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Are fern stomatal responses to different stimuli coordinated? Testing responses to light, vapor pressure deficit, and CO_2 for diverse species grown under contrasting irradiances

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Summary

- The stomatal behavior of ferns provides an excellent system for disentangling responses to different environmental signals, which balance carbon gain against water loss.
- Here, we measured responses of stomatal conductance (g_s) to irradiance, CO_2, and vapor pressure deficit (VPD) for 13 phylogenetically diverse species native to open and shaded habitats, grown under high- and low-irradiance treatments. We tested two main hypotheses: that plants adapted and grown in high-irradiance environments would have greater responsiveness to all stimuli given higher flux rates; and that species' responsiveness to different factors would be correlated because of the relative simplicity of fern stomatal control.
- We found that species with higher light-saturated g_s had larger responses, and that plants grown under high irradiance were more responsive to all stimuli. Open habitat species showed greater responsiveness to irradiance and CO_2, but lower responsiveness to VPD; a case of plasticity and adaptation tending in different directions. Responses of g_s to irradiance and VPD were positively correlated across species, but CO_2 responses were independent and highly variable.
- The novel finding of correlations among stomatal responses to different stimuli suggests coordination of hydraulic and photosynthetic signaling networks modulating fern stomatal responses, which show distinct optimization at growth and evolutionary time-scales.

Introduction

Stomata respond to environmental stimuli to balance hydraulic and photosynthetic needs. Stomatal conductance (g_s) must be high enough for CO_2 assimilation while avoiding lethal dehydration of the mesophyll and xylem (Cowan & Farquhar, 1977). This balancing act influences plant fitness, species distributions, and environmental fluxes of water and carbon (Betts *et al.*, 2007; Berry *et al.*, 2010). Fern stomatal behavior has attracted special attention recently (Franks & Farquhar, 1999; Brodribb & Jordan, 2008; Doi & Shimazaki, 2008; Brodribb *et al.*, 2009; Brodribb & McAdam, 2011; McAdam & Brodribb, 2013), but is still not clearly understood. Fern stomatal control is thought to be much simpler than that observed for angiosperms (Brodribb & McAdam, 2011, 2013), which makes it an excellent system for disentangling stomatal responses to different stimuli. To do this, we need to characterize stomatal responses of given species to particular environmental factors, and the variation in these responses within and across species and environments, as well as the potential coordination of these responses. In particular, the correlation across species of fern stomatal responses to different stimuli has not been examined. A detailed understanding of fern stomatal responses is vital given that ferns represent the second-largest radiation of land plants, and their habitat specificity makes them useful as 'canaries in the coalmine' indicator species of plant responses to environmental change (Page, 2002; Andama *et al.*, 2003; Chang *et al.*, 2009).

Stomatal behavior in angiosperms provides a baseline for hypothesis testing in the ferns. Angiosperm stomata generally show short-term, rapid responses: opening under increasing irradiance and closing under increasing ambient CO_2 concentration (c_a) and vapor pressure deficit (VPD). However, species show strong variation according to their metabolic versus hydraulic needs (Franks & Farquhar, 1999; Brodribb *et al.*, 2009; Aasamaa & Sõber, 2011a; Merilo *et al.*, 2014). Previous studies of ferns found that their stomata closed in response to leaf dehydration and opened in response to red light, as is typical for angiosperms (Brodribb & Holbrook, 2004; Doi *et al.*, 2006), but were relatively or completely insensitive to blue light, CO_2, and VPD (Franks & Farquhar, 1999; Brodribb & Jordan, 2008; Doi & Shimazaki, 2008; Brodribb *et al.*, 2009; Brodribb & McAdam, 2011, 2013; McAdam & Brodribb, 2013). Those studies focused on one to 10 species each (24 species in total) and typically examined responses to a single factor with measurements made

heterogeneously in field, glasshouse and laboratory. We built on these studies with a common garden experiment to test for a 'coordination' of the stomatal responses to different factors – that is, a correlation across species of the magnitude of responsiveness to these factors – and its putative relationship to ecological specialization in ferns.

