

# Herbivory causes increases in leaf spinescence and fluctuating asymmetry as a mechanism of delayed induced resistance in a tropical savanna tree

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**Background and aims** – Leaf spinescence is thought to be a defense against herbivory and may increase in years following high herbivory pressure (DIR – delayed induced resistance). Besides DIR, herbivory also causes plant stress, which can be assessed using fluctuating asymmetry analysis (FA – random departures from leaf bilateral symmetry). In this two-year field study we first investigated the relationships between herbivory, FA and spinescence in *Solanum lycocarpum* trees during an outbreak of moth caterpillars in 2010; then in 2011 we examined whether plants with intense herbivory the previous year presented changes in FA (herbivory induced stress) and spinescence.

**Key results** – In 2010, leaves damaged by moths were significantly more asymmetrical than undamaged leaves. In addition, moths preferentially fed on leaves with fewer and shorter spines. In 2011, plants severely attacked by moths the year before showed significant increases in FA (28%), spine abundance (21%) and spine length (84%), compared to less damaged plants.

**Conclusions** – Our data suggest a delayed response of *S. lycocarpum* to herbivory, in that plants subjected to high leaf damage the previous year maximized their display of anti-herbivore mechanisms, in this case, leaf spinescence. This is one of the few studies to show that FA increased as a response to herbivore attack. We conclude that FA can be used as an indicator of plant stress following leaf damage.

**Key words** – Cerrado, developmental instability, plant physical defense, Saturniidae, *Solanum lycocarpum*, spinescence.

## INTRODUCTION

Herbivorous insects are extremely diverse in the tropics and may feed on virtually any plant structure, thus exerting a negative effect on their host plants (Karban & Strauss 1993, Marquis et al. 2001, Cuda et al. 2008). Nonetheless, plants can rely on several defenses against herbivores, such as tannins (Coley 1986), latex (Agrawal & Konno 2009), trichomes (Løe et al. 2007), secondary metabolites (Lambdon et al. 2003) and silicon accumulation (Korndörfer & Del-Claro 2006), among others (Lucas et al. 2000). However, leaf spines as a mechanical defense against herbivory have rarely been studied worldwide. For instance, leaf spines are only sporadically cited in reviews of plant defenses (Fernandes 1994, Coley & Barone 1996). Spines are not as widely distributed in plants as trichomes or latex (Fernandes 1994, Agrawal & Konno 2009) and their role in herbivore deterrence is controversial and underestimated. Although spines have long been assumed as a barrier against herbivory (Supnick 1983), the role of spines as an anti-herbivory mechanism is conditional. It occurs because spiny plants may also rely

on other anti-herbivory defenses (Potter & Kimmerer 1988) and in addition, some herbivores can avoid spiny leaves and feed on spineless ones in the same plant (Gowda 1996).

Despite the presence of spiny leaves, the neotropical plant species *Solanum lycocarpum* St.Hil. (Solanaceae) may be attacked by herbivorous insects (Stefani et al. 2000, Moreira & Del-Claro 2005). The production of spines varies within *S. lycocarpum* individuals, in that neighboring leaves can have a number of spines or no spines at all. The variable distribution of spines on leaves suggests that these structures require some type of stimulus to be produced. Spine production can be triggered both by abiotic and biotic factors (Gowda & Raffaele 2004), and herbivory is one of the most important influences on spine production (Gowda 1996, Obeso 1997).

Some plants present a peculiar mechanism of protection against herbivory known as delayed induced resistance (DIR) (Zvereva et al. 1997a). Individual plants subjected to severe levels of damage may maximize the production of anti-herbivory apparatus in the following season as a mechanism to reduce herbivory. For instance, defoliation in birch

trees in the previous year decreased the concentration of nitrogen and increased anti-herbivory compounds such as phenols in leaves in the following seasons (Kaitaniemi et al. 1998). In addition, changes in plant metabolism and physiology modify the quality of tissues (Korndörfer & Del-Claro 2006), which in turn negatively affects the performance of herbivorous insects. Zvereva et al. (1997a) noticed that beetles avoided plants that had been subjected to severe herbivory the previous season, and hypothesized that feeding on these plants might reduce beetle growth and survival. In *S. lycocarpum*, it is unknown whether spines act as a defense against insect herbivory, so the investigation of the production of spines following leaf damage may not only clarify whether herbivory elicits leaf spinescence, but also whether increased spinescence is a manifestation of DIR.

