

# Elevated CO<sub>2</sub> decreases leaf fluctuating asymmetry and herbivory by leaf miners on two oak species

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## Abstract

**Fluctuating asymmetry (FA) represents small, random variation from symmetry in otherwise bilaterally symmetrical characters. Significant increases in FA have been found for several species of plants and animals in response to various stresses, including environmental and genetic factors. In this study, we investigated the effects of elevated CO<sub>2</sub> on leaf symmetry of two oak species, *Quercus geminata* and *Q. myrtifolia*, and the responses of three species of leaf miners and one gall-making species to random variation in leaf morphology. Leaf FA decreased with an increase in CO<sub>2</sub> concentration. There were fewer asymmetric leaves and lower levels of asymmetry on leaf width and leaf area on elevated CO<sub>2</sub> compared with ambient CO<sub>2</sub>. Leaf miners responded to leaf asymmetry, attacking asymmetric leaves more frequently than expected by chance alone. Differences in secondary chemistry and nitrogen (N) content between symmetric and asymmetric leaves may be responsible for these results due to lower levels of tannins and higher levels of N found on asymmetric leaves of *Q. myrtifolia* and *Q. geminata*.**

*Keywords:* elevated CO<sub>2</sub>, Kennedy space center, leaf miners, leaf quality, *Quercus geminata*, *Quercus myrtifolia*

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## Introduction

In recent years, it has been suggested that developmental instability reflects the inability of organisms to control developmental processes during ontogeny and to achieve a predetermined phenotypic optimal expression (Moller & Swaddle, 1997). One measure of developmental instability is fluctuating asymmetry (FA), which represents small, random deviation from symmetry in otherwise bilaterally symmetrical characters. Significant increases in FA have been found for several species of plants and animals in response to various stresses, including environmental factors such as nutrition, temperature, and radiation, and genetic factors such as mutation, inbreeding, and interspecific hybridization (Moller, 1995). Nevertheless, developmental instability of plants and FA has, contrary to the situation in animals, not been adequately assessed, with the total number of studies not reaching 20 until 1997 (Moller & de Lope, 1998), with a slight increase since then (reviewed by Moller & Shykoff, 1999).

In addition to being an indicator of plant stress, some studies have shown correlations between FA and insect herbivore attack, suggesting that leaf FA may also serve as an indicator of plant susceptibility to herbivory (e.g., Moller, 1995; Wiggins, 1997; Zvereva *et al.*, 1997). According to this hypothesis, plants with more asymmetric leaves or higher levels of leaf asymmetry should experience increased levels of herbivory due to higher nutritional quality of asymmetric leaves compared with symmetric leaves (Sakai & Shimamoto, 1965; Lempa *et al.*, 2000). Since the left and the right sides of a bilaterally symmetrical trait develop under the control of the same genes, minor deviations from perfect symmetry actually represent developmental instability and may be responsible for differences in nutritional quality or secondary chemistry between asymmetric and symmetric leaves. High levels of fluctuating asymmetry are associated with biochemical and developmental factors, although little is known as to how developmental disorders are connected to plant metabolism and the associated biochemical changes exhibited by asymmetric leaves (Lempa *et al.*, 2000). It is well known that insects choose oviposition sites according to a wide number of factors such as

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host-plant nutritional quality, morphology, phenology, and secondary chemistry (Strong *et al.*, 1984) and we suggest that morphological variation in leaf symmetry may also be used as a cue to plant quality. Moller (1995) suggested that foliar asymmetry in elm trees directly influenced the abundance of the leaf-mining beetle *Rhynchaenus rufus*. These results may indicate either that plants with asymmetric leaves are, on average, more susceptible to attack by herbivores, or that herbivory itself acts as a stressor and directly increases the level of leaf asymmetry. Positive correlations between levels of herbivory and FA are consistent with both hypotheses. Although some authors favour the idea that herbivores themselves can act as stressors increasing leaf asymmetry (e.g., Moller, 1995; Zvereva *et al.*, 1997), correlations between leaf FA and herbivory are unlikely to be causal (Lempa *et al.*, 2000). Instead, chemical and nutritional differences between symmetric and asymmetric leaves probably influence leaf selection by herbivores, which leads to positive correlations between herbivory and FA.

Besides environmental and genetic stressors, another frequently stated factor causing fluctuating asymmetry in plants is pollution. Several studies have shown that pollutants such as nickel (Kozlov *et al.*, 1995), sulphur dioxide (Valkama & Kozlov, 2001), and heavy metals such as copper (Kozlov *et al.*, 1996) increase levels of plant FA. Such changes in FA could result in an increase in herbivory. However, just one study to date has tried to relate FA, aerial pollution, and subsequent defoliations by the leaf beetle *Melasoma lapponica* (Zvereva *et al.*, 1997).

Currently, concern is great over the increasing concentration of CO<sub>2</sub> in the atmosphere, which is expected to lead to global changes in the physical environment of terrestrial organisms (Lincoln *et al.*, 1993). To date, research on the possible impact of elevated CO<sub>2</sub> has concentrated on plant growth and physiology, but several recent studies have been carried out on the impact of elevated CO<sub>2</sub> on insect herbivores (e.g., Bezemer & Jones, 1998; Stiling *et al.*, 1999, 2002). Increased fluctuating asymmetry as a response to aerial pollution has been observed in some plant species (e.g., Kozlov *et al.*, 1996 studying birch plants, Zvereva *et al.*, 1997 studying willow plants), but until now none of the studies that have looked at the relationship between increases in CO<sub>2</sub> concentration and herbivory rates has focused on random variations in leaf symmetry. Elevated CO<sub>2</sub> conditions may have different impacts on plant developmental instability due to the positive effects of CO<sub>2</sub> on plant growth and productivity. Therefore, contrary to other aerial pollutants, we hypothesized that elevated CO<sub>2</sub> conditions may decrease the frequency of occurrence of leaf asymmetry or

levels of asymmetry compared with ambient CO<sub>2</sub> conditions.

