DEVELOPMENTAL INSTABILITY AND GAS EXCHANGE RESPONSES OF A HEATHLAND SHRUB TO EXPERIMENTAL DROUGHT AND WARMING

Laura Llorens,1* Josep Peñuelas,* and Bridget Emmett†

*Unitat d’Ecofisiologia CSIC-CEAB-CREAF, Center for Ecological Research and Forestry Applications (CREAF), Universitat Autònoma de Barcelona, Edifici C, 08193 Bellaterra, Spain; and †Centre for Ecology and Hydrology, Deiniol Road, Gwynedd LL57 2UJ, Bangor, United Kingdom

The effects of predicted climatic changes on the physiological stress of bilberry (Vaccinium myrtillus L.) were investigated in a field experiment in a heathland of northern Wales (U.K.). In the experiment, drought was increased during the growing season by excluding precipitation, and night warming was created by reducing heat loss. To assess physiological stress, leaf size and leaf fluctuating asymmetry (FA) as integrative measures of stress during leaf development and leaf gas exchange as a classical instantaneous method to detect physiological stress, were measured. Since leaf FA increased with increasing size, comparisons of asymmetries were made for relative rather than absolute asymmetry. Relative leaf FA was calculated as the absolute difference between leaf size (area or width) of left and right halves, corrected for trait size. Drought treatment increased the relative leaf area FA in 2000 and not in 1999, in agreement with a stronger treatment in 2000. Conversely, the warming treatment decreased the relative leaf area FA in 1999 and not in 2000, coinciding with the lower minimum temperatures in the growing season of 1999. Differences in water availability and temperature between years were related with changes in the relative leaf area FA, which were consistent with the treatment effects. In contrast, leaf gas exchange rates and leaf size showed no significant response to the environmental manipulations, although there was a slight decrease of photosynthetic values and leaf size in drought treatments in both years. Leaf FA therefore appeared to be a more sensitive indicator of physiological stress than leaf size or gas exchange measurements. Our results indicate that a future increase in the severity of drought during the growing season will increase physiological stress of V. myrtillus, whereas warming will decrease physiological stress during leaf development because of the alleviation of temperature constraints.

Keywords: climate change, drought, warming, developmental instability, gas exchange, fluctuating asymmetry, leaf size, Vaccinium myrtillus.

Introduction

Climate change from greenhouse gas emissions is predicted to raise the globally averaged surface temperature by 1.4° to 5.8°C over the period 1990 to 2100 (Intergovernmental Panel on Climate Change [IPCC] 2001). The raise in mean temperatures will be largely caused by the increase in minimum air temperatures, i.e., nighttime temperatures (IPCC 2001). Although future precipitation patterns are more uncertain, global climate change is likely to increase the risk of summer drought in central and southern Europe (IPCC 2001).

Warmer and drier conditions are likely to affect the physiological stress of plants. Stress is considered to be a significant and lasting deviation from favorable conditions that leads to abnormal demands and a destabilization of vital processes (Larcher 2000). The detection and quantification of physiological stress in plants has been a major concern of ecophysiological studies. Measures of stress can be grouped into instantaneous physiological measurements such as leaf gas exchange or Chl a fluorescence and integrative physiological measures over larger temporal scales such as growth, productivity, survival, or isotope content. Another integrative process is developmental instability, which refers to the inability of a bilateral organ or organism to buffer its development against disturbances and to produce a predetermined phenotype (Møller and Swaddle 1997). Recently, several authors have suggested that measures of developmental instability as fluctuating asymmetry (FA) may provide a more sensitive indicator of stress than traditional measures of performance (e.g., growth, fecundity, survival) because FA has a measurable response at a lower threshold of stress (Rettig et al. 1997).

Fluctuating asymmetry (FA) is a particularly useful measure of developmental instability because, for bilaterally or radially symmetrical traits, the optimal phenotype is known. Ideally, traits showing fluctuating asymmetry will have a normal frequency distribution of signed left-minus-right trait values with a mean of zero (Palmer and Strobeck 1986). Such small deviations from perfect symmetry are considered to result from environmental or genetically based perturbations of the developmental process (Møller and Shykoff 1999). As stress during development may influence developmental precision, FA has been proposed as a potentially useful tool for monitoring stress levels in natural populations (Graham et al. 1993). Typ
ically, the greater the stress, the greater the fluctuating asymmetry (Palmer 1996).

