

## REPORT

Elevated CO<sub>2</sub> and herbivory influence trait integration in *Arabidopsis thaliana*

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## Abstract

We lack information on how elevated CO<sub>2</sub>, and its interaction with other factors like herbivory, affect levels and patterns of trait integration in plants. We experimentally tested the hypothesis that elevated CO<sub>2</sub> disrupts and restructures functional associations among plant traits, in the selfing annual, *Arabidopsis thaliana*. We tested for these effects both in the presence and absence of herbivory by larvae of the diamondback moth, *Plutella xylostella*. Elevated CO<sub>2</sub>, both alone and combined with moth herbivory, modified integrated trait responses. In addition, integration under different environments was genotype-specific. These results imply that global changes in CO<sub>2</sub> are likely to cause divergent evolutionary outcomes among populations of plants that differ in the initial structure of their quantitative genetic variation.

## Keywords

*Arabidopsis thaliana*, diamondback moth, elevated CO<sub>2</sub>, genotypic variation, herbivory, mouse-ear cress, multivariate analyses, non-parametric statistic, *Plutella xylostella*, trait integration.

*Ecology Letters* (2004) 7: 837–847

## INTRODUCTION

Since Darwin's original curiosity regarding the 'Mysterious laws of correlation of growth' (Darwin 1859), and Thompson's (1917) and Wright's (1918) approaches to studying morphological integration, several scientists have been inspired to investigate the complex relationship of plant and animal features. Of particular relevance were the early studies of Olson & Miller (1958) on morphological integration, Berg (1960) on correlation Pleiades, and Clausen & Hiesey (1960) on variation and coherence. Based on these studies, the concept of integration can be expressed as the totality of developmentally and functionally linked characters, which evolve together as a coordinated-whole.

Several examples are found in the literature regarding the effect of abiotic and biotic factors on phenotypic integration. Some of these factors included water, nutrients and clipping (Schlichting 1989a), light and temperature (Endler 1995; Callahan & Waller 2000), pollinators (Herrera *et al.* 2002), and flooding (Kolodynska & Pigliucci 2003). However, although several studies have assessed individual plant responses to elevated CO<sub>2</sub> and herbivory (Bidart-Bouzat 2004, and references therein), we lack information

regarding how trait integration is affected by elevated CO<sub>2</sub>, either alone or combined with herbivory. To our knowledge, only one study has performed a preliminary analysis of integrated trait responses to elevated CO<sub>2</sub>, which showed that CO<sub>2</sub> enrichment changed the genetic correlation structure of six plant traits in *Abutilon theophrasti* (Thomas & Jasienski 1996). Moller *et al.* (1989) pointed out that the understanding of the evolution of phenotypes in different environments requires the assessment of both genetic and phenotypic correlations. Genetic correlations provide information about potential constraints and enhancements on rates of adaptive evolution. However, natural selection directly acts on phenotypes and thus, on phenotypic correlations among interacting traits (Cheverud 1982; Rollo 1994).

Why apply the concept of phenotypic integration to elevated CO<sub>2</sub> studies? Since environmental factors act on entire phenotypes rather than single traits, it is important to base predictions regarding future responses to CO<sub>2</sub> enrichment on multivariate integration patterns. At the phenotypic level, traits may differ in their responses to a particular environment (phenotypic plasticity). Bradshaw (1965) was one of the first recognizing the importance of phenotypic

plasticity in plants and its evolutionary implications. For example, while some morphometric traits such as number of floral parts can be quite canalized (i.e. phenotypically stable) despite environmental variation, fitness-related traits such as total fruit number or vegetative biomass usually show more variation in their responses to different environments (Sultan 1995). A study by Tousignant and Potvin (1996) revealed that changes in CO<sub>2</sub> levels and temperature induced 'trait-specific' responses in *Brassica juncea*. Distinct patterns of trait integration in a given environment emerge 'because some combination of traits work much better together and have higher fitness values compared with other combinations' (Endler 1995). Therefore, gene expression for different traits has to be adjusted so that integration is maintained despite changes in the environment (Schlichting 1989b). Differences in gene expression and subsequent reorganization of trait associations may be expressed as changes in the magnitude and sign of correlation coefficients across environments (Murren 2002). It has been suggested that natural selection may promote positive (direct) associations among fitness-related traits and that negative (inverse) interactions may emerge in stressful or novel environments (reviewed in Rollo 1994). Whereas positive correlations imply the same direction of trait responses, inverse associations may indicate the existence of tradeoffs among traits, which can potentially constrain plant evolutionary responses to future increases in CO<sub>2</sub> levels.

Kingsolver (1996) suggests that organisms with short life spans and large population numbers are more likely to respond (evolutionarily) to global change. Thus, the plant species selected for this study, *Arabidopsis thaliana*, represents an appropriate organism to assess trait integration under CO<sub>2</sub> enrichment. *A. thaliana* is a short-lived, selfing annual plant, which usually shows a patchy distribution consisting of highly inbred individuals. A study by Bergelson *et al.* (1998) on intra- and inter-population genetic variation in *A. thaliana* revealed that most genetic variation was found among rather than within populations. They suggested that limited intrapopulation genetic variation might result from high selfing rates (>0.99) and short-distance seed dispersal (approximately within 1 m) of this species. Other studies using quantitative traits and molecular markers have also shown larger genetic variation among rather than within natural populations of *A. thaliana* (Kuittinen *et al.* 1997; Mauricio 1998). Thus, it could be expected that local *A. thaliana* populations, which mostly consist of single genotypes, would show strongly divergent responses to elevated CO<sub>2</sub> at the whole phenotype level.