The aim of this study was to determine how plants respond to an increase in CO<sub>2</sub> in terms of leaf FA and how leaf miners respond to these changes. This study was also designed to test the hypothesis that herbivores can use leaf asymmetry as a cue to leaf quality and oviposition sites. Specifically, the objectives of the present study were: (1) to quantify the effects of elevated CO<sub>2</sub> on fluctuating asymmetry of *Quercus* leaves; (2) compare the CO<sub>2</sub>-related variation in FA of two *Quercus* species; (3) test whether *Quercus* species are stressed by an increase in CO<sub>2</sub> concentration in terms of leaf chemistry and nutritional quality, (4) test how leaf miner insects respond to leaf FA; and (5) monitor the differences in nutritional quality between symmetric and asymmetric leaves.

## Materials and methods

### Study system

This study was conducted on a 2 ha section of a native scrub-oak community at Kennedy Space Center (KSC), Titusville, Florida. In 1996, sixteen 3.6 m diameter plots were enclosed by PVC chambers with a clear polyester film and an open-top. The main frame of these octagonal chambers was constructed from PVC pipes and chambers were 3.0 m high. An important feature of scrub forest is its low stature because, being fire maintained, plants rarely grow above 3–5 m in height. This fact allowed the installation of chambers over the mature canopy of the scrub forest and exposure to elevated CO<sub>2</sub> conditions. Vegetation in eight of the chambers was exposed to almost twice the concentration of ambient CO<sub>2</sub> ( $\approx 700 \mu\text{L L}^{-1}$ ), while the other eight chambers were exposed to ambient levels of CO<sub>2</sub> ( $\approx 350 \mu\text{L L}^{-1}$ ). Two sclerophyllous oaks dominated the vegetation: myrtle oak *Quercus myrtifolia* and sand live oak *Q. geminata*. Chapman oak, *Q. chapmanii*, was also found, but in much lower densities, and, as a result, only *Q. geminata* and *Q. myrtifolia* were used to assess the relationship between the increase in CO<sub>2</sub>, leaf FA, and insect herbivory rates. *Quercus* species at KSC are attacked by several species of chewers, gallers, and miners and leaf miners are ideal for herbivory studies because they are the most abundant insect herbivores in the study plots (Stiling *et al.*, 1999). Four different leaf miner species were found attacking the oak species, and the three most common species were examined in this study: *Stigmella*, *Cameraria*, and *Stilbosis*. We also studied eyespot galls (Diptera, Cecidomyiidae), due to their high abundance in *Q. geminata* leaves.

### Data collection

This study was performed from March to August 2002. This period incorporated the time when senescent *Quercus* leaves (March–April) are replaced by new leaves and the time when the new leaves suffer the heaviest attack rates by herbivores (May–July). To examine the frequency of occurrence of asymmetric leaves and levels of leaf asymmetry, five nondamaged leaves were collected from each plant species from each chamber, in March and again in April 2002. Leaves sampled during March represented leaves formed in 2001 and leaves sampled in April were the first new leaves from 2002. Because herbivores themselves can act as stressors on plants, we compared FA between ambient and elevated CO<sub>2</sub> leaves before the beginning of mine initiation. Using this approach, we could observe if asymmetric leaves were present in both types of chambers before leaf miner attack, and we could also compare asymmetry of leaves produced during a 2-year interval.

The five leaves were sampled haphazardly, regardless of the direction of branches or the position of shoots on the branch, oven-dried between sheets of filter paper and mounted as herbarium specimens. To quantify *Quercus* fluctuating asymmetry, the widths of all leaves were measured on both the right and the left side, from the leaf edge to the midrib, at the widest point of the leaf. We also quantified asymmetry of leaf area, by measuring right and left areas of each leaf. These distances were measured by photographing each leaf with a digital camera, and analysing leaf length, leaf area, and right and left lengths and areas using the software UTHSCSA Image Tool (University of Texas, USA). All the digital pictures were calibrated to the nearest 0.01 mm before measurements were taken. Absolute asymmetry in leaf width was defined as the unsigned difference between right width (RW) and left width (LW) of a particular leaf as  $FA_{\text{width}} = |RW - LW|$ . Absolute asymmetry on leaf area was defined as the difference in area between right (RA) and left (LA) sides of each leaf as  $FA_{\text{area}} = |RA - LA|$ . The absolute value of right–left traits is a good estimator of variance in FA among leaves assuming that there is no directional asymmetry (consistently larger left or right side) or antisymmetry (consistent lack of symmetry, but in no particular direction). To compare *Quercus* asymmetry between ambient and elevated CO<sub>2</sub>, two indices of FA were used (Palmer & Strobeck, 1986):

$$\text{index 1} = \frac{\sum |R_i - L_i|}{N},$$

$$\text{index 2} = \frac{\sum \left[ \frac{|R_i - L_i|}{(R_i + L_i)/2} \right]}{N},$$

where  $R_i$  is the value of the right side,  $L_i$  is the value of the left side and  $N$  is the number of measurements taken.

Index 1 is the most intuitive asymmetry measure (Roy & Stanton, 1999), and index 2 is size-scaled, correcting for the fact that asymmetry can be size-dependent. These indices were calculated for leaves of both plant species collected from each chamber and averaged for chambers representing ambient and elevated CO<sub>2</sub> conditions.