Fluctuating asymmetry (FA) is widely used as a measure of plant stress and it refers to small and random deviations from the bilateral symmetry of leaves (Cornelissen & Stiling 2005, Alves-Silva & Del-Claro 2013). Several factors, such as sun/shade, pollution, parasitism, soil salinity, habitat fragmentation, nutrients and competition, among others, might be related to high levels of leaf FA (Møller 1995, Kozlov et al. 1996, Puerta-Piñero et al. 2008, Cornelissen & Stiling 2011, Komac & Alados 2012, Cuevas-Reyes et al. 2013). In addition, the effect of herbivores on plants can also be assessed by FA analysis and high levels of FA can be an indication of DIR (Zvereva et al. 1997a, Olofsson & Strengbom 2000). For instance, FA in willows increased following a large infestation of herbivorous beetles in the previous years, indicating that some plant species can exhibit FA as a delayed response to herbivore pressure (Zvereva et al. 1997a, Olofsson & Strengbom 2000). Herbivory can cause plant stress because the damage inflicted on leaves may lead to perturbations in developmental stability, that is, the processes responsible for the normal pattern of growth are under stress in damaged leaves compared with undamaged leaves (Martel et al. 1999). In such situations, injured leaves may present small departures from perfect symmetry, indicating elevated levels of stress, which can be directly assessed by FA analysis (Parsons 1990, Møller & Shykoff 1999). Usually the higher the asymmetry in a population, the higher is the stress it is under (Cornelissen & Stiling 2011). As FA is an indication of plant quality/health/stress (Alados et al. 2002), increases in FA following herbivory may indicate that early leaf damage has caused detrimental effects on plant development.

In this two-year field study we evaluated changes in leaf parameters (FA, spine abundance and spine size) following herbivory by moth caterpillars in a natural setting. In 2010, during a massive infestation of caterpillars on *S. lycocarpum*, we investigated whether spinescence deterred moth herbivory and the relationship between leaf damage and FA. Then, in 2011, we investigated whether herbivory the previous year affected the abundance and size of spines per plant, as well as the FA levels. These parameters were used as a measure of DIR in *S. lycocarpum*. We hypothesized that plants subjected to high levels of herbivory in a year would display increased spinescence and FA in the following year.

## MATERIALS AND METHODS

### Study site and species

Fieldwork was carried out at the Panga Ecological Station (PES: 19°10'S 48°24'E), a savanna reserve 30 km away from Uberlândia city, Brazil, in the region known as the Cerrado biome (Brazilian savanna). Cerrado *strictu sensu* is the main vegetation type in the reserve (400 ha). This vegetation is dominated by trees and shrubs, ranging from 2 to 4 meters tall, with an understory of herbaceous vegetation. The climate is characterized by two well-defined seasons, a dry winter (April to September) and a rainy summer (October to March) (more details in Cardoso et al. 2009).

*Solanum lycocarpum* is one of the most common species in pastures and disturbed areas in the Cerrado (Moreira & Del-Claro 2005). The trees (1–3 m) have round and open crowns, long thorns on the branches, and simple, alternate, and coriaceous leaves (Clerici et al. 2011). Mature leaves are pale green in color and may reach up to 16 cm in length and 9 cm in width. The margin is smooth, ranging from entire to undulate in young and mature leaves, respectively; the apex is acute and the base is cordate. Sharp spines occur all over both sides of the leaf blade of some leaves, but are more concentrated on the midrib. Spines can be up to 7 mm in length and are slightly curved. Leaf flush in *S. lycocarpum* is seasonal and takes place during the wet season, from late October to April (see Stefani et al. 2000). In the dry season trees usually lose most leaves (> 90% leaf senescence).

