

SHADE AND HERBIVORY INDUCE FLUCTUATING ASYMMETRY IN A MEDITERRANEAN OAK

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Fluctuating asymmetry (FA), random nondirectional deviations from perfect symmetry, has been proposed as a useful indicator of environmental stress. Nevertheless, FA acts as a nonspecific indicator of stress; thus, factorial designs are needed to disentangle which of the factors may induce FA. We used an experimental approach in which seedlings of *Quercus pyrenaica* were exposed to two levels of light, water, and simulated herbivory. To determine whether FA may operate as a stress indicator, leaf width and number of lobes were measured. Our predictions were (1) different levels of light, water, and herbivory can bring different levels of FA and (2) fluctuating asymmetry can act as an early stress indicator. As predicted, FA was higher in shade than in sun, both for leaf width and number of leaf lobes. By contrast, pruning affected FA only for number of lobes. FA was higher in pruned seedlings in the sun but lower in pruned seedlings in the shade. The two FA indices used ($|L - R|$ and $\text{Var}(L - R)$, where L and R represent the left and right leaf sides) gave similar results. Because leaf FA in *Q. pyrenaica* increased with low light radiation and simulated herbivory and both are important in the dynamics of Mediterranean communities, FA appears to be a possible tool as an indicator of environmental stress.

Keywords: environmental stress, fluctuating asymmetry, light, *Quercus pyrenaica*, simulated herbivory.

Online enhancements: appendix tables.

Introduction

Fluctuating asymmetry (FA), small nondirectional deviations from bilateral symmetry, is thought to represent a direct measure of the ability of individuals to control their development (Van Valen 1962; Polak 2003). Many studies have associated FA measurements with individual fitness components (Møller 1997; Polak 2003) and environmental stresses (Parsons 1990; Tracy et al. 2003). At the moment, one of the main hypotheses within FA-stress studies is that stress contributes to flawed development of individuals—i.e., stress can generate FA—and thus FA can be an indicator of developmental instability (Møller 1995; Zvereva et al. 1997; Hagen et al. 2003). However, FA acts as a nonspecific indicator of stress, and only some prior studies have reproduced natural conditions (Møller 1995; Rettig et al. 1997). Additionally, most consider only a single stress factor at a time (Møller 1995, 1998; Kozlov et al. 1996; Zvereva et al. 1997; Eeva et al. 2000; Zvereva and Kozlov 2001; Cornelissen et al. 2003; Hagen et al. 2003; Kristensen et al. 2003; Sinclair and Hoffmann 2003; Cornelissen and Stiling 2005), while much less attention has been paid to the interaction between simultaneous stresses (Leung et al. 2000; but see Hochwender and Fritz 1999; Roy and Stanton 1999). In our opinion, this implies that potential studies should consider natural conditions, in addition to factorial designs, which are crucial to disentangling which factors induce FA and thus enhance the relevance of the use of FA as a stress indicator.

In the Mediterranean region, herbivory, drought, and light (as shading or photoinhibition) emerge as the most common stresses affecting plant performance (Valladares 2004). Previous studies have demonstrated that light, water, and herbivory, which affect morphological and chemical plant traits, can act as stressful factors in *Quercus pyrenaica* both in the field (Gómez et al. 2003) and in experimental studies (Baraza et al. 2004). In fact, Baraza et al. (2004) used the same seedlings as those used for this study. For this study, we define stress as any environmental factor causing a change in a biological system that is potentially harmful (Hoffmann and Parsons 1994). Thus, seedlings with lower growth and less chemical defenses in Baraza et al.'s (2004) study were considered to be under stress. Within this scenario, it is possible for FA to be used to obtain an objective index that assesses the effect of stress on plants (Bultman and Faeth 1987; Møller 1995). Our focal hypothesis was that natural populations suffering from stress should display FA. *Quercus pyrenaica* seedlings were exposed to different levels of light, water, and simulated herbivory, mimicking natural conditions (Gómez et al. 2003; C. Puerta-Piñero, J. M. Gómez, and J. A. Hódar, personal observations). Our major predictions were (1) simulated herbivory and low light intensity (factors that negatively affected seedling performance in Baraza et al. 2004) result in FA, and thus, (2) FA can be used as an early stress indicator of these types of stress for this species.