To test whether or not insects preferred asymmetrical leaves, in May and June of 2002, we sampled 10 mined leaves of each leaf miner species *Cameraria*, *Stilbosis*, and *Stigmella*, and the 10 nearest neighbouring leaves without mines. These leaves were collected from each chamber for asymmetry measurements as described above. We also recorded the mean number of mines per leaf and average mine size of *Cameraria*, *Stilbosis*, *Stigmella* in each chamber. For eye-spot galls, 10 galled leaves and the 10 nearest ungalled leaves of *Q. geminata* were collected in June 2002 and same measurements were taken.

To examine the differences in nutritional quality between symmetrical and asymmetrical leaves, 10 nondamaged leaves of each *Quercus* species were collected in each chamber in July 2002, digitalized for further measurements and placed immediately on ice. The tannin concentration of all leaves later classified as symmetric and asymmetric based on analysis of measurements were quantified using the radial diffusion assay (Hagerman, 1987). Leaves were oven-dried, milled to a fine powder, and tannins were extracted from 50 mg of dry tissue with 0.25 mL of methanol 50%. Twenty microlitres of plant extract were placed in a Petri dish containing a mixture of agar and protein (bovine serum albumin). Tannin in the extracts binds with the protein to form an opaque precipitate, and the squared diameter of the precipitate ring is proportional to the tannin concentration of the extract (for details see Hagerman, 1987). A regression equation relating the square of the precipitate-ring diameter to tannic acid concentration was calculated using a series of tannic acid standards ( $\text{diameter}^2 = 0.38 + 5.504 \times \text{tannic acid concentration}$ ; adjusted  $r^2 = 0.98$ ,  $P < 0.001$ ,  $n = 21$ ), and used to calculate the tannin concentration on *Quercus* leaves. The average of three replicates per leaf was used for statistical analysis. To examine the differences in nitrogen (N) content between symmetric and asymmetric leaves, 3 nondamaged leaves of each plant species were collected in each chamber on June 2002. These leaves were digitalized for further measurements, oven-dried, milled to a fine powder, and N content was determined using a CHN analyser.

### Data analysis

Leaf characters demonstrate FA if signed right-minus-left character values are normally distributed with

a mean value of zero, reflecting randomly directed deviations from the optimal symmetrical phenotype. One-sample *t*-tests and Lilliefors' tests (Wilkinson, 1999) were used to test whether the mean values of signed right-minus-left character values differed significantly from zero.

Asymmetry was calculated as the absolute difference between right and left width and right and left areas of a particular leaf. Given that asymmetry increased with leaf size ( $r = 0.39$ ,  $P < 0.05$ ), measurements of asymmetry on leaf width and area were corrected for leaf size according to the formulas  $FA_{\text{width}} = 2 \times |RW - LW| / (RW + LW)$  and  $FA_{\text{area}} = 2 \times |RA - LA| / (RA + LA)$ . An important consideration in asymmetry studies is measurement error (Palmer, 1996). Because measurement errors will resemble FA, it is crucial that the symmetry differences measured are larger than the measurement error, or that subsequent measurements taken on the same leaf are highly correlated. We estimated the measurement error by remeasuring 20% of the leaves collected on each sampling date 10 days after the first measurements were taken and the two measurements were correlated using an index of repeatability (Falconer, 1981).

All the variables analysed were first submitted to Lilliefors' test for data normality (Wilkinson, 1999). Given that chambers, *Quercus* species, and leaf types (symmetric and asymmetric, mined and nonmined) were not independent, we used split-plot ANOVAS (Wilkinson, 1999) to assess the effects of elevated CO<sub>2</sub> on leaf area, fluctuating asymmetry, and tannin concentration. For split-plot ANOVAS, treatments (CO<sub>2</sub> levels) were the plots, each one replicated eight times. Subplots consisted of leaf categories (mined, nonmined) and leaf types (symmetric, asymmetric) and sub-sub plots consisted of plant species (*Q. geminata* and *Q. myrtifolia*). Effects of elevated CO<sub>2</sub> on mine abundance and size were tested using one-way ANOVAS, because mines and plants were independent due to our sampling design: *Stilbosis* mines and eye-spot galls were collected just on *Q. geminata* and *Cameraria* and *Stigmella* mines were collected just on *Q. myrtifolia*. To analyse the differences in tannin concentration and N content between symmetric and asymmetric leaves, we used two-way ANOVAS, where leaf type (symmetric and asymmetric) and CO<sub>2</sub> levels were treatments and chambers were replicates. These last analyses were conducted separately for each plant species, due to the differences in tannin concentration and N of *Q. myrtifolia* compared with *Q. geminata*. To examine herbivore distributions between symmetric and asymmetric leaves, for each species of leaf miner each pair of leaves collected on each chamber were categorized as 1 = mined leaf: symmetric, nonmined: symmetric,

2 = mined leaf: symmetric, nonmined: asymmetric, 3 = mined leaf: asymmetric, nonmined: asymmetric and 4 = mined leaf: asymmetric, nonmined: symmetric. Differences in the frequency of these categories in ambient and elevated CO<sub>2</sub> were tested using a chi-square test. Preference for each leaf type was assumed if mines were found more frequently in a particular leaf type than would have been expected as a result of a simple chance encounter with leaves of both types (symmetric, asymmetric).

Some authors (e.g., Moller & de Lope, 1998) have also claimed that herbivores themselves may cause leaf asymmetry due to their feeding activities. If leaf miners themselves cause asymmetry, we would expect that mined sides of the leaves would be larger/wider than unmined sides of the leaves when the entire mine is encountered on a particular side (right or left) of the leaf. To test this hypothesis, we performed a one-tailed paired *t*-test with all the mines sampled that were located on a particular side of the leaf of *Q. geminata* and *Q. myrtifolia*.

