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# Leaf phenotypic variation and developmental instability in relation to different light regimes

**Henrique Venâncio[1,2], Estevao Alves-Silva[2] and Jean Carlos Santos[1*]**

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## ABSTRACT

For pioneer plants, shaded habitats represent a stressful condition, where sunlight exposure is below the optimum level and so leaves expand in order to intercept a greater amount of light. We investigated changes in both phenotypic variation and stress of *Bauhinia brevipes* in sunny and shaded microhabitats. Leaf area was used as a measure of phenotypic variation, whereas leaf asymmetry (difference between right and left sides of leaves), was used as a measure of stress. We hypothesized an increase in leaf area and stress in shaded locations, which might indicate that *B. brevipes* was compensating for low light absorption, and elevated levels of stress, respectively. Plants in the sun fitted a fluctuating asymmetry pattern (normal distribution of *right minus left* sides), while shaded plants were clearly antisymmetric (bimodal distribution of leaf side differences). Leaf asymmetry and area were 5% and 26.8% higher in plants in the shade compared to plants in the sun, respectively. These results were expected since *B. brevipes* is found predominantly in open areas; so sunlight exposure is important for its development. The presence of antisymmetry is rare in studies of developmental instability, and here it might indicate higher stress compared to plants with fluctuating asymmetry.

**Keywords:** antisymmetry, *Bauhinia brevipes*, fluctuating asymmetry, leaf morphometry, sunlight exposure

## Introduction

Organisms in nature can be subjected to unfavourable developmental conditions that affect their growth and persistence (Jan *et al*. 2012). For instance, plants living in salty, shaded or poor nutrient habitats usually present increased stress levels in comparison to plants in favourable environments (Palmer & Strobeck 1986; Møller & Dongen 2003; Cornelissen & Stiling 2011; Alves-Silva 2012). To persist in stressing habitats, plants have evolved, by natural selection, the ability to respond through morphological and physiological modifications, which is reflected in their phenotypic variation (Cardoso & Lomônaco 2003; Miner *et al*. 2005; Rozendaal *et al*. 2006). These modifications may occur rapidly and in several structures (e.g., leaves) as a response to adverse conditions (Alpert & Simms 2002).

Sunlight is an essential resource for the growth and performance of plants; however, plants can sometimes occur in places where sunlight exposure is below the optimum level (Niinemets 2010). For instance, many species in the Cerrado (Brazilian savanna) occur predominantly in open areas (e.g., Melastomataceae; Myrtaceae; Malpighiaceae; Solanaceae – EA Silva unpubl. res.), where they are exposed to high amounts of solar exposure. In fact, the germination and performance of some Cerrado plants can depend largely on sunny habitats (Lima *et al*. 2014; see also Toledo-Aceves

[1] Instituto de Biologia, Universidade Federal de Uberlândia, Av. Pará, 1720, 38405-320, Uberlândia, MG, Brazil
[2] Pós-Graduação em Ecologia e Conservação de Recursos Naturais, Universidade Federal de Uberlândia, Av. Pará, 1720, 38405-320, Uberlândia, MG, Brazil
* Corresponding author: jcsantos@ufu.br

& Swaine 2008). Therefore, when light is minimum (*i.e.*, shady conditions), plants can respond accordingly (Marques *et al.* 1999; Rossatto *et al.* 2010; Ronquim *et al.* 2013), and approaches such as the assessment of phenotypic variation have been used to evaluate the performance of plants in different microhabitats (Marques *et al.* 2000; Ackerly *et al.* 2002; Niinemets 2010). Usually, plants enhance the leaf area/length in order to intercept higher amounts of light (Valladares *et al.* 2007). In this context, it is expected that plants present intraspecific variation when individuals are exposed to sun or shade (Marques *et al.* 2000; Valladares & Niinemets 2008).

Intense responses to environments such as sun or shade can generate a phenomenon called developmental instability (DI) (Alves-Silva & Del-Claro 2013; Venâncio *et al.* 2016), which is the inability of organisms to cope with stressing conditions (Alpert & Simms 2002; Alves-Silva 2012). Developmental instability is commonly assessed through the fluctuating asymmetry (FA) analysis, which measures the imperfect growth of supposedly bilateral structures, such as leaves (Graham *et al.* 2010). Large deviations from perfect symmetry are evidence of high stress, and so this technique is widely used to compare stress levels in several habitats (reviewed by Møller & Shykoff 1999).