Material and Methods

Seeds and Seedlings

In autumn 1997, several thousand ripe *Quercus pyrenaica* acorns were collected from at least 20 trees (37°05'N, 3°26'W,

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Parque Nacional de Sierra Nevada, Spain). Acorns were pooled to avoid problems related to among-individual differences in acorn traits and were stored in a cold room (2°–4°C) for several weeks. In December 1997, acorns were planted individually in cylindrical pots (7 cm wide × 20 cm deep) in a fenced nursery ~4 km from the forest, at the same altitude (1650 m above sea level). Acorns germinated in February–March 1998, and seedlings emerged in late May and June, as they would under natural conditions. The seedlings were kept for the summer in the nursery. Weeds were periodically removed. In April 1999, 400 seedlings were randomly selected, pruned to a root length of 20–25 cm, and maintained in plastic pots (2.5 L) containing peat, vermiculite, and topsoil (1 : 1 : 2) underneath parental trees.

Our experiment involved the three following factors, with two levels each, in a factorial design.

(1) *Light*. Levels were (i) “sun,” where plants were illuminated with full sunlight, and (ii) “shade,” where plants were set under a black shading net, receiving only 5% of the available sunlight. The two levels were selected to mimic open and understory spaces, respectively. Photosynthetic photon flux density was 2033.0 ± 3.2 and $96.2 \pm 8.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ for sun and shade, respectively (using a Li-Cor LI-200 sz Pyranometer Sensor connected to a LI-1000 data logger; Li-Cor, Lincoln, NE).

(2) *Water*. Plants were watered with 1.5 L (i) weekly (“1-watering”) or (ii) biweekly (“2-watering”). During the summer of 2000, the soil humidity of the experimental seedlings was measured to check the effect of the watering (soil moisture sensor ThetaProbe ML2x, error $\pm 1\%$). These measurements showed that the soil in the 1-watering treatment ($18.83\% \pm 0.85\%$) had significantly more humidity than the 2-watering treatment ($10.26\% \pm 0.58\%$; one-way ANOVA: $F_{1,398} = 69.5$, $P < 0.0001$).

(3) *Pruning*. Levels were (i) “pruned,” where 50% of the aboveground biomass of the seedlings was removed to simulate ungulate damage (Gómez et al. 2003), and (ii) “unpruned,” where seedlings were left untouched. Removal was performed in fall 1999, 6 mo after the seedlings were acclimatized to the other treatments, and was timed to mimic ungulate browsing under natural conditions in the study area.

Fifty seedlings were randomly assigned to each of the eight treatments. Random assignment resulted in no initial between-treatment differences in seedling traits, such as plant height, trunk basal diameter, or number of leaves (three-way ANOVAs: $P > 0.1$ for all cases; Baraza et al. 2004).

Data Collection

Seedlings were harvested in September 2000. The number of seedlings surviving the experiment to study FA was 300, with no between-treatment differences in mortality (see Baraza et al. 2004). They were carefully washed to avoid the loss of fine roots, placed in a plastic bag, and carried to the laboratory. Leaves were then pressed and dried. In 2001, six leaves from each seedling were randomly taken and measured by C. Puerta-Piñero, who was unaware of their origin. If seedlings had fewer than six leaves, all of them were measured. In total, 1748 leaves were measured. As recommended by Van Valen (1962), Palmer (1994), and Hoffmann and Woods (2001), two different morphological traits were used. For the first estimator, maximum width of the left and right halves of the leaf was measured from

the midrib, taking the perpendicular distance to the nearest 0.01 mm using a digital caliper; these values are denoted L_{width} and R_{width} , respectively. For the second estimator, we counted number of lobes on the left and right sides without considering the lobe on the top of each leaf, which is just above the midrib; these values are denoted L_{lobes} and R_{lobes} , respectively. For both traits, we took the mean value of each individual seedling for further statistical and FA analysis.