## Results

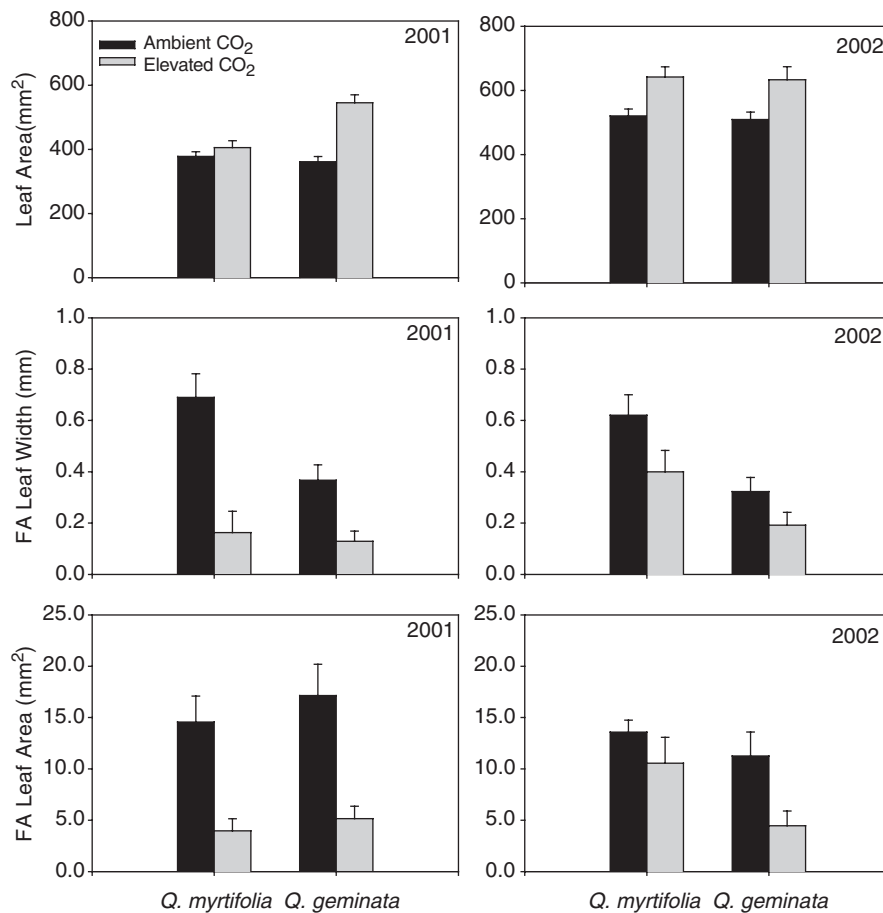
### *Tests for FA in ambient and elevated CO<sub>2</sub>*

The two oak species demonstrated similar patterns of leaf asymmetry. Leaf width and leaf area showed FA for both *Q. geminata* and *Q. myrtifolia*, as signed right-minus-left character values were normally distributed (Lilliefors' tests,  $P = 0.29-0.76$ ) and did not deviate significantly from zero in all data sets (one-sample *t*-tests,  $P = 0.43-0.98$ ). Also, 97% of the leaves that were asymmetric in width were also asymmetric in leaf area, although levels of asymmetry were higher when leaf area was used as the measurement of FA. The mean repeatability of FA measurements was 0.948 ( $F_{31,32} = 26.85$ ,  $P < 0.001$ ), indicating small measurement errors and the reliability of FA measurements.

### *Effects of elevated CO<sub>2</sub> on leaf asymmetry*

We observed a significant increase in leaf area in elevated CO<sub>2</sub>, for both plant species, in 2001 and 2002 (split-plot ANOVAS, 2001:  $F_{1,14} = 13.69$ ,  $P < 0.005$ ; 2002:  $F_{1,14} = 13.07$ ,  $P < 0.005$ ; Fig. 1). On average, *Q. myrtifolia* leaves under elevated CO<sub>2</sub> were 15.0% larger than in ambient CO<sub>2</sub> and *Q. geminata* leaves were 38.0% larger in elevated CO<sub>2</sub> conditions.

Effects of CO<sub>2</sub> on leaf asymmetry showed similar patterns for both plant species in 2001 and 2002. Leaf FA decreased with an increase in CO<sub>2</sub> concentration and there were fewer asymmetric leaves and lower levels of asymmetry in leaf width and leaf area in



**Fig. 1** Differences in leaf area, FA on leaf width, and FA on leaf area of *Quercus myrtifolia* and *Q. geminata* under ambient and elevated CO<sub>2</sub> conditions during 2001 and 2002. Bars indicate SE.

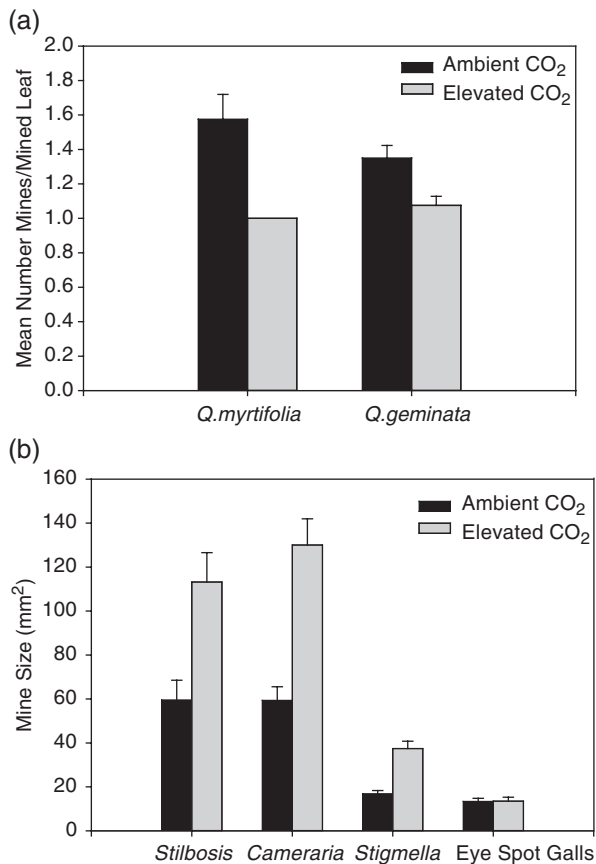
elevated CO<sub>2</sub> compared with ambient CO<sub>2</sub> (Split plot ANOVAS,  $F_{1,14} = 6.64\text{--}59.67$ ,  $P = 0.022\text{--}0.0001$ ; Fig. 1). FA indices for both plants in 2001 and 2002 showed similar results: asymmetry was lower under elevated CO<sub>2</sub> compared with ambient CO<sub>2</sub> using data from absolute or relative asymmetry. Values of index 1 for FA on leaf width under elevated CO<sub>2</sub> conditions ranged from 0.12 to 0.39 mm, while on ambient CO<sub>2</sub> conditions values for the same index ranged from 0.32 to 0.69 mm.