In this study, we present an experimental approach to evaluate trait integration in three *A. thaliana* genotypes, which were exposed to diamondback moth herbivory and CO<sub>2</sub> enrichment. Univariate responses of *A. thaliana* genotypes to elevated CO<sub>2</sub> and herbivory have been

reported elsewhere (Bidart-Bouzat 2004). We specifically addressed the following question: does elevated CO<sub>2</sub> alone, and combined with herbivory, alter levels and patterns of trait integration across different genotypes of *A. thaliana*? In other words, do these environmental factors affect the magnitude, sign and structure of trait correlations among distinct genotypes? Using non-parametric univariate and different multivariate approaches (i.e. PCA and matrix correlation), we found that elevated CO<sub>2</sub> and herbivory can disrupt and restructure patterns of trait associations in *A. thaliana*, and that integration under different environments is genotype-specific. To our knowledge, this is the first study assessing the effect of elevated CO<sub>2</sub> alone, and combined with herbivory, on multiple trait integration. Variability in trait integration within and among genotypes is important since it may be associated with fitness and thus, could result in differential evolutionary outcomes in future enriched CO<sub>2</sub> environments.

## MATERIALS AND METHODS

### Selected plant and insect herbivore species

Studies of trait integration under elevated CO<sub>2</sub> and insect herbivory were conducted on the selfing annual plant *A. thaliana* (Capparales: Brassicaceae). Self-fertilization is a common reproductive strategy not only in annuals such as *A. thaliana* but also in biennials and short-lived perennials (Hamrick & Godt 1997; Ingvarsson 2002). A previous survey including 67 genera and 33 families (129 plant species) showed *c.* 23% were predominantly selfing and 17% were partially selfing (reviewed in Barrett & Harder 1996). Three *A. thaliana* genotypes, Cvi-0, Can-0 and Edi-0 (accession numbers: CS6675, CS6660 and CS6688), were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University). These genotypes represent highly inbred lines from three natural populations of *A. thaliana* with different selective histories. Genotypes originated from Canary Islands, Spain (Can-0); Edinburgh, UK (Edi-0); and Cape Verde Islands (Cvi-0). These *A. thaliana* genotypes exhibited marked differences in chemical (i.e. glucosinolates) defences (Bano 1993), which have been previously shown to influence herbivory in natural plant populations (Mauricio & Rausher 1997). They are also known to differ in their flowering phenologies, growth and reproductive characteristics (Jones 1971; Bidart-Bouzat 2004).

The insect herbivore used in this study was the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), a common insect specialist that feeds exclusively on members of the Brassicaceae family. Diamondback moth adults used to start a colony for the greenhouse experiment were obtained from larvae collected in cabbage plots at the

University of Illinois agricultural fields. The colony was maintained in rearing cages and adults emerging were fed with 10% (v/v) honey solution. Crucifer seedlings of canola (*Brassica napus*) were used to feed the larvae and for adult oviposition.

### Experimental design and growth conditions

To evaluate integration among traits under elevated CO<sub>2</sub> and insect herbivory, a growth chamber experiment (split-split plot design) was performed using four CO<sub>2</sub> chambers located in the Plant Biology Laboratory at the University of Illinois, Urbana-Champaign. Two CO<sub>2</sub> treatments, elevated (720 p.p.m.) and ambient CO<sub>2</sub> (360 p.p.m.), two chambers per CO<sub>2</sub> level, two herbivory treatments (i.e. herbivory and control), three genotypes, and six replicates per genotype accounted for a total of 144 plants. Individual plants were grown from seeds in 750 cm<sup>3</sup> pots, which underwent a 10-day cold treatment to eliminate dormancy and ensure uniform germination. Afterwards, temperature was set at 20 °C and light/dark periods were set at 14 h/10 h. Groups of plants assigned to each treatment combination were constantly rotated within (every 2–3 days) and among (weekly) chambers. This strategy usually decreases standard deviations of sampling distributions and prevents chamber effects. Two second-instar moth larvae were placed at bolting time on each of the plants, randomly assigned to the herbivory treatment, and removed at pupation. This herbivory load resulted in an average of 20% of each plant's total leaf area removed, as measured through a qualitative index of plant damage on rosette leaves (adapted from McCloud and Berenbaum, 1999). This qualitative index was calculated by assigning leaves to four size categories (1, 6–8 cm; 2, 4–6 cm; 3, 2–4 cm; 4, 0–2 cm) and three damage classes dependent upon the amount of tissue removed (25% or less, 25–75%, or more than 75%). Each plant (from both control and herbivory treatments) was protected with a flexiglass cage with holes for aeration, which were covered with a 135 µ<sup>2</sup>-mesh fabric to prevent both larval dispersal and infestation by other insects from the greenhouse.

A variety of phenological, growth-related and reproductive traits were measured for each plant to assess phenotypic integration under CO<sub>2</sub> enrichment and insect herbivory. Flowering phenology was recorded as the number of days required to first flower. At bolting time, rosette diameter was measured. The following traits were measured at harvesting time: total number of fruits, seed number per fruit, total height and aboveground vegetative biomass. To determine biomass, plant material (leaves and stems) was oven-dried (80 °C) for 72 h and weighed. Selected traits are considered important life history characteristics, and are usually directly related to fitness (e.g. total fruit number,

seed number per fruit, flowering time) or considered surrogates of plant fitness (e.g. vegetative biomass, height and rosette diameter). These traits are, therefore, appropriate to evaluate phenotypic integration and its potential evolutionary significance.