In plants, environmental stress factors that have been associated with leaf developmental instability include high altitude (Wisley et al. 1998), pollution (Zvereva et al. 1997), UV-B radiation (Midgley et al. 1998), competition (Rettig et al. 1997), herbivory (Lempa et al. 2000), pathogens (Møller 1999), high boron and low light (Roy and Stanton 1999), and electromagnetic fields (Freeman et al. 1999; recent review, Møller and Shykoff 1999). To date, few investigations have related developmental instability in plants with warming (Hutter 1969) or drought (Hochwender and Fritz 1999; Alados et al. 2001), even though they are two important factors of predicted climatic change. To our knowledge, only Valkama and Kozlov (2001) have investigated specifically the impact of both temperature and precipitation on developmental instability.

In contrast, the effects of changes in temperature or water availability on leaf gas exchange (stomatal conductance, photosynthetic and transpiration rates) have been extensively studied (reviews, Berry and Björkman 1980; Yordanov et al. 2000). In general, stress produces a decrease in leaf gas exchange (Farquhar et al. 1989). Thus, it is expected that a reduction in water availability will lead to stomatal closure, decreasing transpiration and photosynthetic rates. It is also well known that an increase in drought can cause a significant reduction in growth (Long and Hutchin 1991; Pereira and Chaves 1995). By reducing growth, plants can mitigate stress through a decrease of water and nutrient demands (Alados et al. 2001). Then, if water limitation occurs in the beginning of the growth cycle, leaf area is expected to be reduced (Nilsen and Orcutt 1996). In contrast, predicted elevated temperatures may increase leaf size, carbon assimilation, and transpiration rates in sites where low temperatures constrain physiological activity of plants. Climate change effects on nutrient availability (Grogan and Chapin 2000) may also indirectly influence leaf growth and leaf gas exchange.

Recently, a number of field experiments that simulate some of the predicted climatic changes have been conducted, mostly in Arctic and sub-Arctic ecosystems (Shaver et al. 2000, a review of warming experiments). However, most of the techniques used to experimentally warm ecosystems or ecosystem components (heat-resistance ground cables, overhead infrared lamps, vented and unvented field chambers, greenhouses) alter additional environmental conditions (e.g., light, humidity, soil structure, wind). Moreover, most of these experiments simulate a general diurnal increase in temperature rather than the observed increase in $T_{\text{min}}$ (nighttime temperature) (IPCC 2001). To reduce these drawbacks, we have performed a field experiment using a nonintrusive technique to reduce the loss of IR radiation from the Earth’s surface to the atmosphere. This approach imitates the effect produced by greenhouse gases accumulation and results in an increase in $T_{\text{min}}$ or nighttime warming. In addition, we have simulated prolonged drought events by excluding rain during the growing season (Beier et al. 2003).

The aim of our study was to test whether predicted climatic changes (warming and prolonged drought) would produce physiological stress in Vaccinium myrtillus. In Europe, V. myrtillus is a common species in moorlands, and it has a vast distribution area that extends from midlatitude mountain ranges to the Arctic region (Gerdol et al. 2000) and is a characteristic type of moorland. Moorlands, typically dominated by heather (Calluna vulgaris [L. Hull], cover ca. 15% of the land area of the United Kingdom (Anderson and Hetherington 1999), and they are considered to be of high conservation value (Whitehead et al. 1997). Heather moorlands are under threat from changes in land use and management, the potential effects of atmospheric nitrogen deposition, and climate change (Anderson and Hetherington 1999).

In order to detect environmental stress during the growing season in V. myrtillus, we used fluctuating asymmetry and leaf size as integrative measures of stress during leaf development and leaf gas exchange measurements as an instantaneous measure of stress. We hypothesized that if water availability was a limiting factor for V. myrtillus development, our experimental water removal would increase stress conditions during leaf development, resulting in an increase in leaf fluctuating asymmetry and a decrease in leaf size. Moreover, fully developed leaves would decrease stomatal conductance and photosynthetic CO$_2$ fixation. However, we expected that increases in minimum temperatures would alleviate temperature constraints during leaf development, since biotic processes in such ecosystems are usually directly or indirectly constrained by low temperatures (Körner and Larcher 1988). Hence, we hypothesized that our warming treatment would increase leaf gas exchange rates and would decrease low temperature stress during V. myrtillus leaf development, reducing leaf fluctuating asymmetry and increasing leaf size.