#### *Effects of elevated CO<sub>2</sub> on fluctuating asymmetry and herbivory*

Elevated CO<sub>2</sub> decreased mine abundance per mined leaf for both *Q. geminata* ( $F_{1,14} = 9.308$ ,  $P < 0.05$ ) and *Q. myrtifolia* ( $F_{1,14} = 16.03$ ,  $P < 0.005$ ; Fig. 2a), but there were no significant differences in the mean number of galls per galled leaf in ambient and elevated CO<sub>2</sub>. We also observed a significant increase in mine size from ambient to elevated CO<sub>2</sub> (one-way ANOVAS, *Stilbosis*  $F_{1,14} = 11.19$ ,  $P < 0.05$ ; *Cameraria*  $F_{1,14} = 27.59$ ,  $P < 0.0001$ ,

*Stigmella*  $F_{1,14} = 31.12$ ,  $P < 0.0001$ ; Fig. 2b). On the other hand, there was no difference in gall size between ambient and elevated CO<sub>2</sub> ( $P = 0.97$ ).

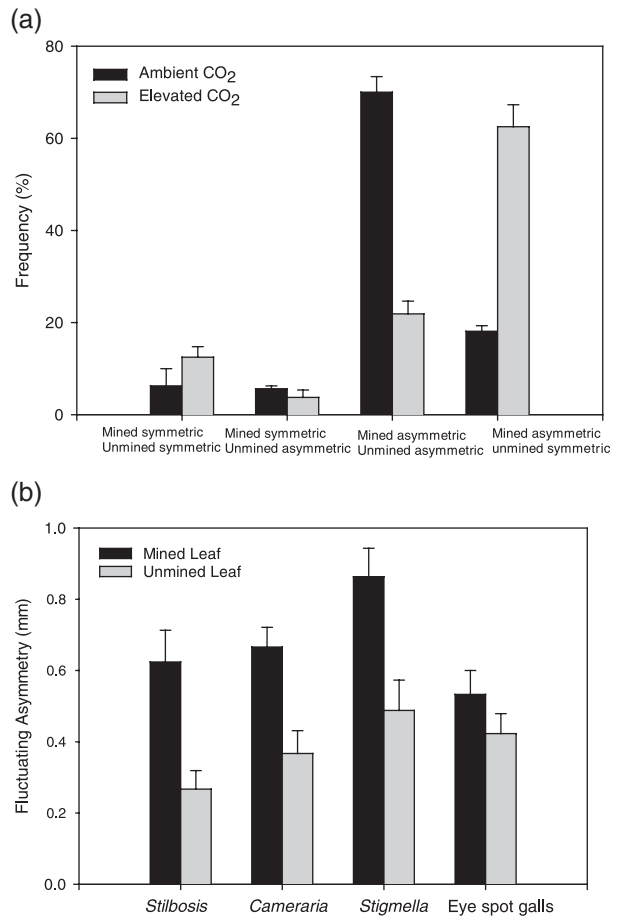
Leaf miners attacked asymmetric leaves more frequently than symmetric leaves in both chamber types ( $\chi^2 = 44.7$ ,  $P < 0.0001$ ; Fig. 3a), although the frequency of occurrence of categories of leaf types was significantly different for ambient and elevated CO<sub>2</sub> ( $\chi^2 = 23.44\text{--}82.16$ ,  $P < 0.001$  in all cases). In ambient CO<sub>2</sub>, in 70% of the cases, both mined and nonmined leaves were asymmetric, indicating no preference of herbivores for asymmetric leaves. On the other hand, in elevated CO<sub>2</sub>, in 62% of the cases, mines were found in asymmetric leaves, while the nearest nonmined leaf was symmetric. In cases where both leaves were asymmetric, for all leaf miners, mean asymmetry was significantly higher on the mined leaf compared with the nearest nonmined leaf (one-way ANOVAS,  $F_{1,14} = 10.36\text{--}12.65$ ,  $P < 0.005$  for all leaf miner species; Fig. 3b). Nevertheless, when effects of treatment (CO<sub>2</sub> concentration) were incorporated in the analyses, significant differences in



**Fig. 2** (a) Average abundance of leaf miners (*Stilbosis*, *Cameraria* and *Stigmella*) on *Quercus myrtifolia* and *Q. geminata* leaves under ambient and elevated CO<sub>2</sub> conditions. (b) Differences in mine size between ambient and elevated CO<sub>2</sub>. Data on size of eye-spot galls on *Q. geminata* leaves are also shown. Bars indicate SE.

asymmetry between mined and nonmined leaves were found only for *Cameraria* and *Stigmella* mines (two-way ANOVAS, *Cameraria*  $F_{1,28} = 13.92$ ,  $P < 0.001$ , *Stigmella*  $F_{1,28} = 19.18$ ,  $P < 0.001$ , *Stilbosis*  $F_{1,28} = 2.46$ ,  $P = 0.128$ ). For eye-spot galls, no significant differences in asymmetry were found between the galled and the nearest nongalled leaf, in both chamber types.