### Statistical analyses

A split-split linear mixed model (Proc Mixed, SAS) was used to analyse univariate responses of the measured variables (i.e. reproductive, growth-related and phenological). CO<sub>2</sub>, herbivory, genotype and their interactions were considered fixed effects, and chamber and their interactions were assumed random. Fixed main effects and interactions were tested against the appropriate error terms as shown by Anderson & McLean (1974, p. 199). Response variables were logarithmically or squared-root transformed when necessary to satisfy parametric model assumptions. Contrasts were performed to assess mean phenotypic differences between CO<sub>2</sub> treatments (ambient vs. elevated CO<sub>2</sub>) for each combination of herbivory and genotype levels.

To evaluate trait integration, the ANOVA-type statistic test (ATS; Brunner *et al.* 2002) was used to assess the effects of elevated CO<sub>2</sub>, herbivory and genotype on the magnitude (absolute value) and sign of Pearson's correlation coefficients. The non-parametric ATS test is based on an approximated F-distribution. This approximation is reasonably accurate and highly conservative when sample sizes are very small (see Brunner *et al.* 2002 for details on this test). In addition, more conservative tests are needed when split-plot restrictions on randomization are not considered (Anderson & McLean 1974). Thus, the ATS test is appropriate to evaluate the magnitude of correlation coefficients, since these coefficients are not replicated and, therefore, the split-split plot structure of the design can not be maintained. The magnitude or level of integration in each group (12 combinations of CO<sub>2</sub>, herbivory and genotype) was estimated as the averaged rank sum of the correlation coefficients among the six response variables, with the exclusion of those corresponding to the correlation of each variable with itself (mean of 15 coefficients per group;  $n = 180$ ). To evaluate how elevated CO<sub>2</sub> affected the strength of integration at each genotype and herbivory level, non-parametric pre-planned contrasts (i.e. ambient vs. elevated CO<sub>2</sub>) based on mean rank sums were performed. The same non-parametric approach was utilized to assess elevated CO<sub>2</sub>-induced changes in mean ratios of positive to negative signs of significant correlation coefficients at each herbivory level. The ratio of the number of positive to negative correlation coefficients provides information on how the environment affected the nature of trait relationships, in terms of their potential direct or inverse associations. In addition, the number of neutral associations (non-significant

correlation coefficients) across treatment combinations was estimated.

Patterns of integration among traits across treatments were graphically evaluated by performing principal components analyses (PCA). Since chamber effects were not significant, chambers were pooled within CO<sub>2</sub> treatments for performing PCA, which increased sample sizes within treatment groups ( $n = 12$ ). Principal components were derived from the within-group correlation matrices. This method is preferred for variables differing on their units of measurement, and for providing equal emphasis to all variables despite their differences in absolute variances (McGarigal *et al.* 2000). Two PCs were retained in each of the 12 groups (based on Mineigen criterion; SAS Institute 1994). Significance of PCs was based on a benchmark of 0.55 (i.e. absolute values of PC loadings) suggested by Tabachnick & Fidell (1989). As for any other parametric multivariate analysis, PCA must meet assumptions of multivariate normality and homogeneity of within-group covariance matrices. Multivariate normality was assessed by plotting ordered squared mahalanobis distances against the quantiles of a chi-square distribution (Khattri & Naik 1999). The plot should be approximately a straight line with a slope equal to 1. Homogeneity of within-group covariance matrices for each genotype and for all groups was tested by a modification of Bartlett's likelihood ratio test (Morrison 1976). Although the assumption of multivariate normality was met, results from the likelihood ratio test indicated a strong heterogeneity of within-group covariance matrices. This indicated that covariance matrices of genotypes exposed to the same environment were markedly different. Therefore, a separate PCA analysis was performed for each of the 12 groups (instead of pooling genotypes). Due to limited sample size of each group ( $n = 12$ ), results from both PCA and matrix correlation analyses (explained below) should be interpreted with caution. However, it has been previously suggested that only a few observations may suffice to unveil trait associations in homogeneous groups (McGarigal *et al.* 2000). In our study, highly homogeneous experimental groups represented by *A. thaliana* inbred lines exposed to controlled environmental conditions provided an appropriate experimental setting to assess multiple-trait associations.