At PES, *S. lycocarpum* can be attacked by two lepidopteran larvae, *Lophocampa citrina* Sepp, 1843 (Arctiinae) and *Leucanella viridescens* Walker, 1855 (Saturniidae). The latter is one of the most important herbivores, as many larvae can be observed on a single plant provoking severe damage to leaves. A single *L. viridescens* larva can injure up to fifty percent of the leaf blade (E. Alves-Silva, pers. obs.). *Solanum lycocarpum* also supports other herbivores, such as suckers (Moreira & Del-Claro 2005, Alves-Silva & Del-Claro 2011), but they were not considered in this study as their abundance on plants was negligible during the study period. In 2010, an outbreak of *L. viridescens* occurred and some plants supported as many as 20 larvae. This abundance is not commonplace; in the previous and following years the occurrence of this moth species was rare. A large population of *L. viridescens* was not observed in 2011 and thus permitted the evaluation of DIR (FA and spinescence following the high herbivory rates the year before).

### Sampling

In October 2010, during the period of *L. viridescens* infestation, we found and tagged 45 *S. lycocarpum* shrubs at PES and surroundings (the edge of a dirt road that gives access to the reserve). Selected plants were 1.5 to 2 m in height, and presented similar phenological and developmental state, with mature leaves. Each plant was tagged with a small numbered metal plate for identification. All plants selected for this study belonged to the same population and were evenly distributed in an area of ~ 10 ha, and therefore prone to similar biotic and abiotic stresses (following Telhado et al. 2010). Furthermore, all *S. lycocarpum* individuals were located in

open areas and were not shaded by large trees. Shortly after the occurrence of moth caterpillars on plants, a single branch was removed from each shrub for further measurements of FA, herbivory and spinescence, yielding at least seven leaves per plant. All branches were located at the most apical part within plant canopies and subjected to full solar exposure all day long. The total sample size was 554 leaves ( $12.16 \pm 0.55$  leaves per plant, mean  $\pm$  standard error [SE]; range 7–23 leaves per plant).

Spines, when present, were counted on the whole leaf blade and then measured from its base to the apex to the nearest 0.01 mm with a caliper. For a rapid evaluation of leaf damage, herbivory rates were assessed by placing a transparent 1 mm plastic grid on each leaf. An index of herbivory was calculated as the proportion of points in the grid that fell over the leaf area lost (Pires & Del-Claro 2014). For each leaf, the percentage of area lost was ranked as 0 (0% of leaf area lost), 1 (1–6%), 2 (7–12%), 3 (13–25%), 4 (26–50%), and 5 (> 51% of leaf area lost) (following García-Guzmán & Dirzo 2001).

### Fluctuating asymmetry

To assess leaf FA, the width of all leaves ( $n = 554$ ) was measured on the right ( $Rw$ ) and left sides ( $Lw$ ), from the leaf edge to the midrib at the midpoint of the leaf corresponding to its widest part. Leaves were measured with a caliper to the nearest 0.01 mm. To test the accuracy of the measurements, a subsample of 100 leaves was measured again and compared with the original  $Rw$  and  $Lw$  measurements. Repeatability of measurements is required to discard measurement errors (Woods et al. 1998). A two-way analysis of variance was used to determine whether the between-sides variation was significantly larger than the measurement error (Alves-Silva & Del-Claro 2013). The significance of the interaction (individual  $\times$  leaf side) was greater than expected by measurement error ( $F_{1,99} = 7.8893$ ,  $p < 0.0001$ ), indicating that leaves were measured with sufficient accuracy (Cuevas-Reyes et al. 2011).