To investigate short-term stomatal responses to irradiance, CO_2, and VPD, and the associations of these responses with native habitat and growth environment, we cultivated 13 tropical fern species, native to open and shaded habitats, under controlled high- and low-irradiance treatments. We selected species that varied strongly in habitat preference, morphology, and physiology within a high-diversity Costa Rican rainforest (Tuomisto *et al.*, 2002; Watkins *et al.*, 2006, 2010; Moran, 2008). These species arose in lineages from the most basal eusporangiate Ophioglossales up to the most recently derived leptosporangiate Polypodiales, with evolutionary histories that span wide variation in climate and atmospheric CO_2 concentration (Royer, 2006; Smith *et al.*, 2006). We focused on short-term g_s responses (i.e. those achieved in 1–3.5 h) for comparison with most previous studies of angiosperms, for consistency with standard measurement recommendations (Evans & Santiago, 2012), and because in angiosperms and ferns this time frame typically includes the majority of responses (see e.g. Kim & Heinrich, 2003; Barbour *et al.*, 2005; Powles *et al.*, 2006; Brodribb *et al.*, 2009; Vrábl *et al.*, 2009; Woodruff *et al.*, 2010; Pasquet-Kok *et al.*, 2010; Brodribb & McAdam, 2011; Buckley *et al.*, 2011).

We tested two main hypotheses. First, given the potential importance of a photosynthetic signal modulating stomatal control (Messinger *et al.*, 2006; Busch, 2014), we hypothesized that higher flux rates of gas exchange for species adapted in open habitats and for plants grown under high irradiance would support greater responsiveness to changes in all stimuli (Givnish, 1988; Johnson *et al.*, 2000; Ackerly, 2004; Proctor, 2012), especially to cope with wider extremes of irradiance and VPD (Zwieniecki *et al.*, 2004; Sack *et al.*, 2005, 2006; Buckley *et al.*, 2011). Secondly, given the proposed simplicity of fern stomatal control and generally weak responsiveness relative to angiosperms (e.g. Brodribb & McAdam, 2011, 2013), we hypothesized that fern stomatal responses to different factors would be coordinated (i.e. correlated in magnitude across species), as would be expected if ferns lack the complex signaling pathways of angiosperms that would allow distinct modulation of responses to different stimuli. Positive correlations of responsiveness to light, CO_2, and VPD might be expected if responses are transduced by a shared signaling network (Hetherington & Woodward, 2003); if there is substantial 'crosstalk' among different signaling networks, as has been supported in Arabidopsis (Merilo *et al.*, 2014); and/or if stomatal responses for a given species are constrained by anatomy and guard cell mechanics, such that a guard cell that is more responsive to one factor is more responsive to all factors (Franks & Farquhar, 2007; Dow *et al.*, 2014). Alternatively, stomatal responsiveness to different stimuli may be uncorrelated, allowing separate optimization. Further, because photosynthetic rate (A) is fundamentally determined by g_s and may drive feedbacks on g_s via intercellular CO_2 concentration (c_i) (Brodribb *et al.*, 2009; Nobel, 2009), we also tested for a correlation of g_s responses with those of A and c_i to increasing stimuli assuming predictable responses of these variables. In particular, species with higher light-saturated carbon assimilation (A) and dark respiration rates (R_d) under low VPD and ambient CO_2 might show stronger stomatal responses to changes in environmental stimuli associated with a more rapid metabolism.