**Methods**

**Study Site and Plant Species**

The study was carried out in northern Wales (U.K.), within Clocaenog Forest at Craig Bron-banog (53°03′N, 3°28′W), at 490 m altitude. Annual rainfalls were 1655 and 1826 mm in 1999 and 2000, respectively, while annual mean air temperatures were 8.3°C and 7.7°C. The study site is in a heather moorland of ca. 15 ha surrounded by plantation forestry. The soil is an acid peaty podzol. The vegetation is dominated by mature Calluna vulgaris (L. Hull), Vaccinium myrtillus (L.), and Empetrum nigrum (L.), with very sparse Deschampsia flexuosa (L. Trin.).

**Experimental Design**

Two types of climatic manipulations of the ecosystem were performed using automatically sliding roofs: ecosystem drought and ecosystem warming. For ecosystem drought, the roofs (transparent plastics) covered the vegetation during rain events over the growing season. For ecosystem warming, the roofs (reflective curtains) were unrolled across the experimental plots covering the vegetation and soil during the night at a height of 0.6 m, reducing the loss of IR radiation. A rain sensor removed the curtain during rain events to maintain the hydrological cycle.

Both drought and warming covers were removed during periods of high winds to prevent damage to the covers. Nine plots (20 m$^2$ per plot) were established: three untreated controls, three drought, and three warming plots. Before mea-
measurements, drought treatment had excluded rain for ca. 4–5 wk each year, starting on June 18 in 1999 and on June 6 in 2000. Particularly, in 1999, drought treatment excluded 23% of 140 mm of accumulated rainfall over the growing season (June and July), while in 2000, drought treatment excluded 37% of 162 mm of accumulated rainfall. Warming treatment started on May 17 of 1999, and it was working over all the experiment period and increased the temperature ca. 1°C compared with control plots.

**Leaf Sampling and Asymmetry Measurements**

For measuring leaf asymmetry, we collected 10 fully developed, current-year leaves per plot on July 12–14 in 1999 and 2000. In order to avoid within-plant pseudoreplication, all leaves were collected from different plants. Likewise, to minimize within-plant variation in asymmetry, we always sampled the fourth leaf from the apical end of the new shoot, unless this was damaged, eaten, or missing, in which case we collected the third leaf.

Leaf asymmetry was calculated as the difference in leaf width (1) and leaf area (2) between the right (R) and left (L) halves of the adaxial part of a leaf. (1) Right and left widths were the distances from the midrib to the right and left margins, respectively, measured at the widest point of each leaf to the nearest 0.01 mm with a digital caliper. (2) To measure right and left areas, we sliced each leaf down the middle of the midvein and spread and fixed the two halves on a sheet. Thereafter, we digitized half-leaf photocopies and determined their areas using ImagePC (version α9 for Windows, Scion, Frederick, Md.).

Individual signed asymmetry was measured as \( L - R \), where \( L \) is the trait value on the left side and \( R \) is the trait value on the right side. Absolute asymmetry \( |L - R| \) was computed as the absolute left-minus-right values of a particular leaf.

**Leaf Fluctuating Asymmetry Data Analyses**

**Repeatability and measurement errors.** The percent of total variance in a character that occurs among individuals is often referred to as repeatability (Yezerinac et al. 1992). Repeatability of leaf width and leaf area asymmetry was obtained through a one-way analysis of variance with individuals as the independent factor and values of leaf FA as the dependent variable (Yezerinac et al. 1992; Merilä and Björklund 1995).

Conversely, the percent measurement error of a character is the percentage of the total variance attributable to the between-sides (within-individual) variance due to the imprecision of the measurements. Measurement errors (ME) can be particularly important in fluctuating asymmetry measures, since differences between sides are often very small; generally <5% and often <1% of the size of the traits being measured (Palmer 1994). Because ME may substantially bias asymmetry estimates, an important initial step in analyzing asymmetry is distinguishing variance in leaf asymmetry from ME (Palmer 1994; Swaddle et al. 1994). Thus, we estimated ME by measuring each leaf twice for each trait. Leaves were measured just after collection and remeasured several months later without reference to earlier measurements.

Leaf area and leaf width ME for each treatment (control, drought, warming) and year (1999, 2000) were estimated using two-way mixed-model ANOVAs where side was the fixed factor and individual was the random factor (Palmer and Strobeck 1986; Palmer 1994; Merilä and Björklund 1995). The last procedure partitions out directional asymmetry (side effect) from nondirectional asymmetry (interaction effect) and identifies whether the measurement error was smaller than other nondirectional asymmetry.