According to Palmer & Strobeck (1986) it is necessary to discriminate FA (random departures from symmetry with a mean value of zero) from other kinds of asymmetry. Directional asymmetry (DA, mean ( $Rw$  minus  $Lw$ ) not equal to zero) was checked using a one sample Student's t-test. To check for antisymmetry (AS, significant deviance of the distribution of ( $Rw$  minus  $Lw$ ) from the normal curve), the normality of ( $Rw$  minus  $Lw$ ) was tested using Lilliefors' normality test. The mean of the ( $Rw$  minus  $Lw$ ) measurements was not significantly different from zero, consequently DA was rejected ( $t_{335} = 0.7944$ ,  $p > 0.05$ ). AS was also rejected because the distribution of ( $Rw$  minus  $Lw$ ) was normal ( $p > 0.05$ ). Thus in this study FA was confirmed in *S. lycocarpum*. FA dependence on leaf size was tested through a linear regression with the absolute difference of the right minus left measurements  $|Rw$  minus  $Lw|$  and leaf length (Santos et al. 2013), but there was no statistically significant relationship ( $R^2 = 0.0381$ ,  $df = 43$ ,  $p > 0.05$ ; mean values per plant were

used). Therefore the measurements were considered reliable, indicating that FA is not dependent on leaf size and thus can be assessed unambiguously in subsequent tests (Alves-Silva 2012).

### Delayed induced resistance in *Solanum lycocarpum*

In 2011, after examining the data collected the year before, we returned to the same population of plants studied in 2010 and divided them in two groups, each containing ten *S. lycocarpum* individuals, according to herbivory pressure the previous year. The reduction in sample size was necessary since some plants in the study area had been cut down to make way for a road. The groups, 'low herbivory' and 'high herbivory', were defined according to the intensity of leaf damage by moth caterpillars the previous year. Plants ranked as low herbivory presented mean leaf area loss of 0.74, which corresponds to 1–6% of leaf area loss (herbivory index  $< 1$ , following García-Guzmán & Dirzo 2001), while in the high herbivory group leaves had mean area loss of 1.82 (herbivory index  $> 1$ , [7–12% of leaf area loss] following García-Guzmán & Dirzo 2001). Choosing plants with distinct herbivory intensities allowed us to evaluate whether herbivory truly affected leaf parameters, such as increases in FA and spinescence. A single branch was removed from each plant for measurements of FA, spine size and spine abundance per leaf ( $n = 268$  leaves,  $13.4 \pm 0.82$  leaves per plant, mean  $\pm$  SE, range 7–21 leaves per plant). Data from 2010 and 2011 were compared and it was expected that plants with high herbivory rates in 2010 would present more and longer spines in 2011, as well as increased FA levels.

### Statistical analyses

Quantitative data are presented as the mean  $\pm$  SE. In cases when data did not satisfy the assumptions of normal distribution, data were log transformed to achieve normality. First, the relationship between spine abundance and size (average per plant) was tested with a Pearson correlation test, and as the variables were not correlated ( $r_{45} = -0.0613$ ,  $p > 0.05$ ), we could then evaluate unambiguously the effect of each variable on herbivory. The relationship between herbivory levels (dependent variable) and spines (abundance and size, independent variables) was examined with a general linear model, including the effect of each independent variable and their interaction. In this test, the average values per plant were used (following Venâncio et al. in press) and original data was log-transformed. The relationship between FA and herbivory, spine abundance and spine size was examined with Pearson correlation tests (with  $\alpha = 0.05/3$  as corrected  $p$  value). Paired Student's t-tests were used to compare (i) spine abundance; (ii) spine size and (iii) FA in leaves with and without herbivory of the same individual plants (average values were used). In these tests, we used the mean values per plant. For the investigation of DIR, we compared the values of spine abundance, size and FA of 2010 and 2011 from the low herbivory and high herbivory groups using paired Student's t-tests. All statistical procedures were performed in Systat 12 and GraphPad 5.0 software.

## RESULTS

**Leaf parameters of *Solanum lycocarpum* in 2010**

Most leaves sampled from *S. lycocarpum* presented spines ( $68.39 \pm 2.39\%$ ,  $8.78 \pm 0.16$  leaves per plant;  $n = 377$  leaves). Spine abundance ranged from 0 to 14 per leaf ( $3.09 \pm 0.14$  per leaf) and mean spine height from 1.05 to 5.56 mm ( $2.73 \pm 0.05$  mm). Herbivory levels were neither significantly related to spine abundance nor to spine size (table 1, fig. 1). The interaction effect (spine abundance + size) was also not significant. The relationship between FA and herbivory was negative, but not statistically significant ( $r_{43} = -0.1148$ ,  $p > 0.05$ ). Spinescence was also not significantly related to FA levels in *S. lycocarpum* (spine abundance,  $r_{43} = -0.1506$ ,  $p > 0.05$ ; spine size,  $r_{43} = -0.1468$ ,  $p > 0.05$ ).