Materials and Methods

Common garden experimental design

Thirteen fern species of tropical lowland rainforest were selected to maximize morphological and phylogenetic diversity and cultivated in two light environments at La Selva Biological Station, Costa Rica (84°00′12.922″W, 10°25′52.610″N). From August to September 2010, we collected 16 individuals of each species from the forest along with their local clay loam soil (Eutric Hapludand Andisol; Weitz *et al.*, 1997) to minimize transplant shock. We included seven species from open habitats (i.e. species found in large clearings) and six species from shaded habitats (i.e. species found in understory or small clearings; Table 1), and collected study individuals from at least three subpopulations per species separated by 0.5–2 km. Irradiance treatments were imposed with two replicate shadehouses for each irradiance treatment using layered 50% 'aluminet' shade cloth (Aluminet Reforzado; CNBM, Beijing, China), which improves the proportion of diffuse light, thus favoring net photosynthesis and growth (Markvart *et al.*, 2010), and reflects the nontransmitted light, thus reducing shadehouse cloth and air temperatures (Bailey, 1981). The percentage of daylight photosynthetically active radiation (PAR) transmitted into the shadehouse was determined at noon on a typical cloudy day by averaging light meter measurements (LI-250A; Li-Cor, Lincoln, NE, USA) at six locations across the benches in each shadehouse and dividing by simultaneous measurements taken in a clearing outside the shadehouses. To reduce shock, all plants were acclimated under medium irradiance (10% daylight PAR) for 6 months before transferring to high irradiance (20% daylight PAR) and low irradiance (1.5% daylight PAR) growth environments. Temperature and humidity were similar across the high- and low-irradiance growth treatments, and tracked the climate outside the shadehouses: midday measurements using the Li-Cor 6400 (Li-Cor) of air temperature and relative humidity (RH) averaged for five typical days were (mean ± SE) 28.0 ± 0.8°C and 77.0 ± 6.2% RH, respectively, in the high-irradiance treatment and 28.5 ± 0.8°C and 77.3 ± 6.8% RH for the low-irradiance treatment, and 29.1 ± 0.8°C and 71.6 ± 7.2% RH immediately outside the shadehouses. We randomized plants across benches initially and re-randomized plant locations across benches monthly to avoid any potential block effects within the treatments. Soil was kept moist by daily rainfall and additional watering on days without precipitation. Plants were acclimated for 3 months in the high- and low-irradiance treatments and had produced new leaves before measurements.

Table 1 List of genera and species (family in parentheses), and their habitat type, spanning understory to small clearings for shade habitat low-irradiance species, and up to large clearings for open habitat high-irradiance species

Species	Native habitat	Habitat openness (1–4 ordinal scale)
Shade species		
Danaea wendlandii (Marattiaceae)†	Understory	1
Saccoloma moranii (Saccolomataceae)†	Understory	1
Diplazium striatastrum (Woodsiaceae)†	Understory	2
Cyclopeltis semicordata (Dryopteridaceae)	Understory–small clearings	2
Blechnum occidentale (Blechnaceae)	Understory–small clearings	2
Adiantum latifolium (Pteridaceae)†	Understory–small clearings	2
Higher irradiance species		
Thelypteris nicaraguensis (Thelypteridaceae)†	Understory–large clearings	3
Tectaria lizarzaburui (Tectariaceae)†	Small–large clearings	3
Campyloneurum brevifolium (Polypodiaceae)	Small–large clearings	3
Nephrolepis biserrata (Nephrolepidaceae)†	Large clearings	4
Hemionitis palmata (Pteridaceae)	Large clearings	4
Pityrogramma calomelanos (Pteridaceae)	Large clearings	4
Ophioglossum nudicaule (Ophioglossaceae)	Large clearings	4

Habitat openness was quantified using an ordinal scale of 1–4, with 1 representing the most shaded habitats, and 4 representing the most open. The ordinal scale was calibrated against average measurements of photosynthetically active radiation for the 30 most abundant fern species at La Selva Biological Station, which included seven of the species selected for the common garden study (denoted by †; $R = 0.86$; $P < 0.01$); fern abundance census data were derived from M. Jones (pers. comm.) and Jones *et al.* (2008).

Measurement of gas exchange responses

From June to November 2011, we measured light response curves, A–c_i curves and VPD responses for stomatal conductance, photosynthetic rate per unit leaf area, transpiration rate per unit leaf area, and intercellular CO_2 concentration (g_s, A, E and c_i, respectively) with a Li-Cor 6400 photosynthesis system.

Our approach was designed to allow comparisons of responses among study species, and with species studied previously in the literature, while also providing sufficient acclimation time for robust responses to environmental factors. The majority of previous studies on stomatal responses have tended to report scantily on methodological details (e.g. equilibration times before response curves, whether curves were determined with ascending or descending steps of intensity, the number of steps, and time at each step (but see Cousins *et al.*, 2007; Tazoe *et al.*, 2011). To facilitate comparisons with future studies, we have included detailed information for our light, CO_2, and VPD response methods below and in Supporting Information Methods S1.