**Directional asymmetry and antisymmetry.** Before interpreting our data, we followed Swaddle et al.’s (1994) and Palmer’s (1994) suggestions to evaluate whether leaf asymmetry was FA rather than directional asymmetry or antisymmetry. Because many cases of directional asymmetry and antisymmetry reflect normal development, i.e., they have a genetic rather than environmental origin, such asymmetries are usually not considered to be useful for measuring developmental instability (Leary and Allendorf 1989; Palmer 1994, 1996).

Directional asymmetry is characterized by consistently greater trait development on one particular side, either left or right, resulting in mean values of the distributions of left-minus-right sides (\( L - R \)) deviating from zero (Van Valen 1962; Palmer and Strobeck 1986). To test for directional asymmetry, we used two methods: we used the mean squares generated by the two-way analysis of variance mentioned above to determine whether the leaf sides differed in size (Palmer 1994), and we performed one-sample t-tests on the signed differences (left-minus-right) for each treatment and year to determine whether the mean values differed from zero (Swaddle et al. 1994).

Antisymmetry occurs when one side is consistently larger than the other, but the larger side may be either the right or the left, at random, resulting in a platykurtic (broad-peaked or bimodal) distribution of \( L - R \) differences about a mean of zero (Van Valen 1962; Palmer and Strobeck 1986). To test for antisymmetry, we checked departures from normality of the distributions of \( L - R \) within treatments each year using Shapiro-Wilks tests (Zar 1996). We also examined the normality of the distributions determining whether skewness and kurtosis coefficients deviated from zero, which is the expected value for normal distributions (Palmer 1994; Zar 1996). A significant negative kurtosis indicates possible antisymmetry (Cowart and Graham 1999).

**Leaf size.** Improper interpretations of leaf FA may arise if leaf size differs among treatments and leaf FA co-varies with leaf size (Palmer 1994; Swaddle et al. 1994). To know whether leaf size (area or width) differed among treatments, we performed ANOVAs to compare separately drought and warming leaves with control leaves, with treatment and year the fixed factors. We used one value of leaf size per plot (the average of 10 leaves) and year. To determine whether leaf size differed between years, we performed one-way ANOVAs using only control leaves. Finally, to know whether leaf FA co-varies with leaf size, we obtained nonparametric Spearman’s correlation coefficients for the two studied years between absolute leaf asymmetry \( |L - R| \) and leaf size \((L + R)/2\) \((n = 90)\) (Palmer 1994).

**FA indices.** For both traits (leaf area and leaf width), we calculated a size-corrected or relative leaf FA index: \( |L - R|/\text{size} \). To be sure that the correction was successful, we again correlated the size-corrected indices with size.
Typically, FA indices based on the unsigned left-minus-right character value have half-normal distributions, truncated at zero (i.e., highly skewed to the right) and with some zero values. Because of this, we applied a Box-Cox transformation to relative leaf FA indices (for leaf area and width) to normalize their distributions before performing parametric analyses (Swaddle et al. 1994): $FA = (\text{relative } FA + 0.001)^{0.4}$.

**Data analyses.** We used the statistical software of SPSS 10.0 (SPSS, Chicago) for all the statistical analyses. Since multiple tests were performed with the same variables, significance level ($\alpha = 0.05$) was always adjusted for the number of statistical tests, using a sequential Bonferroni correction to prevent against groupwide type errors (Rice 1989). All tests were two-tailed.

In all the statistical analyses, we used one value of leaf asymmetry per plot (the average of 10 leaves) and year. To examine the effect of treatments on leaf FA, we performed ANOVAs with treatment and year as fixed factors and leaf FA (area or width) as the dependent variable. For leaf FA values, we found a significant interaction between year and treatment. Hence, we analyzed separately the effect of treatments on leaf area FA for the 2 yr. We tested the differences between drought and warming with control leaves separately. To compare leaf FA between years, we performed one-way ANOVAs using only control leaves. Finally, we calculated the Spearman’s correlation coefficients between the average of leaf area and relative leaf area FA and the total rainfall received over the growing season (June and July) by each treatment each year (1999 and 2000).