When damaged and undamaged leaves were compared we found that spines were 9% more abundant in leaves without herbivory, but this difference was not statistically significant ( $t_{44} = 1.2496$ ,  $p = 0.10$ ) (fig. 2). Spines were 13% smaller in leaves attacked by moths, compared to undamaged leaves, and results were marginally significant ( $t_{44} = 1.6232$ ,  $p = 0.0558$ ) (fig. 2). Damaged leaves were significantly more asymmetrical than healthy leaves, yielding differences of 22% between damaged and undamaged leaves ( $t_{44} = 2.7785$ ,  $p < 0.01$ ) (fig. 2).

**Herbivory influence in leaf parameters in 2011**

Herbivory in the previous year affected measures of leaf spinescence and FA of *S. lycocarpum*, but differences were significant only in plants with high herbivory levels. For low herbivory plants, there was no statistical change in leaf FA and spinescence between 2010 and 2011 (FA  $t_9 = 1.6283$ ,  $p > 0.05$ ; spine abundance  $t_9 = 0.6293$ ,  $P > 0.05$ ; spine size  $t_9 = 1.5061$ ,  $p > 0.05$ ). In contrast, for high herbivory plants, the comparison between 2010 and 2011 revealed increases in every leaf parameter — FA increased 28% ( $t_9 = 2.2476$ ,  $p < 0.05$ ), spines were 21% more dense ( $t_9 = 2.7359$ ,  $p < 0.05$ ) and 84% longer ( $t_9 = 2.2709$ ,  $p < 0.05$ ) (fig. 3).

## DISCUSSION

Our results suggest that *S. lycocarpum* presents delayed induced resistance against herbivores, since all leaf parameters measured in 2010 had significantly increased in 2011, the year after herbivore damage. Nonetheless, some aspects related to plant spinescence and herbivory are intriguing. For instance, in 2011 there was a burst in the production of more and longer spines in *S. lycocarpum* that could be attributed to a severe attack by moths the year before. However, our results of 2010 suggest that spines were at best only slightly deterrent to moth herbivory; leaves avoided by moths presented more abundant and longer spines, but pairwise comparisons (attacked and non-attacked leaves) were not significant. In addition, our GLM model showed no significant relationship between herbivory and leaf spinescence. Potter & Kimmerer (1988) also noted no relationship between leaf spinescence and insect herbivory, and some studies suggest that this type of leaf defense is only effective against vertebrates such as browsers (Cooper & Owen-Smith 1986, Gowda 1996, Han-

ley et al. 2007). Indeed, few examples relate leaf spinescence with insect herbivore attack (Potter & Kimmerer 1988).

To the best of our knowledge, *S. lycocarpum* leaves are not eaten by vertebrate herbivores, only by insects. Chewing insects such as the caterpillars of our study can easily circumvent spines and feed on the surrounding leaf tissue (E. Alves-Silva, pers. obs.). In that case, it is puzzling that plants increased spinescence in 2011. We believe that increases in spine abundance and size in 2011 might nevertheless be a defense against herbivores for two reasons. First, large spines could prevent adult female moths from landing on leaves, as the sharp tips of spines might easily injure the insect's body. Second, a high abundance of spines might hinder the locomotion of caterpillars on the leaf blade, and might eventually present insurmountable spine barriers. By way of comparison, leaf trichomes can also act as defense against herbivores (Valverde et al. 2001) and can hinder insect locomotion on the leaf blade (Medeiros & Moreira 2002).