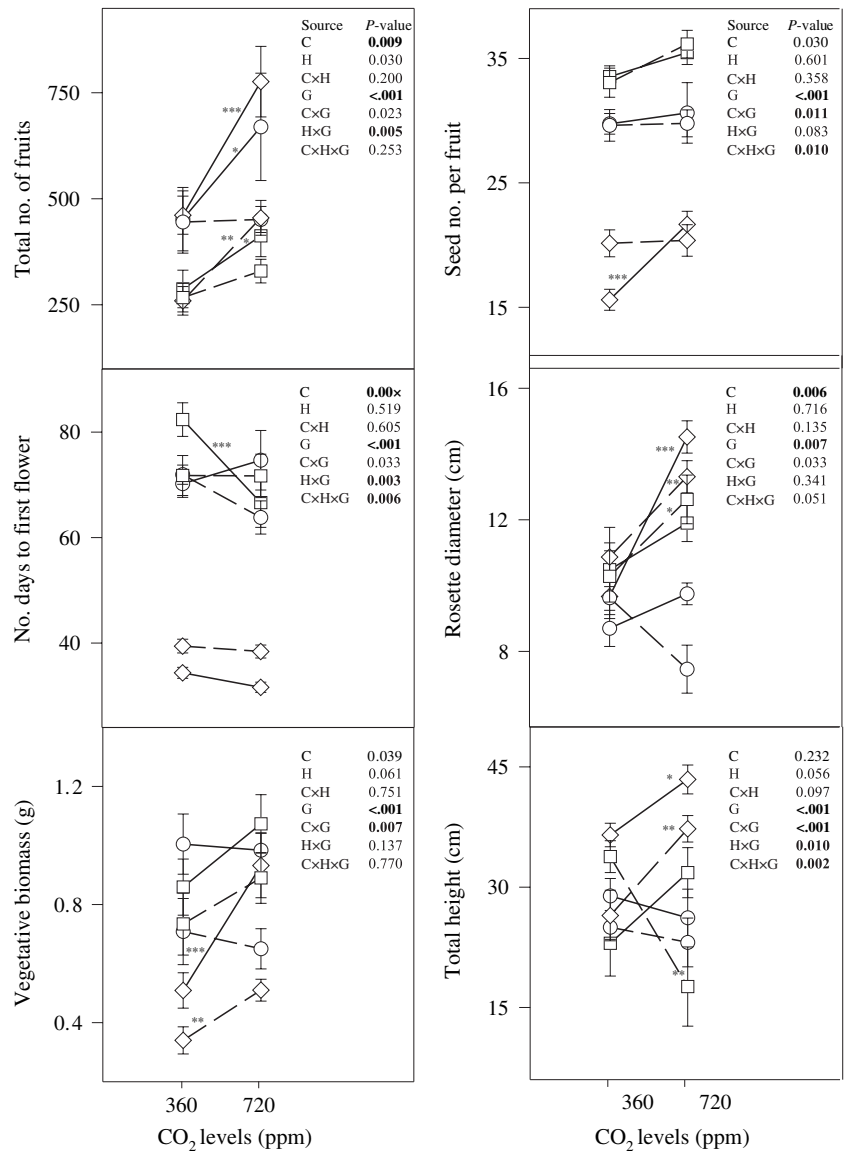
To evaluate potential changes in the patterns of trait associations across environments, Mantel tests of correlation between matrices were performed (Mantel 1967). The Mantel test estimates the relationship between two independent dissimilarity matrices using sampled randomization techniques (Sokal & Rohlf 1995). Dissimilarity matrices were obtained by subtracting correlation coefficients from one. Matrix correlation coefficients measuring structural similarity between matrices vary from  $-1$  to  $+1$ . A matrix correlation coefficient of  $+1$  or  $-1$  indicates identical

correlation patterns or mirror-imaged matrices, respectively. On the other hand, a zero or close to zero value indicates no structural similarity between matrices and thus, a non-significant Mantel's correlation coefficient (Marroig & Cheverud 2001). Significance of the Mantel statistic (i.e. the correlation between the elements of two dissimilarity matrices) was tested by randomly permuting the rows and columns of one of the matrices 10 000 times and each time computing a Mantel coefficient, so that a reference distribution of these coefficients was obtained. Then, the Mantel statistic computed from the original matrices was compared against this reference distribution (Sokal & Rohlf 1995; Marroig & Cheverud 2001). This is a good test to evaluate if the association between two matrices is greater than that expected by chance. A series of Mantel tests were performed to assess the impact of elevated CO<sub>2</sub> and herbivory on patterns of correlations for each of the three genotypes. Specifically, matrix correlations for each genotype exposed to control conditions (i.e. ambient CO<sub>2</sub> and no herbivory) were compared with the correlation matrices obtained at elevated CO<sub>2</sub> only, herbivory only, and both elevated CO<sub>2</sub> and herbivory combined. In addition, matrix correlations among genotypes exposed to the same set of environmental conditions were compared to evaluate whether genotypes differed in their phenotypic response to the same environment.

## RESULTS

Univariate analyses and reaction norms of response variables shown in Fig. 1 revealed that most variables responded significantly to CO<sub>2</sub> effects, although they differed in their degree of response. Herbivory effects were significant only for total fruit number and marginally significant for height and vegetative biomass. In addition, genotypes differed in their trait responses to elevated CO<sub>2</sub> (significant CO<sub>2</sub> × genotype interactions). For example, while the Cvi-0 genotype was the most responsive (i.e. plastic) to increased CO<sub>2</sub> in terms of reproductive and growth-related characteristics, Edi-0 appeared to be more plastic for phenological traits such as flowering and germination time (germination time data not shown). On the other hand, the three-way interaction (CO<sub>2</sub> × herbivory × genotype) was significant only for three of the six variables studied (i.e. seed number per fruit, flowering time, and height).

Non-parametric ANOVA-type-statistics (ATS) were computed to test for main effects (i.e. elevated CO<sub>2</sub>, herbivory and genotype), and their interactions on trait integration levels (Table 1). These tests revealed that, overall, the average strength of correlation coefficients did not differ among genotypes and it was not altered by either elevated CO<sub>2</sub> or herbivory. However, main effects, *per se*, are not as informative as their potential interactions, particularly those