**Leaf Gas Exchange Measurements**

Leaf gas exchange rates were measured in three to four randomly selected plants in each plot on July 12, 1999, and July 14, 2000. Net photosynthetic rate ($A$) and stomatal conductance ($g_s$) were determined at midday afternoon (1200–1700 hours solar time) on one shoot per plant. Only current year shoots in full sun and with fully expanded leaves were considered. Measurements were performed with a portable open-flow gas exchange system (ADC4, ADC, Hoddesdon, Hertfordshire), which also monitored photosynthetically active radiation (PAR) and air temperatures in the leaf chamber. Water vapor pressure deficits between leaf and air (VPD) were calculated on the basis of relative humidity and chamber air temperature. All results are expressed on the basis of area. Leaf areas were measured, using ImagePC, from photocopies of all the leaves of each measured shoot.

**Leaf Gas Exchange Data Analyses**

In all the statistical analyses, we used one value per plot (the average of the three to four plants measured) and year. To test the effects of treatments on leaf gas exchange rates (photosynthesis and stomatal conductance), we performed ANCOVAs comparing drought and warming leaves with control leaves separately. Treatment and year were considered fixed factors, while PAR was the co-variable. To test the effect of year, we performed one-way ANOVAs (since PAR was not significant as covariable) using only control leaves. We also calculated nonparametric Spearman’s correlation coefficients between leaf gas exchange values of plants from the different treatments and the total rainfall received by each treatment over the growing season (June and July) both years (1999 and 2000).

**Results**

**Leaf Fluctuating Asymmetry**

*Repeatability and measurement errors.* Repeatability of leaf width asymmetry measurements was $0.96$ ($F_{179, 180} = 44.9, P < 0.001$) and $0.99$ for leaf area ($F_{179, 180} = 240.9, P < 0.001$). The percentage of measurement error (ME) of leaf width and leaf area measurements within each treatment was less than 4.5% in both years. In all the ANOVAs, the interaction between individual and side was significant ($P < 0.001$), indicating that the ME was negligible compared with the variation between sides (asymmetry; Palmer 1994; Perfectti and Camacho 1999). The high repeatabilities and the relatively low levels of ME indicate that ME accounted for a minor part of the total variance in asymmetry, and therefore, asymmetries were measured with sufficient precision to allow the use of the average measure for each leaf in the following analyses.

*Directional asymmetry and antisymmetry.* We did not find any significant effect of leaf side in any of the two-way mixed-model ANOVAs mentioned above, either for leaf width or for leaf area. Moreover, $t$-tests revealed that mean values of each treatment each year did not differ significantly from zero, indicating a lack of directional asymmetry (table 1). Left-minus-right distributions of leaf width and leaf area for each treatment and year did not deviate from normal distribution ($P > 0.05$). Moreover, none of the skewness and kurtosis values differed significantly from zero (table 1). Therefore, we

<table>
<thead>
<tr>
<th>Year and treatment</th>
<th>Skewness</th>
<th>Kurtosis</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area FA:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>-0.161</td>
<td>-1.338</td>
<td>-0.0178 ± 0.0123</td>
</tr>
<tr>
<td>D</td>
<td>-0.516</td>
<td>1.053</td>
<td>-0.0128 ± 0.0108</td>
</tr>
<tr>
<td>W</td>
<td>0.116</td>
<td>0.390</td>
<td>-0.0085 ± 0.0087</td>
</tr>
<tr>
<td>2000:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>-0.262</td>
<td>0.745</td>
<td>0.0035 ± 0.0092</td>
</tr>
<tr>
<td>D</td>
<td>-0.040</td>
<td>3.496</td>
<td>-0.0358 ± 0.0193</td>
</tr>
<tr>
<td>W</td>
<td>0.034</td>
<td>-0.045</td>
<td>-0.0142 ± 0.0123</td>
</tr>
<tr>
<td>Leaf width FA:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>-0.732</td>
<td>0.231</td>
<td>-0.0630 ± 0.0916</td>
</tr>
<tr>
<td>D</td>
<td>-0.183</td>
<td>-0.151</td>
<td>-0.0950 ± 0.0710</td>
</tr>
<tr>
<td>W</td>
<td>0.161</td>
<td>0.245</td>
<td>-0.0903 ± 0.0592</td>
</tr>
<tr>
<td>2000:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>-0.665</td>
<td>0.260</td>
<td>0.0422 ± 0.0784</td>
</tr>
<tr>
<td>D</td>
<td>-0.505</td>
<td>0.582</td>
<td>-0.1467 ± 0.0889</td>
</tr>
<tr>
<td>W</td>
<td>0.679</td>
<td>2.267</td>
<td>-0.0465 ± 0.0925</td>
</tr>
</tbody>
</table>