According to the literature, FA is the most common estimate of developmental instability (Graham *et al.* 2010; Santos *et al.* 2013). Nonetheless, there is some evidence that other types of asymmetry, such antisymmetry (bimodal distribution of leaf sides, *i.e.*, the population can have both increased left and right sides) and directional asymmetry (one leaf side is invariably greater than the other) also depict developmental instability (Graham *et al.* 1998). However, so far only a few studies have examined this issue (Lens & Dongen 2000; Silva *et al.* 2015). Therefore, any new relationship between the other types of asymmetry and a given stressing factor might promote advances for the studies of DI (McKenzie & O'Farrell 1993; Telhado *et al.* 2016).

In the Brazilian savanna, the legume tree *Bauhinia brevipes* Vogel (Fabaceae) is a good model to investigate the relationship between phenotypic variation and DI according to different sunlight conditions. This species occurs in several phytophysiognomies within the savanna (Vaz & Tozzi 2003) and thus can be expected to occur in habitats with a wide variety of sunlight conditions. *Bauhinia brevipes* presents phenotypic variation and FA according to herbivory levels (Cornelissen & Fernandes 2001; Santos *et al.* 2013), but environmental factors as agents that promote phenotypic variation and DI have not yet been examined. Thus, in this study, we investigated changes in both phenotypic variation and DI of *B. brevipes* in sites with different levels of sunlight exposure. Our measure of phenotypic variation was leaf area and DI was assessed through the differences between the leaf right and left sides (following Santos *et al.* 2013). We hypothesized that increased leaf area and DI would occur in shady locations, which might indicate *B. brevipes* compensation for light absorption and elevated levels of stress, respectively.

# Materials and methods

## Study area

Fieldwork was conducted in November 2015 in a rupestrian field at the Parque Estadual da Serra de Caldas Novas, located in Caldas Novas city, Brazil (17°46'S - 48°40'W). The park has 123 km² of area, and sustains several phytophysiognomies distinctive of the Brazilian savanna (Cerrado biome), especially the Cerrado *strictu sensu* (see Felfili & Silva Jr. 1993; Castro *et al.* 1999 for detailed information about the Cerrados). The park has a tropical climate with two well-defined seasons, a hot-wet summer (October–March) and a (less hot) dry winter (April–September) (Lima *et al.* 2010). The sites chosen for the study supported sparse vegetation with a layer of herbs and grasses and a predominance of shrubs (<1 m tall) and medium trees (2–3 m tall).

## Plant species

*Bauhinia brevipes* Vogel (Fabaceae) is a semi-deciduous tree that is widely distributed in the Brazilian savanna (Santos *et al.* 2013), especially in sites where taller trees in the vicinity possess narrow canopies (Gonçalves-Alvim & Fernandes 2001; Vaz & Tozzi 2003). Adult plants can reach up to 3 m in height, but many individuals in the Cerrado occur as shrubs (1–1.5 m tall), principally in areas where fires are common, such as at the park where the study was carried out (Lopes *et al.* 2009; Frizzo *et al.* 2012). *Bauhinia brevipes* has green and smooth margined subcoriaceous leaves that may reach up to 9 cm in length and 7 cm in width. Small trichomes occur throughout the adaxial leaf surface (Vaz & Tozzi 2003). Leaf flush takes place in October and November at the onset of the rainy season (Santos *et al.* 2013). At the time of sampling some individuals within the population of *B. brevipes* presented different phenological conditions, so we narrowed our search to those plants that had similar patterns of leaf and maturation and growth.

## Study design

All *B. brevipes* selected for this study were located in a 5-ha radius, and we tagged individual plants that were at least 10 m distant from each other. In addition, only plants in the same phenological conditions (with mature leaves) and minimum presence of herbivory (*i.e.*, galls and chewers) were chosen for the study design. Plants were chosen to permit the assessment of different sunlight conditions (e.g., shade and sunlight exposure). Following these criteria, we were able to select 26 *B. brevipes* shrubs (< 1.5 m tall) that fulfilled our requirements. Individual plants were equally assigned as 'shade' (plants that occurred near tall trees with large canopies that prevented *B. brevipes* shrubs from receiving direct sunlight) and 'sun' (plants that were not totally shaded by the canopy of any trees nearby), as adapted from Clark & Clark (1992) and Venâncio *et al.* (2016) (Fig. 1A-B).