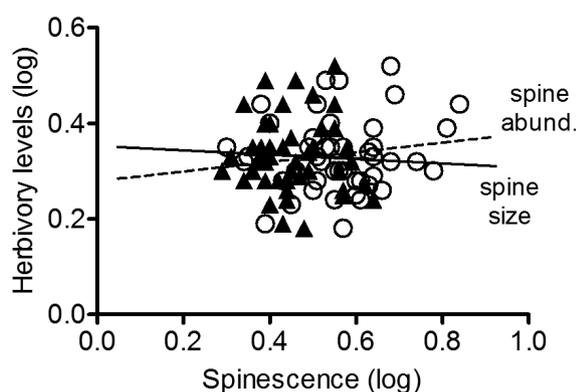
Usually, DIR is also demonstrated through detrimental effects on herbivores, as they can have low performance on previously damaged plants (Zvereva et al. 1997a). Nevertheless, we could not assess moth life-history in *S. lycocarpum*, as these insects did not occur on the plants in 2011. As predicted by DIR theory, changes in plants after herbivory reduce insects' preferences for the damaged plants (see Karban & Myers 1989), and indeed the lack of moths in 2011 may indicate that plants were less favorable to herbivores. An alternative explanation for the absence of moths on *S. lycocarpum* in 2011 is that herbivory the year before may have elicited the production of secondary chemicals such as alkaloids, which are common in *Solanum* species (Mola et al. 1997, Oliveira et al. 2004, Araújo et al. 2010). Alkaloid concentration rapidly increases as a consequence of herbivory (Baldwin 1988, Khan & Harborne 1990) and in *Solanum*, glycoalkaloids have a negative impact on herbivore performance and reproduction (Güntner et al. 1997).

Injured leaves of *S. lycocarpum* presented significant high levels of FA in both years of study. The relationship between FA and herbivory has become an important topic in FA studies. While some studies show a positive association between FA and herbivores, and vice-versa (Møller 1995, Zvereva et al. 1997b), other studies have failed to find any relationship between these variables (Alves-Silva 2012, Costa et al. 2013). Furthermore, a controversial aspect of FA-herbivory studies is that sometimes herbivores do not cause FA, but rather seek the more asymmetric leaves to feed on (see Cornelissen & Stiling 2005).

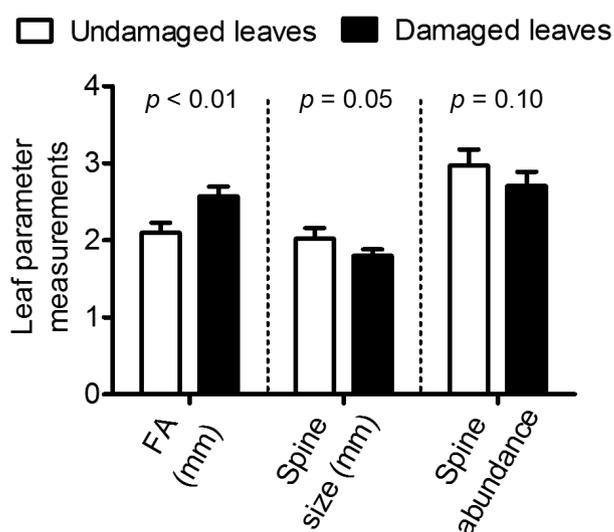
In our analysis of 2010 data, we found that damaged leaves were significantly more asymmetric than undamaged ones, but we cannot state for sure that herbivores caused FA or that they choose the more asymmetrical leaves on the plants (as in Cuevas-Reyes et al. 2011), as both scenarios may occur in nature (Olofsson & Strengbom 2000). Nonetheless, our results from 2011 indicated that the most injured plants the year before presented increased levels of FA, thus demonstrating that herbivory was indeed a stressor to the plant, causing leaf asymmetry.

**Table 1 – Coefficients and analysis of variance of the relationship among herbivory (intercept), spine abundance and spine size.** Herbivory was not related to any of the variables. Std. Error – standard error; MS – mean squares; df – degrees of freedom.