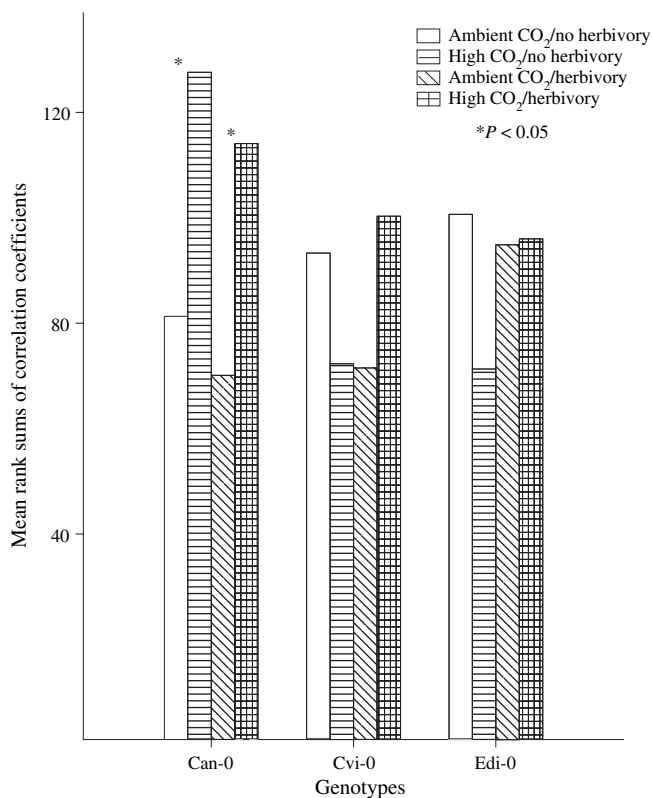
**Figure 1** Univariate responses of *A. thaliana* genotypes (G) exposed to elevated CO<sub>2</sub> (C) and herbivory (H). Boldface indicates significant  $P$ -values after a sequential Bonferroni correction. Solid and dotted lines represent plant responses to elevated CO<sub>2</sub> in the absence and presence of herbivores, respectively. Plant genotypes are denoted by circles (Can-0), diamonds (Cvi-0), and squares (Edi-0). Asterisks on lines symbolize significant contrasts (ambient vs. increased CO<sub>2</sub>) at each herbivory and genotype level. Significance levels: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

evaluating genotypic differences in the response to a given environmental change. In this study, *A. thaliana* genotypes showed differential correlation levels when exposed to elevated CO<sub>2</sub> (i.e. significant CO<sub>2</sub> × genotype interaction). Results of pre-planned contrasts (i.e. ambient vs. elevated CO<sub>2</sub> for each genotype and herbivory level) indicated that elevated CO<sub>2</sub> significantly increased the level of integration in the Can-0 genotype, either with or without herbivory (Fig. 2). On the other hand, the opposite trend was found for genotypes Cvi-0 and Edi-0 under no herbivory, although results of contrasts were not significant ( $P = 0.29$  and  $0.085$ , respectively). In addition, the enriched CO<sub>2</sub>-induced increase in correlation strength in the Cvi-0 genotype under herbivory was not significant either ( $P = 0.097$ ).

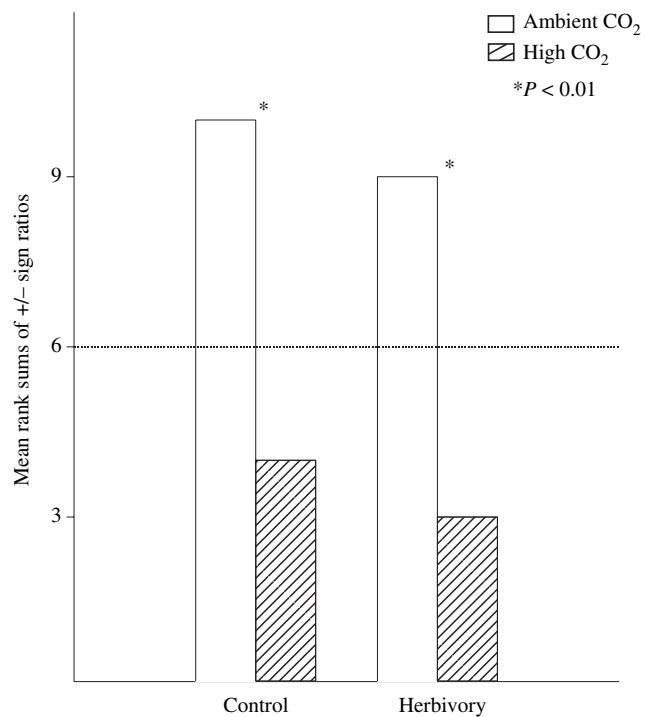
Changes in the proportion of direct vs. inverse associations among traits were evaluated by performing the ATS test on ratios of the number of positive to negative correlation coefficients. These correlation sign ratios differed among treatment groups (ATS = 10.56,  $P = 0.0037$ ,  $n = 12$ ). In particular, these ratios appeared to be specially influenced by CO<sub>2</sub> enrichment rather than by herbivory treatments. Figure 3 shows a higher number of positive correlations under ambient CO<sub>2</sub> (values of +/− sign ratio > 1, equivalent to mean sum ranks > 6) and a greater number of negative correlations at elevated CO<sub>2</sub> (values of +/− sign ratio < 1). This result was validated by non-parametric contrasts (i.e. ambient vs. elevated CO<sub>2</sub> at each herbivory level), which revealed a significant decrease in mean rank sums of +/− sign ratios attributed to elevated

**Table 1** ANOVA-type statistics (ATS) to evaluate CO<sub>2</sub>, herbivory, and genotypic effects on absolute values of Pearson's correlation coefficients

Source of variation	D.f.	ATS
CO <sub>2</sub>	1	2.61
Herbivory	1	0.01
CO <sub>2</sub> × herbivory	1	2.71
Genotype	2	1.13
CO <sub>2</sub> × genotype	2	5.09*
Herbivory × genotype	2	0.67
CO <sub>2</sub> × herbivory × genotype	2	0.98
Error	169	