From each individual plant, five branches located at approximately the same distance from the soil were chosen and one apical leaf was collected from each branch ($n$ = 65 leaves from each plant group). Special care was given to this sampling and we chose only leaves without herbivory marks or hypersensitive reactions to galls (Santos *et al.* 2008). Leaves were photographed under a layer of transparent glass, and then we proceeded to the assessment of leaf area and sides (developmental instability) following Cornelissen & Stiling (2005).

## Leaf measurements

To investigate whether the leaf size varied according to microhabitat conditions (sun/shade), the area ($mm^2$) of all leaves collected was estimated using Image J software from digital images of leaves. In addition, all leaves of *B. brevipes* were measured on both the right and left sides (*Rs* and *Ls*, in mm), and the horizontal middle portion of the leaves was used as the reference in order to account for the widest part of leaves (Fig. 1C). Individual leaf asymmetry was assessed as $d = Rs - Ls$. Trait size for leaf sides was calculated as $(Rs + Ls)/2$ and it was used to verify whether $|d|$ (i.e. unsigned *right minus left* sides) varied according to leaf size. In order to verify whether our measurements were conducted with enough accuracy to eliminate errors, we used the Index of Repeatability of Falconer, which takes into account the individual *versus* side variance to estimate the measurement accuracy (Yezerinac *et al*. 1992; Cornelissen & Stiling 2005). A subsample of 50 leaves was re-measured and compared with the original measurements of *Rs* and *Ls*.

## Statistical analyses

A two-way ANOVA using leaf sides, individuals and measurements was conducted and the variance within and between the samples was used to calculate the Index of Repeatability (Yezerinac *et al*. 1992), which varies from 0 (maximum error) to 1 (absence of measurement error). Before any inference on the role of environmental stress on the developmental instability levels, it is necessary to verify whether the leaf asymmetry measurements fit the pattern of fluctuating asymmetry (FA; *i.e.*, small and random deviations from perfect symmetry), directional asymmetry (DA; *i.e.*, one particular side is significantly larger than the other) or antisymmetry (AS; bimodal distribution of the $Rs - Ls$ measurements). The presence of DA was tested with the Student's $t$ test with the mean being equal to zero. Following Cowart & Graham (1999), we examined measures of skew ($\gamma_1$) and kurtosis ($\gamma_2$) to see whether the data deviated from normality (indication of antisymmetry). According to the tests, normal data should have a skewness value around zero and a kurtosis value around 3. These analyses were conducted with the package 'moments' in *R* statistical software 3.2.3. In addition, Shapiro–Wilk normality tests were also performed to check the normality of the data. We also conducted the Hartigans' Dip test for unimodality (package 'diptest' in *R*), which indicates potential bimodality in our data (evidence of AS). Measures of relative (coefficient of variation) and absolute (mean, standard deviation and median) were estimated for $|d|$ and leaf area ($mm^2$) in both plant groups.

The relationship between $|d|$, and trait size (both leaf width ($(Ls+Rs)/2$) and leaf area ($mm^2$)) was examined with linear regressions. A significant relationship between these variables requires the use of a specific formula to assess developmental instability. Since leaf area was related to $|d|$ (see Results section), it indicated that our data set fitted a multiplicative error according to Cowart & Graham (1999). In this scenario, the values of $|d|$ incur transformations before subsequent analyses. First, a log transformation was