This study focused on relatively short-term stomatal responses to reduce the likelihood that g_s responses to irradiance, CO_2 and VPD would be confounded by changes in other intrinsic conditions. Stomata may continue to respond to a shift in environmental stimulus for several minutes, or for some species for > 60 min, and the response may show diurnal and circadian oscillations (Meidner & Mansfield, 1968; Franks *et al.*, 1997; Franks & Farquhar, 1999; Brinker *et al.*, 2001; Hubbard *et al.*, 2007). We focused our analyses on the overall responses of g_s to a wide range of each stimulus (0–1500 μmol m^{-2} s^{-1} for photosynthetic photon flux density (PPFD), 0–40 Pa for CO_2 and 1–2 kPa for VPD), and present the maximum g_s responses, measured over *c.* 1–3.5 h as needed to ensure stability of g_s (85–100 min, 65–75 min, and 50–210 min, respectively), a time span at least as long as those typically used in previous studies of ferns and angiosperms (c.f. Franks & Farquhar, 1999; Brodribb & Jordan, 2008; Brodribb *et al.*, 2009; Brodribb & McAdam, 2011; Soni *et al.*, 2012). To ease the plants through the large total shift in levels of irradiance and CO_2, after acclimation and stabilization of g_s to initial conditions, we adjusted the stimulus intensity gradually through step-changes (Evans & Santiago, 2012) before allowing stabilization at the end of the response curve for a comparison of minimum and maximum g_s. Typically, the response of g_s to irradiance and CO_2 saturated at steps previous to the highest (or lowest) stimulus level applied. We present plots of responses to the intermediate step-changes (see Figs S1, S2) to illustrate the procedure, and to highlight the gradual responses in g_s, but note that we quantified and analyzed only the *total* responsiveness of g_s across the entire stimulus range and acclimation period (1–3.5 h) within and across species.

Because an extended acclimation at low light may contribute to a longer g_s response time to an environmental stimulus (Gross & Chabot, 1979; Vines *et al.*, 1982, 1983; Stoop *et al.*, 1991; Tinoco-Ojanguren & Pearcy, 1993), we acclimated leaves at high irradiance for all response curves, thus maximizing stomatal opening before beginning measurement of responses to irradiance, CO_2 or VPD.

We measured light response curves of g_s and photosynthetic assimilation rate (A) for five to six leaves from four to six different individuals in each irradiance treatment for all 13 species (see Methods S1, 'Measurement of short-term g_s responses to irradiance' and Table S1 for analysis of variance test results of stomatal responses to irradiance across species, treatments, and habitats).

We measured responses of g_s and A to c_i and VPD for five to six leaves from four to six individuals in each irradiance treatment for 10 species, five from open habitat, and five from shade habitat (see Methods S1, 'Measurement of short-term g_s responses to CO_2' and 'Measurement of short-term g_s responses to vapor pressure deficit' and Table S1). All 10 of these species were measured in the low-irradiance growth treatment, and seven of 10 species were also measured for these responses in the high-irradiance treatment.

After each gas exchange response was measured, the area of leaf in the chamber was traced onto a transparent acetate sheet,

scanned and measured using IMAGE J (US National Institutes of Health, Bethesda, MD, USA) to normalize values for chamber leaf area.

Quantification of responsiveness of flux traits to changing irradiance, CO_2, and VPD

We determined the responsiveness of leaf stomatal conductance (g_s), photosynthetic rate (A) and intercellular CO_2 concentration (c_i) to each environmental factor in absolute and relative terms (Table 2). An absolute difference in responsiveness ($Resp_{abs}$) was calculated as the difference between maximum and minimum flux trait values (e.g. max − min g_s). As this may bias responsiveness toward leaves with higher flux rates, we also calculated a relative response ($Resp_{rel}$) by dividing the absolute difference by the maximum value and multiplying by 100% (e.g. (max − min g_s)/max g_s × 100%).