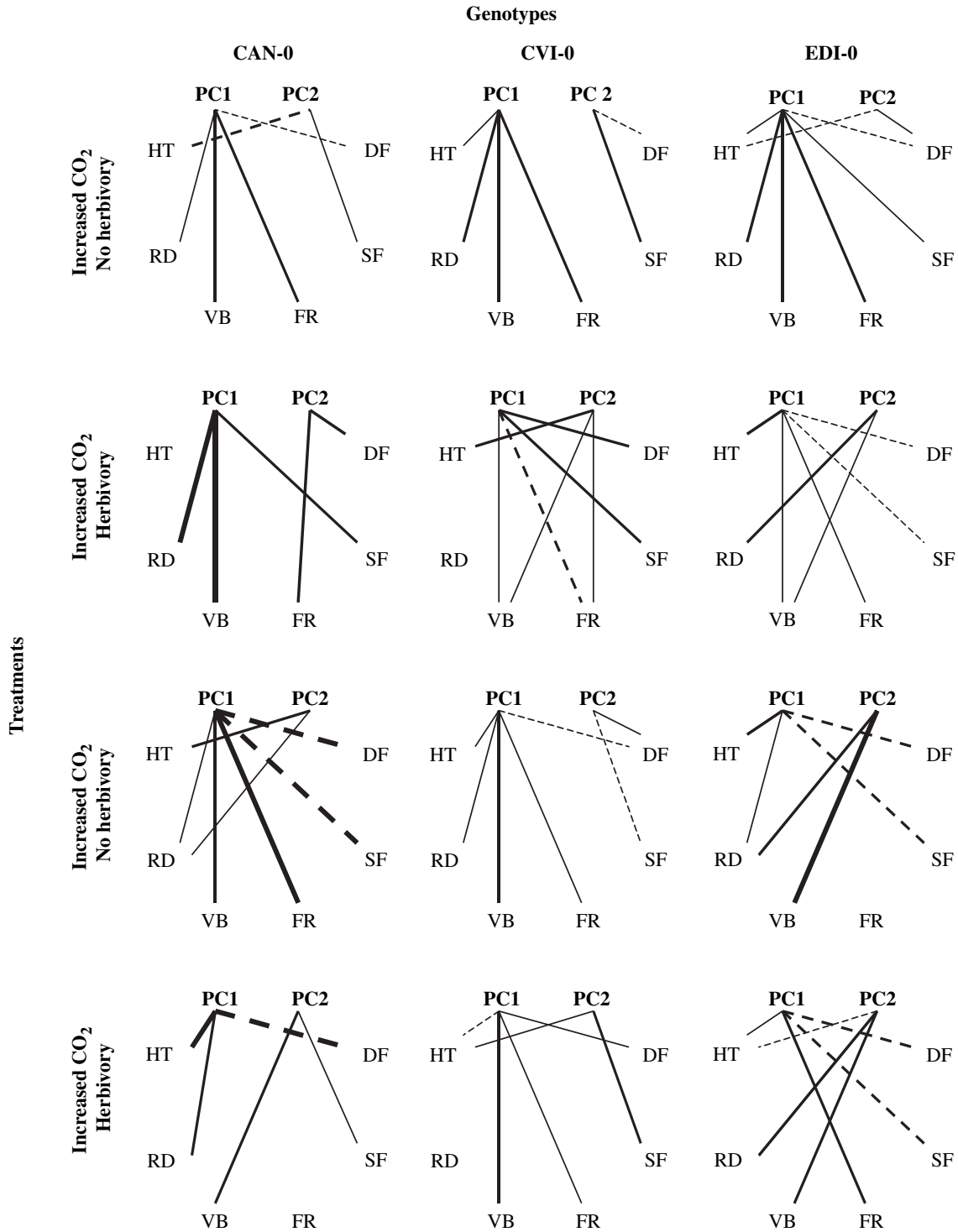
\**P* < 0.01.**Figure 2** Rank sum means (mean Wilcoxon scores) for Pearson's correlation coefficients indicating the level of integration for each *A. thaliana* genotype in different environments. Asterisks represent significant contrasts (ambient versus increased CO<sub>2</sub>) for each herbivory level and genotype.

CO<sub>2</sub> regardless of herbivory levels. The proportion of negative correlation coefficients is useful to evaluate potential costs or tradeoffs among traits that may emerge under elevated CO<sub>2</sub> conditions. In addition, the number of neutral associations (i.e. non-significant correlation

**Figure 3** Rank sum means (mean Wilcoxon scores) for the ratio of positive to negative signs (+/-) of Pearson's correlation coefficients for *A. thaliana* exposed to elevated CO<sub>2</sub> and herbivory. Asterisks represent significant contrasts (ambient versus increased CO<sub>2</sub>) at each herbivory level. The dotted line represents a +/- sign ratio of 1 (equal number of positive and negative correlation coefficients).

coefficients) was not affected by changes in the environment (ATS = 2.07, *P* = 0.18, *n* = 12). Non-parametric contrasts performed for this type of correlation were not significant either.