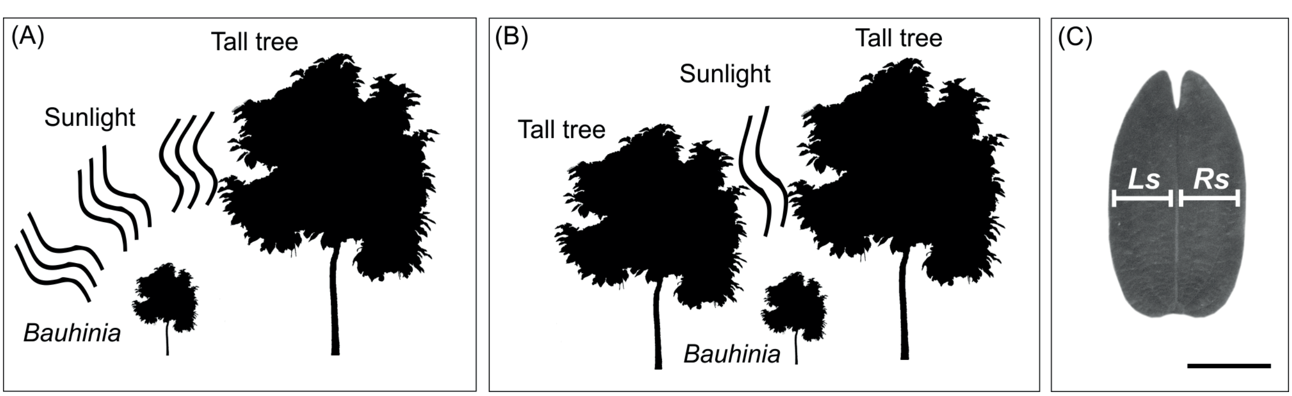


**Figure 1.** Study design and leaf measurements of *Bauhinia brevipes* in a neotropical savanna. (A) plants labelled as 'sun', as they received sunlight most part of day; (B) shaded plants under canopy of tall trees; (C) *B. brevipes* leaf: Ls – left side, Rs – right side. Scale bar = 25 mm.

performed ($|d| = log\ Ls - log\ Rs$) and then data were Box Cox transformed ($d^* = (|d| + 0.00005)^{0.33}$). These series of transformations remove any size scaling from multiplicative error (when leaf asymmetry and trait size are related) and normalize the data (Cowart & Graham 1999). In addition, as we found antisymmetry in our data set (see Results section), the Box-coxed transformed data is more appropriate for statistical tests and comparisons between plant groups.

The DI index in plants was then assessed as DI = $\Sigma(|d^*|)/n$, where '*n*' is the number of leaves measured. The mean DI (i.e. leaf asymmetry) per plant was used for the subsequent statistical analyses. Comparisons of DI levels and leaf area ($mm^2$) between plants in the sun and shade were made with Student's *t* tests.

## Results

The Index of Repeatability showed that our measurements were conducted with enough accuracy, thus eliminating possible errors during the process of leaf morphometry evaluation (IR = 0.98). Our data indicated that plants in the sun presented purely FA, as both DA and AS were discarded, whereas data from shaded plants did not show DA or FA, but fit into AS. Neither kurtosis nor skewness were statistically significant in any plant group (Tab. 1). Density plots showed a clear bimodality in the leaf asymmetry data for plants in the shade (Fig. 2).

Data were skewed to the right for both plant groups (skew > 0 in both plant groups). For the kurtosis, plants in the sun presented a platykurtic distribution. Shaded plants had a bimodal distribution, and so kurtosis interpretation was impractical. Leaf width was not related to leaf asymmetry $|d|$ (sun: $F11 = 0.0260$; $R^2 = 0.0023$; $P > 0.05$; shade: $F11 = 2.8230$; $R^2 = 0.2042$; $P > 0.05$), but was positively and significantly related to leaf area from shaded plants ($F11 = 6.0859$; $R^2 = 0.3562$; $P < 0.05$; sun: $F11 = 1.2464$; $R^2 = 0.1017$; $P > 0.05$) (Fig. 3).

Developmental instability ($d^*$) was roughly 5% (average values) higher in plants in the shade compared to plants in the sun, but results were not significant ($t24 = 0.9413$, $P = 0.1779$) (Fig. 4A). Leaf area, however, was statistically greater in shaded plants by 26.8% ($t24 = 2.1500$, $P < 0.05$) (Fig. 4B). Variation of leaf DI was higher for plants in the sun, while leaf area varied more for shaded plants. The range of DI was also higher for sunny plants and the opposite occurred for leaf area, as shaded plants presented larger variations compared to sunny plants. Values of median indicated that shaded leaves were 14% more asymmetrical and 22% larger than their counterparts in the sun (Tab. 2).