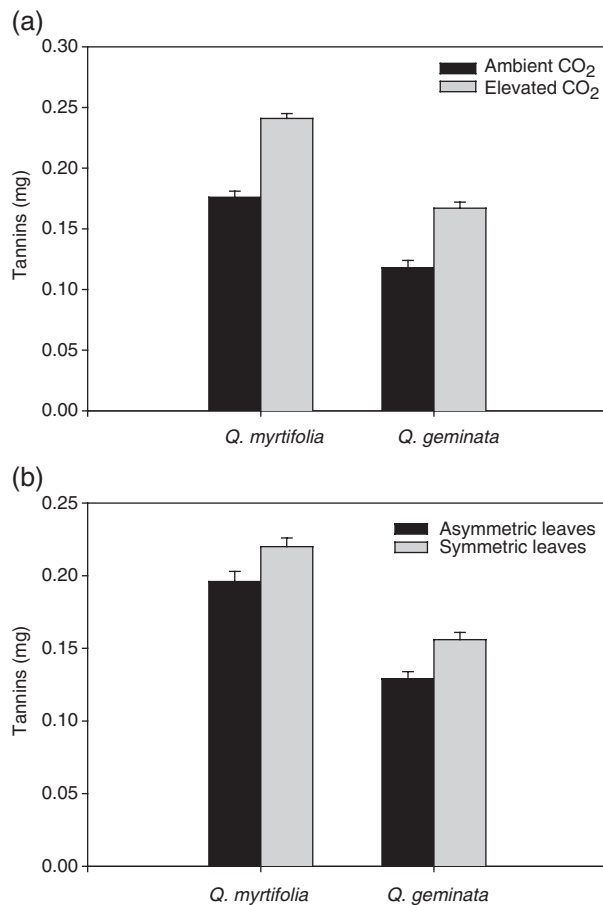
Elevated CO<sub>2</sub> significantly increased tannin concentration for both *Q. myrtifolia* and *Q. geminata* leaves (Split plot ANOVAS  $F_{1,14} = 26.76$ ,  $P < 0.001$ ; Fig. 4a). Also, asymmetric leaves contained significantly lower concentrations of tannins than symmetric leaves for both *Q. geminata* ( $F_{1,14} = 13.44$ ,  $P < 0.001$ ) and *Q. myrtifolia* ( $F_{1,14} = 6.379$ ,  $P < 0.05$ ; Fig. 4b). Nevertheless, when effects of treatment were incorporated in the analyses, significant differences of tannin concentration between symmetric and asymmetric leaves were found only for *Q. geminata* (two-way ANOVA *Q. geminata*  $F_{1,28} = 6.10$ ,  $P < 0.05$ ; *Q. myrtifolia*  $F_{1,28} = 2.97$ ,  $P = 0.091$ ). Also, no



**Fig. 3** (a) Frequency of occurrence of combinations of asymmetric and symmetric leaves on pairs of mined and unmined leaves under ambient and elevated CO<sub>2</sub> conditions. Leaves from all leaf miners studied were combined ( $n = 320$  leaves). (b) Differences in leaf width asymmetry between mined and nearest undamaged leaf for all leaf miners studied. Leaves from ambient and elevated CO<sub>2</sub> were combined to conduct analysis. Bars indicate SE.

significant relationship was found when we correlated the percentage difference in width and area between the two halves of each leaf – an indication of a difference in the levels of asymmetry among asymmetric leaves – and tannin concentration for both plant species in ambient and elevated CO<sub>2</sub> ( $P = 0.098$  for *Q. geminata* and  $P = 0.084$  for *Q. myrtifolia*).

For both plant species, N contents were higher in ambient compared with elevated CO<sub>2</sub> conditions (split plot ANOVA  $F_{1,14} = 30.29$ ,  $P < 0.005$ ; Fig. 5a). Moreover, N concentration was higher in asymmetric leaves compared with symmetric leaves in both chamber types, although the results were statistically significant only for *Q. myrtifolia* (two-way ANOVA *Q. myrtifolia*  $F_{1,28} = 16.909$ ,  $P < 0.05$ ; *Q. geminata*  $F_{1,28} = 3.74$ ,  $P = 0.06$ ; Fig. 5b).