Statistical Analyses

We estimated measurement errors by measuring 50 randomly taken marked leaves one day and remeasuring them 1 wk later (Yezerinac et al. 1992; Hóðar 2002). In order to test whether the observed deviations were FA rather than directional asymmetry or antisymmetry, we tested for normality, mean = 0, and kurtosis (Møller and Swaddle 1997). We used, respectively, the Shapiro-Wilk test, Student *t*-test, and kurtosis and skew of the distribution of the difference between left and right sides for each individual treatment (SAS Institute 1997). Because FA may increase with increasing size of the measured structure (Palmer and Strobeck 1986; Palmer 1994), either linearly or nonlinearly, we computed multiple regressions between absolute asymmetry and character size and the absolute value of the log(left) minus log(right) value for leaf width and number of lobes (Møller et al. 1999; Nachman and Heller 1999; Diaz et al. 2004) for each individual treatment. As size estimators we took left + right width and number of lobes, respectively (see table A1 in the online edition of the *International Journal of Plant Sciences* for results). As Palmer (1994) and Hoffmann and Woods (2001) suggested, we used two different asymmetry indices for testing the effects on FA; these were indices 1 and 4 in Palmer’s (1994) study. These indices were selected because of the structure of our data (with no evidenced size dependence of direct asymmetry). Absolute index (index 1) was calculated as $|L_{\text{width}} - R_{\text{width}}|$ and $|L_{\text{lobes}} - R_{\text{lobes}}|$, while index 4 in Palmer’s study was given by $\text{Var}(L_{\text{width}} - R_{\text{width}})$ and $\text{Var}(L_{\text{lobes}} - R_{\text{lobes}})$. The effects of the different treatments on FA indices were tested by three-way fixed ANOVAs. All data were analyzed using JMP statistical package, version 5.0 (SAS Institute 1997).

Results

The range in absolute bilateral differences for leaf width in the 50 remeasured leaves was 0.030–4.180 mm (mean \pm SE, 0.962 ± 0.121 mm), while the absolute difference between the original and the repeated measures was 0–0.300 mm for L_{width} (0.075 ± 0.010 mm) and 0–0.290 mm for R_{width} (0.062 ± 0.004 mm). The range in absolute bilateral differences in number of lobes was 0–3 lobes (0.520 ± 0.091 lobes), while the absolute difference between consecutive measures was 0–1 lobes (0.080 ± 0.039 lobes) for L_{lobes} and 0–1 (0.020 ± 0.020 lobes) for R_{lobes} . The repeatability of leaf width had $r^2 = 0.998$ for L_{width} and $r^2 = 0.999$ for R_{width} . For number of lobes, the repeatability had $r^2 = 0.955$ and $r_s = 0.975$ for L_{lobes} and $r^2 = 0.986$ and $r_s = 0.988$ for R_{lobes} . The measurement errors were low in both width ($<0.14\%$) and number of lobes ($<2\%$), according to the criteria of Yezerinac et al. (1992). The observed deviations were ideal FA

Table 1
Results of the Three-Way Fixed ANOVA Tests

Source	$ L_{lobes} - R_{lobes} $		$ L_{width} - R_{width} $		$\text{Var}(L_{lobes} - R_{lobes})$		$\text{Var}(L_{width} - R_{width})$	
	F	Power	F	Power	F	Power	F	Power
Light	4.828 [*]	.591	71.015 ^{****}	1.000	1.093	.180	26.157 ^{****}	.999
Water	.767	.141	.306	.085	.968	.165	.021	.052
Pruning	.035	.054	.103	.062	.031	.053	.099	.061
Light × water	.843	.150	.024	.053	.034	.054	.130	.065
Light × pruning	7.299 ^{**}	.768	.024	.053	6.432 [*]	.715	.015	.052
Water × pruning	.261	.080	.863	.152	.080	.059	.781	.143
Light × water × pruning	5.957 [*]	.682	.256	.080	4.051 [*]	.518	.796	.144

Note. Degrees of freedom = 291 for the error. $|L_{lobes} - R_{lobes}|$ = absolute fluctuating asymmetry (FA) for number of lobes; $|L_{width} - R_{width}|$ = absolute FA for leaf width.
^{*} $P < 0.05$.
^{**} $P < 0.01$.
^{****} $P < 0.0001$.

rather than directional asymmetry or antisymmetry because no distribution departed from normality, and mean = 0 at $P > 0.05$ in all cases (see table A1). No correlation existed between absolute FA and leaf size when each treatment was considered individually (see table A2 in the online edition of the *International Journal of Plant Sciences*). Thus, as proposed by Palmer (1994), we took $|L - R|$ and $\text{Var}(L - R)$ for future statistical analysis.