## Discussion

Several plant species have the ability to modify their structures through phenotypic variation to maintain their

**Table 1.** Statistical analyses to check for directional asymmetry (DA, one-sample Student's *t* test), antisymmetry (AS, Shapiro–Wilk and Hartigan's Dip test), skewness and kurtosis in two groups of *Bauhinia brevipes* in a Brazilian savanna. *n.s.* – non-significant; * $P < 0.05$ and ** $P < 0.001$. Values in bold indicate statistically significant differences.

| Analyses | Plant Groups | |
|---|---|---|
| | Sun | Shade |
| Direct. asymmetry | $t = 1.7525$ n.s. | $t = 1.4305$ n.s. |
| Antisymmetry | $w = 0.9628$ n.s. | **$w = 0.9326$ *** |
| Bimodality | dip = 0.0537 n.s. | **dip = 0.0721 *** |
| Skewness | skew = 0.3718 n.s. | skew = 0.3014 n.s. |
| Kurtosis | kurt. = 2.3369 n.s. | **kurt. = 1.9064 **** |
| STATUS | Fluctuating asymmetry | Antisymmetry |

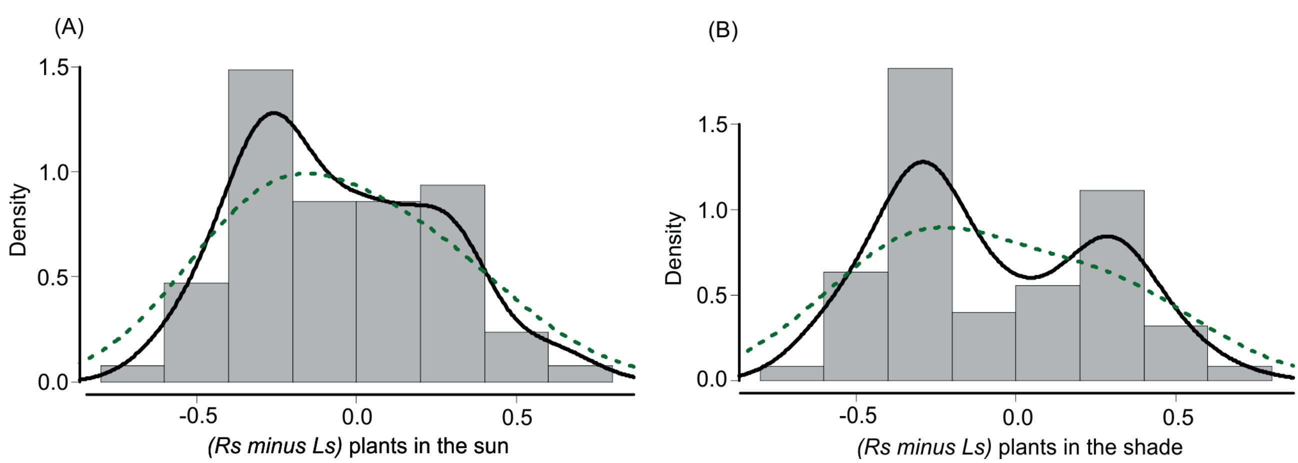


**Figure 2.** Density plots showing the distribution of leaf sides (right and left sides) according to plant location: (A) in the sun and (B) in the shade. Complete lines – adjusted distribution; dashed lines – predicted distribution.

**Table 2.** Measures of relative and absolute variability of developmental instability and leaf area in leaves of *Bauhinia brevipes* according to their occurrence in a Brazilian savanna. C.V. – coefficient of variation.

| Variables | C.V. (%) | Range | Median |
|---|---|---|---|
| Dev. instability (*d**, mm) | | | |
| Sun | 15.68 | 0.14 - 0.26 | 0.25 |
| Shade | 6.59 | 0.19 - 0.24 | 0.29 |
| Leaf area ($mm^2$) | | | |
| Sun | 24.19 | 425.68 - 1025.97 | 646.17 |
| Shade | 29.93 | 518.39 -1435.75 | 828.93 |