PCA graphically revealed pronounced differences in the patterns of trait integration among treatment combinations and genotypes (Fig. 4). Major differences in the representations of the 12 PCA graphs indicated a strong environmentally induced reorganization of trait relationships. This outcome was observed across groups for all phenological, growth-related and reproductive traits. For example, in the Can-0 genotype, flowering time was inversely associated with total number of fruits, vegetative biomass and rosette diameter under control conditions (i.e. ambient CO<sub>2</sub>, no herbivory) but directly associated with total fruit number when this genotype was exposed to herbivory. In addition, an inverse association between seed number per fruit and total fruit number in this genotype was observed only under elevated CO<sub>2</sub>/no herbivory conditions, providing another example of the environmental dependency of trait relationships. Common patterns of



**Figure 4** Principal components analyses on six measured variables (Ht, height; Rd, rosette diameter; Vb, vegetative biomass; Fr, total number of fruits; Sf, number of seeds per fruit; and Df, days to first flower) for each treatment combination of CO<sub>2</sub> and herbivory in three *Arabidopsis thaliana* genotypes. Lines indicate variables with significant component loadings,  $s_{ij} > 0.55$  or  $< 0.55$  (Tabachnick & Fidell 1989; McGarigal *et al.* 2000). Line thickness correspond to three ranges of absolute values of loadings: 0.55–0.73 (thinnest), 0.73–0.91 (intermediate) and  $> 0.91$  (thickest). Solid and dotted lines represent positive and negative loadings, respectively.

trait associations across groups were not visually detected from the PCA graphs.

The strong variation in trait associations among groups was corroborated by results from a series of matrix correlation analyses. None of the matrix correlations comparing patterns of trait associations for each genotype at control conditions (i.e. ambient CO<sub>2</sub> and no herbivory) versus those at the other three treatment combinations (i.e. elevated CO<sub>2</sub>, herbivory, and elevated CO<sub>2</sub> + herbivory) were significant (*P*-values ranged from 0.08 to 0.93). These results indicate marked differences among correlation matrices of each *A. thaliana* genotype when exposed to different environmental settings. Likewise, matrix correlations performed among genotypes at each of the four treatment combinations were not significant either (*P*-values ranged from 0.1 to 0.84). These non-significant associations among matrices suggest pronounced differences in the way that each genotype responded to the same set of environmental conditions. It should be noted that the Mantel permutation test is entirely conditional on the specific matrices observed and thus, it ignores the sampling variability of the correlation matrix estimates. Since the sample sizes within each group are rather small, our results should be interpreted somewhat cautiously. Nonetheless, one would generally expect that incorporating sampling variability would increase *P*-values. Thus, the non-significance of the tests observed here should not be highly affected by the conditional nature of the permutation test.

## DISCUSSION

Since environmental factors act at the level of the entire phenotype, studies of phenotypic integration are fundamental for understanding plant responses to global climate change. While most studies evaluating environmentally induced changes in phenotypic integration have been carried out at the species level (Schlichting 1989b; Nicotra *et al.* 1997; Kolodnynska & Pigliucci 2003), little information exists regarding how individual genotypes or populations differ in their multi-trait responses across environments. Interpopulation variation in integrated trait responses to environmental change may contribute to genetic differentiation of populations, and possibly to speciation events (Agrawal 2001). This variability in integration patterns represents underlying quantitative genetic variation among populations and may have implications for the conservation of genetically distinct populations, which are considered a significant component of biodiversity (Hughes *et al.* 1997). It has also been proposed that genetic differences in the response of phenotypes to global change may affect abundance and diversity of organisms/species and their complex interactions, which in turn can

impact community and ecosystem dynamics (Whitham *et al.* 2003).

Our results revealed that genotypes of *A. thaliana* differed in their responses to elevated CO<sub>2</sub>, in terms of strength and direction of trait associations. The significantly higher level of integration in the Can-0 genotype under CO<sub>2</sub> enrichment (regardless of herbivory level) may indicate tighter linked functional processes operating in this particular genotype when exposed to novel environments such as elevated CO<sub>2</sub>, which may constrain adaptive plastic responses. In addition, it is interesting to note that in a previous study, Bidart-Bouzat (2004) found that this genotype had the highest fitness value under both ambient and elevated CO<sub>2</sub>. Schlichting & Pigliucci (1998) have previously showed a positive link between phenotypic integration and fitness in *Phlox drummondii*, and suggested that this pattern may reflect a balance between the requirement for trait integration and the need for independence of trait responses to cope with environmental change. Although our study was not designed to test the relationship between fitness and integration, a positive trend was noticed between mean fitness (product of total fruit number and seed number per fruit) and integration levels (average of correlation coefficients' absolute values for each treatment group) in the Can-0 genotype (Table 2). However, our experiment has not provided statistical power to test this regression and thus, no conclusive information can be inferred from this data either at the genotype level (*n* = 3) or within genotypes (four treatment combinations per genotype, *n* = 4). This topic certainly deserves further attention to improve our understanding of how natural selection might act on a multitude of traits under future environmental challenges.