Light significantly affected index 1 (table 1), although it was only marginally significant for FA_{lobes} ($P = 0.029$, power =

0.591). Seedlings exposed to full light showed less FA than those growing in the shade, both for FA_{lobes} and for FA_{width} (fig. 1). The interaction between light and pruning was also marginally significant for FA_{lobes} (table 1). Seedlings increased their asymmetry when unpruned in low light (fig. 1). The significance of both light and the light × pruning interaction for FA_{lobes} withdrew after sequential Bonferroni correction. The results for index 4 showed analogous patterns (table 1). First, for FA_{lobes} , it gave significant results for light treatments and marginally significant results for the light × pruning interaction; unpruned

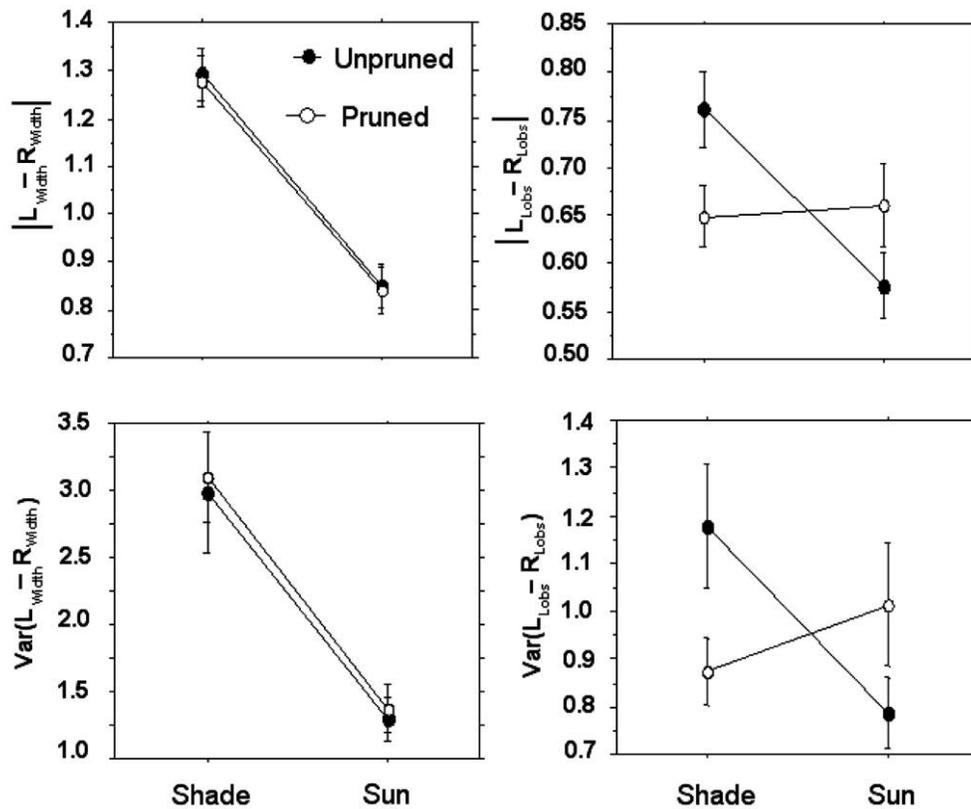


Fig. 1 Differences in fluctuating asymmetry (FA) indices as a result of light and pruning factors (two-way ANOVAs). Values are mean ± SE. $|L_{lobes} - R_{lobes}|$ = absolute FA for number of lobes; $|L_{width} - R_{width}|$ = absolute FA for leaf width.

seedlings attained higher FA_{lobes} when growing in the shade (fig. 1). As for index 1, in both cases the significance disappeared after the sequential Bonferroni correction. Second, for FA_{width} , light again showed a high significance ($P < 0.0001$, power = 0.999). As illustrated in figure 1, plants in the shadow also showed higher levels of FA_{width} .