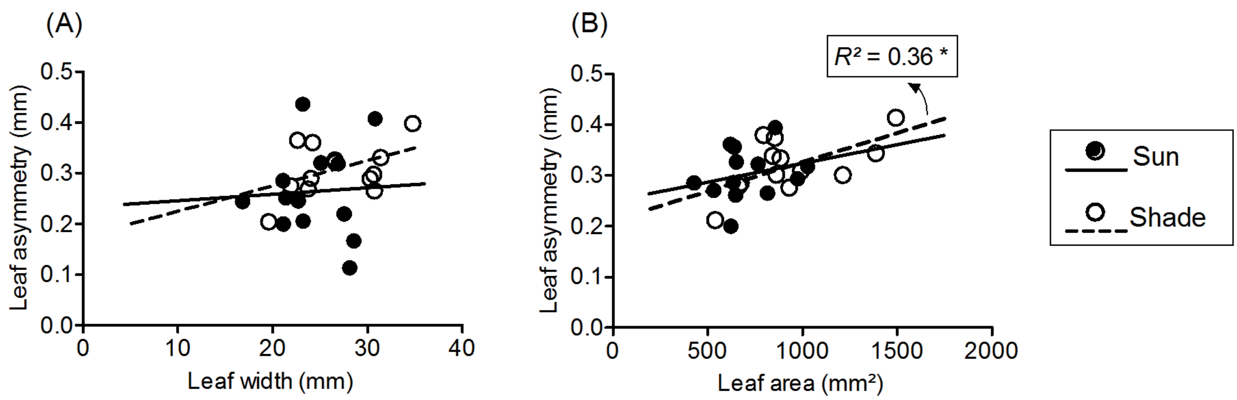


**Figure 3.** Relationship between leaf asymmetry |d|, leaf width and leaf area. Results were positive in all cases, but significant only for the relation between leaf asymmetry and area in shaded plants.

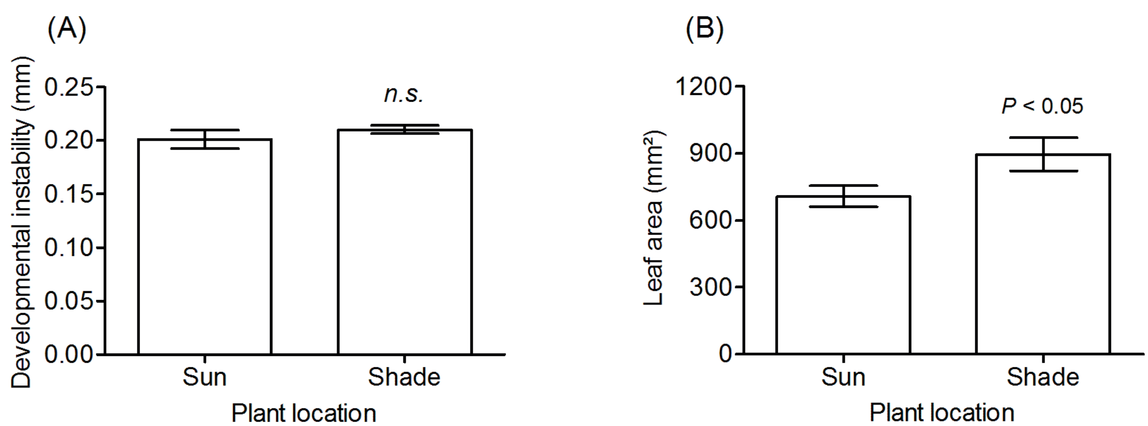


**Figure 4.** Leaf asymmetry as a measure of developmental instability, and phenotypic variation in *Bauhinia brevipes* according to location, i.e. sun or shade in a Brazilian savanna. (A) Leaf asymmetry; (B) leaf area. *n.s.* – non-significant.

performance in the face of stressing conditions (Cardoso & Lomônaco 2003). This modification is fairly common in light-dependent plant species growing in shaded habitats (Markesteijn *et al.* 2007). In our study, the legume shrub *B. brevipes* presented marked differences in leaf area in response to the microhabitat, as shaded plants had significantly larger leaves in comparison to plants in sunny sites. The modification of leaves is implemented by some plant species in order to increase the absorption of sunlight (Valladares *et al.* 2000; Baesse *et al.* 2014). We have no data to show that *B. brevipes* is a pioneer species, but this plant is observed to resprout rapidly on the edges and in areas subjected to disturbances such as fire (EA Silva unpubl. res.), and so we are inclined to believe that it is fully adapted to open/sunny sites. Therefore, increased leaf area in shaded plants, as found in our study, can be a countermeasure of plants to persist in the late stages of the succession processes (Valladares *et al.* 2000; Valladares *et al.* 2002; Rozendaal *et al.* 2006). Within the area where the study was carried out, fires are frequent and produce clearances for the colonisation of pioneer plants (Lopes *et al.* 2009). However, as the succession process advances, pioneer plants are subjected to high levels of light heterogeneity created by the canopy of large trees (Valladares *et al.* 2000; Poorter *et al.* 2004). This microhabitat heterogeneity might have been responsible for the increased levels of variability in leaf area from shaded plants, which might allow for a better exposure to sunlight and postpone the persistence of *B. brevipes* in shaded habitats.