The evaluation of trait relationships in each genotype is especially important in highly selfing plants such as *A. thaliana*, in which natural populations usually consist of patches of single genotypes (multilocus haplotypes) adapted to local conditions (Bergelson *et al.* 1998). Local selective pressures in future environments (i.e. elevated CO<sub>2</sub> × local environment interactions) acting on these populations may result in distinct evolutionary outcomes. These locally adapted populations are known to differ in flowering phenology and other characteristics related to growth, reproduction and defence (Jones 1971; Bano 1993). Our results (Fig. 1; Bidart-Bouzat 2004) and previous CO<sub>2</sub> research in *A. thaliana* (Ward & Strain 1997; Andalo *et al.* 2001) have shown differences in individual responses of traits to elevated CO<sub>2</sub>, which also differed across genotypes. For example, the Cvi-0 genotype has shown more pronounced responses to elevated CO<sub>2</sub>, in terms of growth and reproduction than in phenological traits (Norton *et al.* 1995; Ward & Strain 1997; Bidart-Bouzat 2004). Conversely, the genotype Edi-0 was more plastic for phenological than for fitness-related traits (Bidart-Bouzat 2004). Since differential



**Table 2** Mean fitness\* values and integration† levels of *Arabidopsis thaliana* genotypes exposed to elevated CO<sub>2</sub> and herbivory

Treatment	Genotype					
	Can-0		Cvi-0		Edi-0	
	Fitness	Integration	Fitness	Integration	Fitness	Integration
Ambient CO <sub>2</sub> -herbivory	11243 ± 1244	0.27 ± 0.06	5074 ± 622	0.26 ± 0.05	8733 ± 1021	0.37 ± 0.06
Ambient CO <sub>2</sub> -no herbivory	13237 ± 2097	0.32 ± 0.07	7058 ± 699	0.37 ± 0.07	9780 ± 1581	0.39 ± 0.05
Elevated CO <sub>2</sub> -herbivory	13427 ± 1043	0.48 ± 0.07	9247 ± 1033	0.41 ± 0.07	11778 ± 892	0.40 ± 0.07
Elevated CO <sub>2</sub> -no herbivory	19198 ± 2384	0.56 ± 0.07	16776 ± 2102	0.27 ± 0.05	14557 ± 1733	0.29 ± 0.07

\*Product of total fruit number and seed number per fruit (± SE).

†Average of correlation coefficients' absolute values for each treatment group (± SE).

trait responses to elevated CO<sub>2</sub> can result in changes in trait integration, variable trait associations, such as those found in our study, may not be rare in natural populations of *A. thaliana* exposed to future CO<sub>2</sub> enrichment.

Differences in trait associations among environments are reflected not only in the strength but also in the sign of correlation coefficients. The sign of correlation coefficients provides information regarding the relationship between two traits such as their positive, negative, or neutral association. While positive correlations indicate the same direction of response, negative correlations denote potential tradeoffs among traits, which may constrain plant evolutionary responses (Murren 2002). For example, Etterson & Shaw (2001) showed that inverse correlations among plant traits constrained adaptive responses to global warming. Our results revealed an increase in the number of negative correlations under elevated CO<sub>2</sub>, both in the presence and absence of herbivory, which may denote an augmented potential for tradeoffs among traits under CO<sub>2</sub> enrichment. It is worth noting that neutral correlations were not significantly affected by changes in elevated CO<sub>2</sub> or herbivory. Therefore, the increase in negative correlations under elevated CO<sub>2</sub> derived from a decrease in the number of positive correlations, rather than from a change in the proportion of neutral ones. These results appeared to be consistent with the hypothesis that character correlations tend to be positive under optimal environmental conditions but negative in response to stress or new selective regimes, and may, therefore, constrain adaptive responses (reviewed in Rollo 1994). On the other hand, other studies suggest that the previous hypothesis may not hold true under novel environmental conditions (Bell 1984; Service & Rose 1985). For example, Service & Rose (1985) found that a laboratory population of *Drosophila melanogaster* showed increased positive correlations among fitness components when flies were exposed to a novel environment.

It is well known that integration among traits can result from shared resources within an organism, which is

usually expressed as tradeoffs among traits. In addition, integrated trait responses can also emerge as a result of common genetic control (i.e. pleiotropy or linkage), and from traits responding to a common external environment (Stearns 1992). Changes in multiple trait integration across environments may arise as a result of the differential success of various trait combinations in terms of maximizing fitness in each environment (Endler 1995). In the present study, patterns of trait integration strongly differed among genotypes and treatment combinations of CO<sub>2</sub> and herbivory levels. This was visualized in the graphical representation of PCAs, which summarized the correlation structure matrix of each group and thus, the patterns of trait associations in each genotype and environment. Matrix correlations corroborated the dissimilarity of matrices, which resulted from the exposure of each genotype to various environments. In addition, integration patterns were not stable among genotypes grown in the same environment. These results are in marked agreement with the hypothesis that the environment is highly responsible for disrupting and restructuring functional or developmental associations among traits (Marroig & Cheverud 2001).

The application of the concept of integration to study global change is essential to better predict what phenotypes may be selected in future changing environments. Results from our experiment suggest a strong environmentally induced and genotype-specific reorganization of trait associations, which could result in divergent evolutionary outcomes among populations of plants differing in the initial structure of their quantitative genetic variation. More information is definitely needed regarding how local environmental factors interacting with CO<sub>2</sub> levels may affect patterns of phenotypic integration in the field. Nonetheless, our study provides an initial step towards a better understanding of how elevated CO<sub>2</sub> and its interaction with insect herbivory may influence multiple-trait responses in fast growing plants.

## ACKNOWLEDGEMENTS

We are very grateful to Juan L. Bouzat, Oswald Schmitz, Don Waller, and two anonymous reviewers for constructive comments on previous versions of this manuscript, and the Arabidopsis Biological Resource Center for supplying seeds for this experiment. This work was funded by a Sigma Xi Grant-in-Aid of Research, and by the Maria Pia Gratton graduate fellowship (University of Illinois at Urbana-Champaign) to M.G. Bidart-Bouzat.

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Editor, Don Waller

Manuscript received 15 March 2004

First decision made 23 April 2004

Second decision made 21 May 2004

Manuscript accepted 7 June 2004