Note. FA = fluctuating asymmetry. After sequential Bonferroni correction, no skewness or kurtosis values differed significantly from zero; neither did mean values.
Fig. 1 Leaf size (L + R) expressed as leaf area (cm$^2$) and leaf width (mm) for control, drought, and warming plants of *Vaccinium myrtillus* in 1999 and 2000. Error bars indicate the standard errors of the mean (plot averages of 10 leaves each). Significant differences in control plants between years are indicated by different letters. Statistical significance of differences between treatments and control also is depicted. NS = not significant.

Fig. 2 Relative leaf area fluctuating asymmetry (FA) index for control, drought, and warming treatments in 1999 and 2000. Error bars indicate the standard errors of the mean (plot averages of 10 leaves each). Significant differences (\(P < 0.05\)) in control plants between years are indicated by different letters, while significant differences (\(P < 0.05\)) between treatments and controls (within years) are indicated by an asterisk.

concluded that observed deviations from symmetry are fluctuating asymmetry rather than antisymmetry.

Leaf size. Neither treatments nor year significantly affected leaf size (area or width), although there was a tendency to decrease leaf area and leaf width with drought (fig. 1). The interaction between treatment and year was never significant, which indicates that the effects of treatments on leaf size followed the same trends both years.

Absolute leaf asymmetry \(|(L - R)|\) was correlated with leaf size \((|L + R|/2)\) for leaf area \((P < 0.01\), both years\) and for leaf width \((P = 0.064\) in 1999 and \(P = 0.038\) in 2000). The positive values of the correlation coefficients both years indicated an increase in leaf FA with increasing size. Thus, comparisons of asymmetries were made for relative rather than absolute asymmetry (Møller and Swaddle 1997). No significant correlation was found between the relative index of fluctuating asymmetry and size in any of the studied traits.

Leaf fluctuating asymmetry. Mean relative (or size-corrected) FA values in *Vaccinium myrtillus* leaves were 0.056 (SE = 0.004) for leaf width and 0.06 (SE = 0.004) for leaf area. The Box-Cox transformation of the relative FA values succeeded in normalizing their distributions (Shapiro-Wilks, in all groups, \(P > 0.2\)). Correlation between leaf area and leaf width normalized indices of FA was significant \((P < 0.01)\), with a Pearson’s correlation coefficient of \(r = 0.51\) \((n = 180)\).

Values of relative leaf area FA (fig. 2) for control leaves in 2000 were significantly lower than values in 1999 \((F_{1,4} = 21.2, P = 0.01)\), while there were no differences in leaf width FA between years (data not shown). Treatments did not significantly affect leaf width FA, and the interaction between treatment and year was never significant (data not shown). In contrast, although drought treatment did not affect leaf area FA values in 1999, drought leaves in 2000 showed higher leaf area FA values than did control leaves \((F_{1,4} = 17.8, P = 0.01)\). However, warming treatment significantly decreased leaf area FA values in 1999 \((F_{1,4} = 28.9, P = 0.006)\), while no significant effect was found in 2000 (fig. 2).

The leaf area did not correlate significantly with the accumulated rainfall received during the growing season by each treatment each year \((r = -0.14, P = 0.79, n = 6)\). In contrast, the Spearman’s correlation between the relative leaf area FA index and rainfall was significant \((r = -0.829, P = 0.04, n = 6)\) (fig. 3).

Leaf Gas Exchange

Average values of photosynthetically active radiation (PAR) in the leaf chamber, leaf temperature, and water vapor pressure deficit (VPD) were 1346 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), 31.4°C, and 2.83 kPa in the 1999 measuring date, while they were 402 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), 14.3°C, and 0.6 kPa in the 2000 measuring date. Thus, the sampling date in 1999 was much warmer, lighter, and drier than in 2000. PAR, leaf temperature, and VPD did not differ among treatments in either of the 2 yr.

Leaf photosynthetic rates of control leaves (fig. 4) were significantly lower in 1999 than in 2000 \((F_{1,4} = 8.72, P = \)
Fig. 3  Relative leaf area fluctuating asymmetry (FA) index in relation to accumulated rainfall (mm) over the growing season (June and July) for control (white circle), drought (white square), and warming (white triangle) treatments in 1999 (white symbols) and 2000 (black symbols). Each point is the mean of three plot averages of 10 leaves each. Bars are the standard errors of the mean.