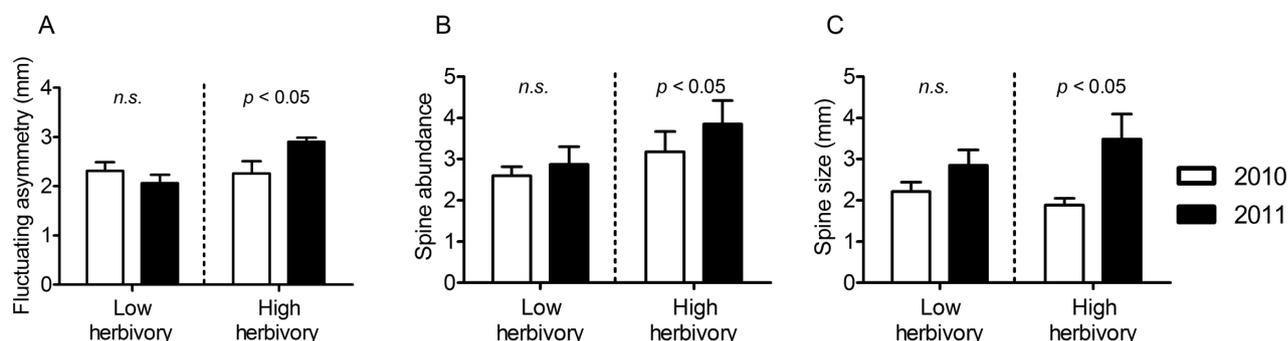
Variables	Coefficients	Std. Error	t-value	P-value
Constant (herbivory levels)	0.4053	0.3933	1.0305	0.3088
<b>Main factors</b>				
Spine abundance	0.0405	0.7169	-0.0565	0.9552
Spine size	-0.3066	0.8280	-0.3703	0.7131
<b>Interaction effects</b>				
Spine abundance × size	0.3441	1.4662	0.2347	0.8156
<b>Analysis of Variance</b>				
Regression (df = 3,41)	MS	F-Ratio	R <sup>2</sup>	P-value
	0.0089	0.4722	0.0334	0.7033



**Figure 1 – Relationship between herbivory levels and spinescence (spine abundance and size) in *Solanum lycocarpum* leaves.** Herbivory was not significantly related to leaf spinescence. The figure shows the average values per plant, which were also log transformed to fit normality assumptions. Triangles and complete line – spine abundance; circles and dashed line – spine size (mm).



**Figure 2 – Parameters investigated in *Solanum lycocarpum* in 2010 after herbivory by moth larvae.** Leaves damaged by moths (black bars) had fewer and smaller (mm) spines compared to undamaged leaves (white bars), but differences were not significant (*ns*, paired Student's *t*-test). Fluctuating asymmetry (FA, mm) was significantly higher in damaged leaves ( $p < 0.01$ , paired Student's *t*-test). All bars show mean  $\pm$  SE.



**Figure 3 – Comparison of leaf parameters in plants with low and high herbivory rates.** *Solanum lycocarpum* trees with high herbivory levels the year before presented (A) increased FA, (B) more spines and (C) longer spines. *n.s.* – not significant;  $p < 0.05$  – statistically significant, paired Student's *t* tests.

Delayed FA has been demonstrated in plants experiencing high herbivory levels in previous seasons. It might be evident that herbivores can provoke stress in plants and that the effects of herbivory can persist for longer periods (Zvereva et al. 1997a, 1997b, Møller & De Lope 1998, Martel et al. 1999). In these specific cases, the delayed induced FA can be detrimental to insect herbivores in the years following herbivory, because stressed leaves (high FA) causes slower growth (Cornelissen & Stiling 2005) and higher mortality rates in insects (Zvereva et al. 1997a). In this context, FA can be considered as DIR, as plants stressed the previous year/season might be less favorable for herbivore development (Zvereva et al. 1997a).

Our data provides evidence that herbivory pressure triggered a strong response in *S. lycocarpum*, as spinescence and FA significantly increased following leaf damage. In summary, we assume that this plant presents DIR. However, we are still to investigate the effects of DIR on herbivore performance and whether such an anti-herbivore mechanism is related to biochemical changes in leaves.

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