Discussion

Interestingly, Baraza et al. (2004) used the same *Quercus pyrenaica* seedlings of this study to test for other supplementary common morphological and chemical stress indicators. This provides the eminent opportunity to compare the reliability of our FA estimates with that of more direct trait measures. Of the morphological traits, shade-treated plants showed the worst seedling performance in all cases. It was also found that the effect of herbivory was significant only for shade-grown seedlings. For chemical traits, it was found that seedlings in the shade had the highest nitrogen levels and lower levels of phenols and tannins than seedlings in the sun (Baraza et al. 2004). These findings indicate that low light may also produce seedlings more vulnerable to herbivory (Larsson et al. 1985; Tipler et al. 2002; Hagen et al. 2003; see Cornelissen et al. 2003 and Cornelissen and Stiling 2005 for similar results with chemical traits and FA in other *Quercus* species). If we take our results together with those of Baraza et al. (2004), we can assume that our results agree with the first hypothesis previously mentioned, that stress can generate FA (Møller 1995; Hagen et al. 2003). On the other hand, some authors have suggested that FA can generate more susceptibility to stress because organisms use FA as a sign of “lower-quality” individuals and an indication to attack at lower costs (Cornelissen et al. 2003; Cornelissen and Stiling 2005). Although our results do not reject this second hypothesis, we consider it more likely that organisms may distinguish between chemical traits than between FA measurements.

Leaf width and number of lobes in *Q. pyrenaica* seedlings displayed fluctuating asymmetry as a response to light and simulated herbivores. Because both water treatments were supplied to advocate seedling survival, we consider the 2-watering treatment not to have been stressful in our study, and thus we conclude that applying more water does not entail better seedling performance (see also Baraza et al. 2004 and a review in Polak 2003). Shade always resulted in higher levels of FA_{width} . While light had a clear effect either alone or together with pruning, the latter factor was more difficult to interpret because its effect was reversed between light treatments. It seems that seedlings suffering from light stress are more susceptible to suffering from herbivory. These results agree with previous studies on fo-

liar asymmetry in other oak (Cornelissen et al. 2003; Díaz et al. 2004; Cornelissen and Stiling 2005) and plant species (Møller and Eriksson 1994; Møller 1995; Zvereva et al. 1997; Roy and Stanton 1999; Hagen et al. 2003) that found that FA increases with stress. On the other hand, as Hoffmann and Parsons (1994) and Møller (1995) have suggested, organisms frequently suffering from one kind of stress are more susceptible to suffering from additional ones.

In our study, different plant traits seem to behave differently (Hoffmann and Woods 2001). Number of lobes appears to be more sensitive to stress than leaf width (table 1). The interaction among factors also appears to be more important for number of lobes than for leaf width. While seedlings exposed to full light conditions always showed lower FA_{width} , number of lobes interacts differently with stress compared to leaf width. Because different traits showed different patterns in FA, it seems that it is not possible to state a single, general relationship between asymmetry and environmental stress in this oak species (Roy and Stanton 1999; Hoffmann and Woods 2001).

Since we found a clear cause-effect relationship between light and herbivory stresses and FA, we can propose the use of FA as a stress indicator in this and possibly other plant species. However, we also argue that FA may act as an imprecise signal as a result of a group of different undistinguishable stresses operating simultaneously. Thus, there is no single, general relationship between FA and environmental stress, so experimental designs with interactions should be considered. On the other hand, different plant traits can give different results, and stresses can interact; thus, the results may sometimes be confusing. We propose that future studies should include several plant traits as well as clear cause-effect relationships between FA and stress. This could clearly unravel whether FA is acting as a cause or a consequence of environmental stress in natural communities.

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