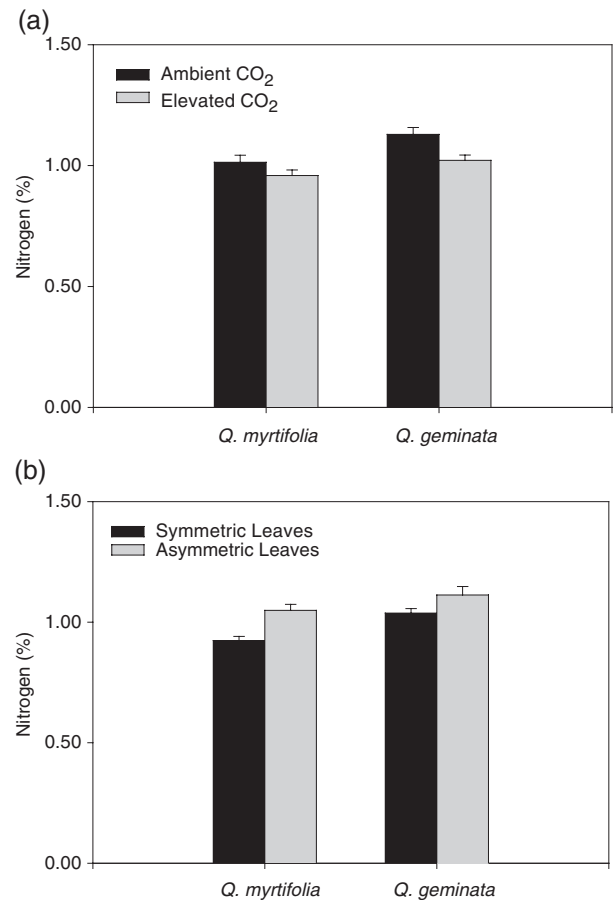


**Fig. 4** (a) Differences in tannin concentration of undamaged *Quercus myrtifolia* and *Q. geminata* leaves between ambient and elevated CO<sub>2</sub>. (b) Differences in tannin concentration between symmetric and asymmetric leaves of *Q. geminata* and *Q. myrtifolia*. Owing to differences in frequency of occurrence of symmetric and asymmetric leaves in different chambers, leaves from ambient and elevated CO<sub>2</sub> were pulled together to conduct analysis. Bars indicate SE.

A paired *t*-test revealed no significant differences in leaf width between mined and unmined sides of the leaf (mean width of mined side of leaf = 8.142; mean width of unmined side of leaf = 8.254;  $t = -1.552$ ,  $P = 0.123$ ,  $n = 134$ ). These results suggest that there was no direct relationship between the presence of leaf miners studied and changes in the width of the mined side of the leaves.

## Discussion

Some aspects of plant responses to elevated CO<sub>2</sub> are well documented, such as changes in plant morphology (Ceulemans & Mousseau, 1994), growth, and physiology (McDonald *et al.*, 1999, reviewed by Curtis & Wang,



**Fig. 5** (a) Differences in N content of undamaged *Quercus myrtifolia* and *Q. geminata* leaves between ambient and elevated CO<sub>2</sub>. (b) Differences in nitrogen content between symmetric and asymmetric leaves of *Q. myrtifolia* and *Q. geminata*. Bars indicate SE.

1998), and leaf chemistry (Lincoln *et al.*, 1993; Watt *et al.*, 1995). In general, plants growing in elevated CO<sub>2</sub> conditions are larger and have increased C:N ratios (Agrell *et al.*, 2000; Yin, 2002), affecting, therefore, higher trophic levels. An increase in C:N ratio, known as the dilution effect, reduces foliar quality for herbivores by decreasing N and increasing the availability of C-based chemical compounds. Our results suggest that mine size and differences in tannin concentration between the two treatments are in agreement with this hypothesis. Leaf size is also an important criterion for leaf miner colonization and survival. Elevated CO<sub>2</sub> caused leaf miners to increase the area of leaf they consume, compensating therefore for poorer leaf quality. Similar results regarding mine size were found at KSC for the same leaf miners in previous studies (Stiling *et al.*, 1999) and for other different insect species feeding on plants under elevated CO<sub>2</sub> conditions (e.g., Lincoln *et al.*, 1986; Salt

*et al.*, 1995). Increases in plant secondary chemical levels have also been found under elevated CO<sub>2</sub> conditions, although this is not always the case (e.g., Roth & Lindroth, 1994; Williams *et al.*, 1997). Clearly, the effects of elevated CO<sub>2</sub> will vary among plant species, plant traits studied, and differences in CO<sub>2</sub> concentrations among experimental designs.

To our knowledge, this is the first study addressing the effects of CO<sub>2</sub> on leaf asymmetry and how herbivores respond to these changes. Individual and population levels of FA have been shown to relate positively to a wide range of biotic and abiotic stresses, although the strength of this relationship varies considerably between taxa, traits studied, and the types of stress (Lens *et al.*, 2000). Asymmetry in leaves of several plant species has been shown to increase with the proximity to potential stresses, such as copper-nickel smelters (Zvereva & Kozlov, 2001) and industrial pollution (Kozlov *et al.*, 1996).

Supporting our initial prediction of an inverse relationship between elevated CO<sub>2</sub> and leaf fluctuating asymmetry, we observed that asymmetric leaves were less frequent in elevated CO<sub>2</sub>, and, when encountered, they were less asymmetric than leaves growing under ambient CO<sub>2</sub>. A possible explanation for this pattern is the fact that, in contrast to other environmental stresses, which can cause negative effects on plant growth, the predominant effect of elevated CO<sub>2</sub> on plants is to promote growth with consequent reallocation of resources (Docherty *et al.*, 1996). Martel *et al.* (1999) experimentally induced leaf growth in birch *Betula pubescens* by breaking apical dominance and found an increase in leaf FA of clipped trees compared with control ones. An increase of FA with rapid leaf or plant growth can occur if faster growth rates create more opportunities for developmental errors. However, this seems not to be the case in our study, since, although *Quercus* leaves growing under elevated CO<sub>2</sub> conditions showed an increase in leaf area in 2001 and 2002, they did not show more frequency of asymmetric leaves or higher levels of asymmetry. Another possible explanation for the negative relationship between CO<sub>2</sub> and FA is the fact that CO<sub>2</sub> acts as a plant fertilizer, and, as a result, elevated CO<sub>2</sub> ameliorates plant stress compared with ambient levels of CO<sub>2</sub>. Moreover, leaf developmental instability can be caused by factors other than stress such as accelerated and less controlled growth in plant modules (Martel *et al.*, 1999), thus long-term exposures to elevated CO<sub>2</sub> levels can have positive effects on plant growth. Asymmetry on *Quercus* leaves growing under ambient CO<sub>2</sub> conditions could be influenced by several factors not addressed in this study such as nutritional deficiencies and water shortage, plant competition, or even higher herbivory rates

during previous years. Furthermore, levels of asymmetry of *Quercus* leaves under ambient CO<sub>2</sub> (calculated by both indices 1 and 2) are within the reported range of FA found for other plant species growing under natural conditions (reviewed by Moller & Erickson, 1994), including other *Quercus* species, such as *Q. rotundifolia* (relative FA: 0.081–0.102, Moller & de Lope, 1998).