As we hypothesized, leaves in the shade presented increased levels of stress (but not statistically significant), as measured by the difference between the leaf sides. However, these differences were of only 5% (mean values; median = 14% differences). Leaf asymmetry was positively related to leaf area, but the data were significant only for shaded plants. This result may indicate that as the leaves grow larger to intercept more sunlight, they tend to become more asymmetric. A significant relationship between leaf asymmetry and area was not found for plants in the sun, indicating that leaf growth is not followed by developmental instability.

Some studies have found a relationship between sunlight and leaf developmental instability (Puerta-Piñero *et al.* 2008; Alves-Silva & Del-Claro 2013; Venâncio *et al.* 2016). For instance, developmental instability in leaves of the pioneer shrub *Miconia fallax* (Melastomataceae) was 25% higher in shaded plants than their co-specifics growing on the edges and exposed to sunlight (Alves-Silva 2012). All these studies found that leaves presented fluctuating asymmetry, and in fact, in a recent study, Santos *et al.* (2013) found true patterns of fluctuating asymmetry in *B. brevipes*. Fluctuating asymmetry has long been considered as a phenotypic indicator of developmental instability and a biomarker of plant stress (Kozlov *et al.* 2001), and it is related to several biotic (e.g., herbivory, competition, parasitism) and abiotic factors (e.g., pollution, climate, nutrients) (Rettig *et al.* 1997; Cuevas-Reyes *et al.* 2011; Beasley *et al.* 2013; Uhl 2014; Ivanov *et al.* 2015; Alves-Silva & Del-Claro 2016). For instance, *B. brevipes* with high parasitism by galls have increased fluctuating asymmetry (Santos *et al.* 2013). Other types of leaf asymmetry (e.g., directional asymmetry and antisymmetry) in relation to plant stress have rarely been examined, both because they do not seem to be common in nature and because researchers are still unsure whether they depict developmental instability (Graham *et al.* 1993; Silva *et al.* 2015; Telhado *et al.* 2016).

In the present study, we found two patterns of asymmetry in *B. brevipes*. Plants in the sun, despite the high variability in bilateral morphometry, presented true patterns of fluctuating asymmetry, whereas leaves from shaded plants were rigorously antisymmetric. Leaf asymmetry was 5% higher in shaded plants (although this was not significant), revealing that these plants were, presumably, in a more stressed habitat compared to plants in the sun (see Puerta-Piñero *et al.* 2008; Alves-Silva & Del-Claro 2013; Venâncio *et al.* 2016). This makes us inclined to believe that antisymmetry can also depict stress, because if we assume that leaf asymmetry is related to stress levels, shaded plants were unable to buffer the developmental processes, thus giving rise to increased leaf asymmetry. Nonetheless, as differences in leaf asymmetry were low (5%) between plant groups, we cannot rule out the fact that leaf asymmetry may rather reflect the acclimation potential of the species over a wide host range with a diverse array of abiotic conditions (*i.e.*, sun, shade, wind, edaphic conditions, relief, etc). In order to conclude that shade environment was stressful, additional investigations should be performed.

According to Graham *et al.* (1993 and references therein), antisymmetry can arise from 'symmetry-breaking phase transitions', which occur when organisms are under severe stress, resulting in nonlinear processes of development (see also Lens & Dongen 2000, and Graham *et al.* 1998, for similar considerations on directional asymmetry). However, most studies on antisymmetry have been conducted with animal populations (Pratt & McLain 2002; Will & Liebherr 2015), and so the stress-antisymmetry relationship is lacking for plants. According to Sakai & Shimamoto (1965), both fluctuating asymmetry and antisymmetry can manifest in the same plant species. In *B. brevipes*, we found both fluctuating asymmetry and antisymmetry in the same population, and Santos *et al.* (2013) found only fluctuating asymmetry in another distant population. This indicates that in some cases, different types of leaf asymmetry might depend on both spatial scales and stressing factors.

Here we showed changes in the phenotypic variation of *B. brevipes* in response to different microhabitats, and we also provide evidence that a plant outside its supposedly optimum habitat presents increased levels of leaf asymmetry, which fits the antisymmetry pattern. Whether other types of asymmetry (directional asymmetry and antisymmetry) reflect developmental instability still remains to be discussed (Graham *et al.* 1993; Graham *et al.* 2010; Telhado *et al.* 2016) and more studies may clarify the relationship between plant stress and leaf asymmetry.

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