Drought Effects

In relation to control plots, drought treatment was stronger in 2000 than in 1999, since the treatment excluded 37% of the accumulated rainfall over June and July in 2000 and 23% in 1999. Accordingly, whereas in 1999 we did not find any significant effect of drought treatment on relative leaf area FA, in 2000, drought leaves showed higher leaf area FA values than did control leaves (fig. 2). However, values of leaf area FA of control plants in 1999 were higher than in 2000, in agreement with the lower rainfall experienced by control plots during the growing season of 1999. Thus, our results indicate that a reduction in water availability increased stress conditions, affecting stability during leaf development, which is corroborated by the significant correlation between the relative leaf area FA values and the total rainfall over the growing season (fig. 3).

To date, supporting evidence for an increase in leaf FA in response to decreased water availability during the growing season was limited. Hochwender and Fritz (1999) found that even though Salix plants developing in dry sites tended to present higher levels of FA than those in wet sites, the differences were not statistically significant. In another study, even though plants that were habituated to long periods of aridity tended to present greater developmental stability against extreme drought conditions than plants of the same species growing in more benign habitats, no significant differences of FA between locations were observed (Alados et al. 2001). Further investigation is needed to understand the mechanisms underlying these effects.

Discussion

The detected relative fluctuating asymmetry (FA) values in Vaccinium myrtillus (fig. 2) were within the range of variability observed in previous studies of floral and foliar asymmetry (0.01–0.10; Møller and Eriksson 1994). Likewise, detected measurement errors (ME) of leaf area and leaf width were also within the range of variability (3%–20%) observed in previous studies (Møller and Shykoff 1999). Finally, repeatabilities of leaf asymmetries were high and were comparable to those reported in similar studies, which indicates that methods for studying FA were properly applied.
thermore, in a study over a period of 8 yr, Valkama and Kozlov (2001) did not find any effect of the amount of precipitation on birch leaf FA.

It is well known that an increase in drought can cause a significant reduction in growth (Long and Hutchin 1991; Pereira and Chaves 1995) and a decrease in leaf photosynthetic rates, mostly as a consequence of stomatal closure (Chaves 1991). Our drought treatment tended to decrease leaf size (area or width) and leaf gas exchange (photosynthetic rates and stomatal conductance) of plants, though the differences with control plants were not statistically significant (figs. 1, 4). It is likely that the absence of drought effects on leaf gas exchange in 1999 were due to the low treatment intensity. In 2000, the still high water availability in drought plots and especially the low evaporative demand (low VPD, PAR, and temperature during the measuring date) probably hindered any differentiation in stomatal conductance between treatments. When comparing control leaves between the 2 yr, the lower leaf photosynthetic rates that we found in 1999 than in 2000 are in agreement with the lower rainfall registered in the growing season of 1999 and are consistent with the higher leaf asymmetry of control leaves in 1999.

Warming Effects

In our study, warming treatment decreased leaf area FA during the first year (fig. 2), which indicates a positive effect of nocturnal warming on the development of V. myrtillus leaves. Conversely, no effect of warming on leaf FA was found in 2000. Such differences between years were probably related with the differences in minimum temperatures during the growing season of both years. Indeed, the average of minimum temperatures for June and July in 1999 was 4.5°C, while in 2000 it was 5.7°C. Thus, our results support the hypothesis that low temperatures throughout the growing season may be a stress factor for leaf development in V. myrtillus. Havström et al. (1993) pointed out that, during particularly cold years, temperature might be a limiting factor for the leaf growth, while during years with warmer summers, other factors (e.g., nutrients or water) can become limiting. The lower FA of control leaves in 2000 in relation to 1999 values also matches with the higher minimum temperatures experienced by leaves during June and July in 2000. Using a data set collected over a period of 8 yr, Valkama and Kozlov (2001) found that asymmetry in birch leaves was highest during cold summers and lowest during warm summers.