Besides abiotic stresses, significant increases in leaf asymmetry have been found for several species of plants and animals in response to biotic factors including herbivory (e.g., Zvereva *et al.*, 1997; Moller & de Lope, 1998). Although some studies assume that herbivores are responsible for stress and developmental instability, it may be more likely that FA can provide herbivores a cue to plant quality. However, sampling leaves after herbivory has occurred can confound the relationship between herbivory and FA. Four main findings of our study reinforce the idea that herbivores may use leaf fluctuating asymmetry as a cue to plant quality and therefore, selection for oviposition sites: (1) Asymmetric leaves were available to insects in both ambient and elevated CO<sub>2</sub> before leaf miners oviposit, (2) Leaves in which mines were encountered were more asymmetric than the nearest nonmined leaf, (3) Asymmetric leaves were of better nutritional quality, i.e., presented reduced chemical defenses and higher N content, than symmetric leaves, and (4) No differences in leaf width were observed between mined and unmined sides of a particular leaf. Also, our results showed that leaf miners were encountered in asymmetric leaves more frequently than expected by chance alone, even when effects of treatments were considered in the analyses. A few mines were found in cases where both leaves of the mined-nonmined pair were symmetric (less than 15% on both CO<sub>2</sub> treatments), or in cases where mined leaf was symmetric and nonmined leaf was asymmetric (less than 10% in both CO<sub>2</sub> treatments). These findings suggest that herbivores under elevated CO<sub>2</sub> conditions are under strong selection to find suitable leaves for oviposition, due to the fact that asymmetric leaves were less frequent than symmetric leaves in elevated compared with ambient CO<sub>2</sub>. Our results also corroborate previous studies conducted with the same leaf miners (Stiling *et al.*, 1999, 2002), which found higher abundance of leaf miners in ambient CO<sub>2</sub>, where asymmetric leaves are abundant. The higher frequency of leaf miners on asymmetric leaves and higher levels of asymmetry on mined leaves compared with undamaged ones suggest that herbivores may prefer asymmetric leaves and use FA as a cue to leaf quality and suitable oviposition sites. An alternative hypothesis for the higher frequency of the leaf miners studied on asymmetric leaves not addressed



by our study is the possibility that herbivores may indiscriminately oviposit in symmetric and asymmetric leaves, but egg hatching or larval survivorship is higher on asymmetric leaves of both *Quercus* species. Mopper *et al.* (1995a, b) have shown that *Stilbosis* eggs and early stage larvae usually suffer little natural enemy attack, resulting in low mortality rates, since females place eggs among the dense trichomes on the ventral leaf surface. Data on mortality rates of eggs and early stage larvae of *Cameraria* and *Stigmella* deserve future investigations and studies relating the oviposition behaviour of *Stilbosis*, *Stigmella*, and *Cameraria* in both leaf types may assist in clarifying this point in the future. Moreover, studies of preference-performance of insects on asymmetric leaves, such as parasitism and predation rates between leaf types after larval establishment would help to determine whether insects select asymmetric leaves due to higher nutritional quality (preference) and/or higher survivorship (performance) of larval stages due to faster growth and reduced chances of mortality.

Differences in nutritional quality between asymmetric and symmetric leaves reinforce the idea that the relationship between asymmetry and herbivory is not causal, i.e., herbivory does not cause asymmetry in our study system. Instead, herbivores may use asymmetry as a cue to plant quality and suitable oviposition sites. According to Lempa *et al.* (2000), in addition to being an indicator of plant stress, leaf FA may also serve as an indicator of plant susceptibility to herbivores and 'correlations between leaf FA and indices of herbivory are unlikely to be causal; they presumably operate via biochemical or physical characteristics, which modify leaf value for herbivores.' Although the relationship between FA and foliar chemistry is not clear, deviations from normal development may disturb enzymatic regulation in pathways of primary and secondary metabolites, modifying therefore their concentrations (Lempa *et al.*, 2000). Yet again, better nutritional quality of asymmetric leaves (i.e., lower secondary chemistry and higher N content) may be responsible for higher attack rates on asymmetric leaves compared with symmetric ones. These differences in nutritional quality between symmetric and asymmetric leaves were first described by Sakai & Shimamoto, (1965), who demonstrated that asymmetric leaves of *Nicotinia tabacum* presented lower nicotine concentrations than symmetric leaves. Nevertheless, few studies have addressed differences in nutritional quality between symmetric and asymmetric leaves since then (but see Lempa *et al.*, 2000). In our study, we showed that asymmetric leaves presented lower tannin concentration and higher N content than symmetric leaves for both *Q. geminata* and *Q. myrtifolia*. Nevertheless, the increase in leaf quality

due to a reduction in secondary chemistry does not appear to be dosage-dependent in our study systems, since there was no significant relationship between tannin concentration and different levels of leaf asymmetry. The absence of a relationship between CO<sub>2</sub> and gall abundance, gall size, and frequency of occurrence of galls in symmetric and asymmetric leaves suggests that galling insects may not be as influenced by plant aspects that change under elevated CO<sub>2</sub> conditions as leaf miners are, since their special mode of feeding within the gall allows them to manipulate plant characteristics, and possibly avoid defensive strategies of the host and circumvent differences in nutritional quality between symmetric and asymmetric leaves.

Future studies should monitor the long-term effects of CO<sub>2</sub> on leaf asymmetry and address the differences in mine growth and survivorship between symmetric and asymmetric leaves, to corroborate the hypothesis that herbivores can use random differences in leaf morphology to assess plant quality and select oviposition sites where performance is enhanced.

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