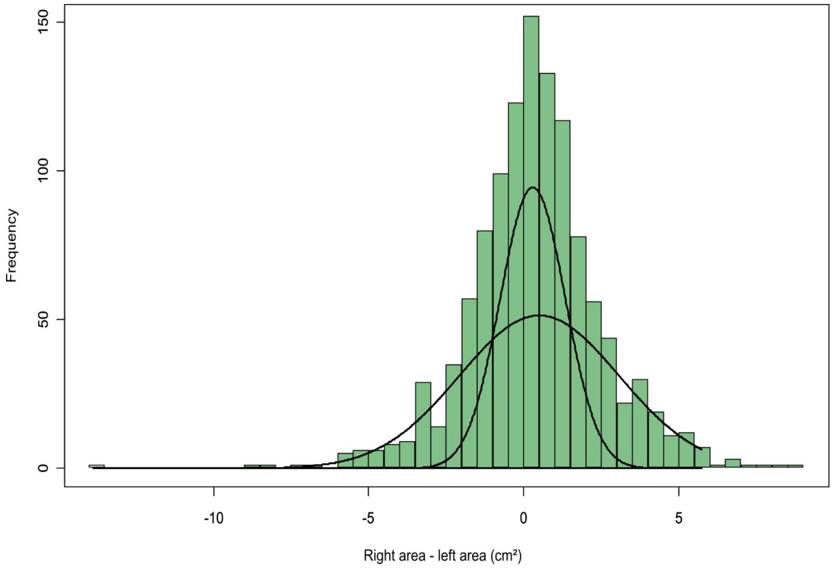

Figure 3. Mixture analysis of the differece between the right and left areas of the leaves of *Miconia albicans* (Melastomataceae), with two normal curves (means of 0.22 and 0.10 and standard deviations of 2.83 and 1.16) adjusted to the data. Results for only one researcher (ALB) are shown.

Table 1. Slope (b) and p values of linear, mixed and quantilic models between leaf fluctuating asymmetry and plant and leaf size, with statistically significant relationships highlighted in bold.

Response variable	Explanatory variable	ALB*	LHA*	PD*	Mean
FAtot	Total basal area	NA**	NA	b = -0.0074 p = 0.142	NA
	Maximum height	NA	NA	**b = 0.0041** **p = 0.028**	NA
FArel	**Total basal area**	**b = 0.0002** **p = 0.010**	**b = 0.0002** **p = 0.027**	NA	**b = 0.0002** **p = 0.014**
	Maximum height	**b = -0.0001** **p = 0.009**	**b = -0.0001** **p = 0.016**	NA	**b = -0.0001** **p = 0.007**
FArel	**Total leaf area**	**b = -0.0002** **p = 0.005**	**b = -0.0002** **p = 0.005**	**b = -0.0002** **p = 0.03**	**b = -0.0002** **p = 0.02**
FArel - quantile 0.10		b = -0.00007 p = 0.13	b = 0.00001 p = 0.79	b = -0.00004 p = 0.45	b = -0.00002 p = 0.84
FArel - quantile 0.25		b = -0.00002 p = 0.69	b = -0.00006 p = 0.37	b = -0.00004 p = 0.59	b = -0.00003 p = 0.64
FArel - quantile 0.50		b = -0.00004 p = 0.13	b = -0.00008 p = 0.26	b = 0.00002 p = 0.87	b = -0.00005 p = 0.49
FArel – quantile 0.75		b = -0.0002 p = 0.13	b = -0.0002 p = 0.15	b = -0.0001 p = 0.15	b = -0.0002 p = 0.16
FArel – quantile 0.90		**b = -0.0006** **p = 0.036**	**b = -0.0008** **p = 0.0004**	**b = -0.0005** **p = 0.013**	**b = -0.0007** **p = 0.0010**

*Analyses for the fluctuating asymmetry measured by three researchers (ALB, LHA, PD) and for the mean values. **This explanatory variable was not in the final model selected by the stepwise regression.