In this study, an increase of ca. 1°C did not affect leaf size (area or width) of V. myrtillus (fig. 1). Other studies have not found significant effects of warming on leaf size either (Wookey et al. 1994). In contrast, Callaghan et al. (1989) found a positive correlation between the temperature of the growing season and the leaf length in Cassiope tetragona from the Arctic. Accordingly, an experimental warming of a sub-Arctic heath in Sweden increased leaf area in V. myrtillus (Parsons et al. 1994). Some studies have reported contrasting effects of increases in temperature on leaf size of different species. For example, while the graminoid Eriophorum vaginatum increased its leaf length after an experimental rise in temperature (+3.5°C) in tussock tundra of Alaska, the evergreen shrubs Ledum palustre and Vaccinium vitis-idaea were relatively insensitive to the treatment (Chapin and Shaver 1996). In addition, although C. tetragona increased leaf length in response to an experimental increase in air temperature by 2°C–4°C at a sub-Arctic fellfield site (1150 m a.s.l.), there was no change in leaf length of the same species in response to the treatment at a sub-Arctic tree-line heath site (450 m a.s.l.) (Havström et al. 1993).

Finally, leaf gas exchange rates were not affected by warming treatment (fig. 4). Several other studies also failed to find significant effects on leaf CO₂ assimilation and stomatal conductance after performing different climate warming manipulations (Wookey et al. 1994; Teskey 1997; Loik et al. 2000). On the contrary, other studies found significant effects of warming on photosynthesis, e.g., an increase of 3.5°C in air temperature during summer stimulated photosynthesis in Eriophorum and Vaccinium in Arctic Alaska (Chapin and Shaver 1996). In our study, the absence of a direct warming effect on leaf gas exchange rates may result from the fact that during the diurnal hours, when photosynthesis takes place, the warming treatment is very low (Beier et al. 2003).

Final Remarks and Conclusions

The ecophysiological significance of increases in leaf asymmetry, such as the ones found in our study, is difficult to assess, since the relationship between leaf asymmetry and fitness is not clear. Several authors have proposed different physiological consequences of leaf asymmetry, such as a less efficient use of light for photosynthesis among leaves on a branch (Møller 1995), a less even distribution of drag by wind causing an increase in damage during strong winds (Møller 1995), and a less even distribution of resources from the midvein throughout the leaf that limits leaf growth (Welby and Yalouni 1999). None of these hypotheses has been demonstrated. However, Møller found that increased leaf FA in Ulmus glabra gave rise to higher infestation by leaf-mining insects (Møller 1995) and higher disease susceptibility (Møller 1999). In contrast, other authors failed to find a significant relationship between leaf asymmetry and some fitness components, such as reproductive effort, biomass accumulation, or flower production (Roy and Stanton 1999; Andalo et al. 2000) while others have found equivocal results (Evans and Marshall 1996).

Our results support the idea that the measure of leaf FA provides a sensitive and inexpensive means for quantifying developing stresses in plants in response to an increase in the severity of drought and warming. Thus, fluctuating asymmetry could be used as a tool for monitoring the impact of climatic change. In contrast, measurements of leaf size and instantaneous leaf gas exchange rates failed to provide evidence of physiological stress as a result of our environmental manipulations.

The reduction in water availability applied during the growing season increased the leaf fluctuating asymmetry in V. myrtillus, which indicates enhanced physiological stress during leaf development. Conversely, the experimental warming reduced leaf fluctuating asymmetry, which indicates a decrease in the physiological stress during leaf development. A great interannual variability in the response of leaf FA to the treatments...
has been observed, so it appears that stressing environmental factors can change from one year to another. Differences between years supported the results obtained from the environmental manipulations.

Finally, this long-lived, slow-growing plant may not have acclimatized fully after only 2 yr of manipulations. Measurements need to be continued if the long-term effects of climatic change on the physiological stress of this species are to be elucidated.

**Acknowledgments**

J. González-Solís kindly commented on previous versions of this article. This research was financially supported by the European projects CLIMOOR (Climate Driven Changes in the Functioning of Heath and Moorland Ecosystems) (UE- DG XII ENV4-CT97-0694) and VULCAN (EVK2-CT-2000-00094) and the Spanish MCYT (Ministerio de Ciencia y Tecnología) REN2000-0278/CLI and REN2001-0003/GLO.

**Literature Cited**


Rettig JE, RC Fuller, AL Corbett, T Getty 1997 Fluctuating asymmetry indicates levels of competition in an even-aged poplar clone. Oikos 80:123–127.


Wookey PA, JM Welker, AN Parsons, MC Press, TV Callaghan, JA Lee 1994 Differential growth, allocation and photosynthetic responses of Polygonum viviparum to simulated environmental change at a high Arctic polar semi-desert. Oikos 70:131–139.