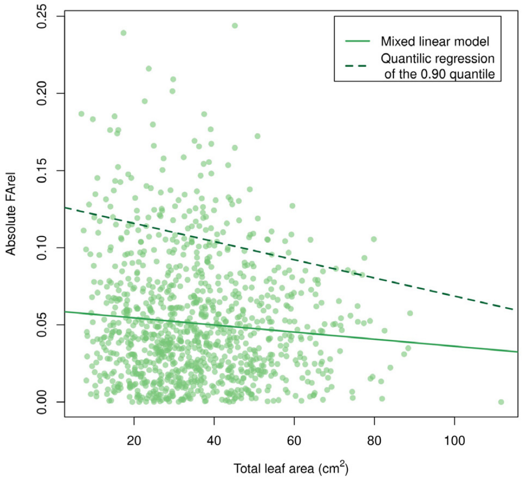

Figure 4. Relation between relative fluctuating asymmetry (calculated as the absolute difference between the right and left leaf areas divided by total leaf area) and total leaf area. The solid line represents a mixed linear model, with plant included as a random factor, whereas the dashed line represents a quantilic regression of the 0.90 quantile. The data shown are the mean of the values calculated by the three researchers for each leaf.

deviations from a bilateral pattern and may have a small genetic component (Palmer and Strobeck, 1992). Still, we believe this does not preclude the assessment of how leaf asymmetry may be related to other plant characteristics, such as branching and venation patterns (Freeman et al., 1993) as well as plant and leaf size, considering that many studies highlight that developmental instability can be better evaluated when using several traits for the same individual and seeking to prove the fluctuating asymmetry in all traits (Tomkins and Kotiaho, 2001). However, it is challenging to choose traits that evolve under the same selective pressures and respond to environmental stress in similar ways.

Our expectation regarding plant size effects of leaf fluctuating asymmetry was not confirmed, as we did not find any clear relations between plant size and fluctuating asymmetry. This may indicate that physiological stress faced by the individual during its development does not affect leaf fluctuating asymmetry, which may be affected mostly by the microclimatic conditions faced by the individual leaves (Cowart and Graham, 1999). It could be argued that we did not have a large enough gradient in plant size to detect a response; however, the range of plant sizes observed in our study is representative of the variation in the size of this species in the study site and elsewhere (Dodonov et al., 2014; Hoffmann and Solbrig, 2003). Conversely, as this study species is well adapted to the environment, it is possible that the physiological stress faced by it was not sufficient to elicit a response in leaf fluctuating asymmetry, as all plants were growing in a natural environment without direct influence of stress-inducing factors such as high air pollution levels. Another plausible explanation would be the fact that plants tend to respond to stressful conditions using plasticity in the development of morphological structures (Komac and Alados, 2012). Thus, these compensatory physiological responses can prevent us from identifying stress (Kozlov et al., 2002; Freeman et al., 2005) and camouflaging the existence of fluctuating asymmetry. Finally, an important source of stress of plants is herbivory and parasitism by galling invertebrates, which can significantly impair the performance of this species (Viana et al., 2013). In our study, we deliberately minimized this effect by selecting leaves with little damage by herbivores and few galls because we were interested in other sources of stress. Thus, it is possible that stronger effects would be observed if the effects of the number of galls were assessed alongside those of plant and leaf size.

The relation between leaf size and asymmetry was reflected mostly in the upper limits to leaf fluctuating asymmetry, as larger leaves had smaller maximum FArel values. On one hand, it may be related to measurement error, as small measurement errors are relatively more important in smaller leves (Graham et al., 2015). However, this relation was consistent for all three researchers as well as for the mean values calculated for the three researchers. Assuming that measurement errors are unbiased, positive and negative errors would be cancelled out when the mean values are calculated, reducing the influence of measurement error. We therefore believe it can be validly stated that, in the study species, larger leaves tend to be relatively less asymmetrical than smaller leaves, at least regarding the maximum asymmetry values observed for each leaf size. There may be much variation in leaf characteristics on the same tree (Suomela and Ayres, 1994; Bruschi et al., 2003), possibly because of variation in stress factors such as exposure to sun, drought and herbivory. Our results indicate that the leaves that are able to reach a greater size tend to be less asymmetric, possibly indicating that they were subjected to less stressful conditions during their development. It thus appears that leaf fluctuating asymmetry is related more closely to the conditions faced by an individual leaf than by the plant as a whole.

It is known that factors such as shade (Alves-Silva, 2012), galling invertebrates (Viana et al., 2013), pollution (Ivanov et al., 2015) and other factors may affect plant performance and/or plant fluctuating asymmetry. Although we did not assess variation in these variables, given the plants' locations and our selection of leaves with little herbivore damage and none or few galls, it is likely that these variables did not have great variation among our study plants and leaves. Nevertheless, we detected a relationship between leaf size and fluctuating asymmetry. The smaller fluctuating asymmetry in larger leaves, when accounting for variation in leaf size, may indicate that they may have been subjected to less stress during their development even in the absence of obvious stress factors.

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