Notably, in the response curves, maximum and minimum g_s values did not always occur at the extreme levels of the environmental parameters. Thus, we determined absolute and relative responses in two ways. First, the maximum responsiveness (*MaxResp*) was determined using absolute minimum and maximum g_s across the range tested for the environmental variable (e.g. *MaxResp* = max g_s − min g_s). Secondly, the standardized responsiveness (*StdResp*) was determined using trait values extracted from the same start and end points of response curves, that is at minimum and maximum environmental stimulus intensity (e.g. *StdResp* = g_s at 1500 PAR – g_s at 0 PAR). For responses to irradiance and CO_2 concentration, the differences in flux traits at minimum and maximum environmental signal strength were taken, respectively, at 0 versus 1500 μmol m^{-2} s^{-1} PAR and at 0 versus 200 Pa. For VPD, because a lower VPD reflects higher air moisture, 1 kPa VPD was considered the maximum value, and 2 kPa VPD the minimum. Because g_s responses to VPD were measured using two VPD levels (Franks & Farquhar, 1999), standard and maximum responses of g_s to VPD were the same. Maximum and standardized responsiveness variables tended to be strongly positively correlated, and in the description of results we refer to both standardized and maximum responses unless otherwise noted.

Statistical analysis

To test for the overall significance of within-species stomatal responsiveness to changes in irradiance, CO_2, and VPD, we used paired *t*-tests to compare g_s at minimum and maximum values of the environmental factor across plants of each species in each growth irradiance treatment.

To test for significant differences in stomatal responsiveness across species, habitats and irradiance treatments, we used a three-level nested general linear model analysis of variance (MINITAB 16 Statistical Software; Minitab, Inc., State College, PA, USA). Before analyses of variance, to improve normality (especially because some species showed responses that were opposite in direction), we added to all values for a given response the lowest species mean plus a constant of 0.0001 to ensure that all species' values were positive, then applied log-transformation.

To analyze potential correlations across species of stomatal responses to irradiance, CO_2, and VPD, and to test *a priori* hypotheses of mechanistic associations between g_s and other gas exchange variables, we tested Pearson and Spearman correlations on species means (R Development Core Team; R Foundation for Statistical Computing, Vienna, Austria). We considered trait relationships to be significant if $P \leq 0.05$ for at least two of the three

Table 2 Variable categories, symbols, units and definitions

Category	Variables	Units	Definition
Environmental stimulus	Irradiance	μmol m^{-2} s^{-1}	Light response curve irradiance from 0 to 1500 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)
	CO_2	Pa	A–c_i curve CO_2 concentration from 0 to 200 Pa
	VPD	kPa	Stepped VPD response from 1 to 2 kPa
Flux traits	g_s	mol m^{-2} s^{-1}	Stomatal conductance
	A	μmol m^{-2} s^{-1}	Photosynthetic rate
	c_i	Pa	Intercellular CO_2 concentration
	R_d	μmol m^{-2} s^{-1}	Dark respiration rate
Standard flux trait responsiveness	$StdResp_{abs}$		Standard absolute response: difference between standardized minimum and maximum flux trait values taken from respective start and end of response curves, that is, minimum and maximum stimulus intensity, for example, g_s at 1500 PAR – g_s at 0 PAR
	$StdResp_{rel}$	%	Standard relative response: difference between minimum and maximum flux trait values normalized by the maximum value to avoid potential bias toward species with high flux rates, for example, g_s at 1500 PAR – g_s at 0 PAR/g_s at 1500 PAR
Maximum flux trait responsiveness	$MaxResp_{abs}$		Maximum absolute response: difference between true maximum and minimum flux trait values from across the response curve, for example, max g_s − min g_s
	$MaxResp_{rel}$	%	Maximum relative response: difference between true maximum minimum and maximum flux trait values normalized by the maximum value to avoid potential bias toward species with high flux rates, for example, max g_s − min g_s/max g_s

Short-term responsiveness traits were calculated for changes in stomatal conductance (g_s), photosynthesis (A), and intercellular CO_2 concentration (c_i) under respective increases in photosynthetically active radiation (PAR; μmol m^{-2} s^{-1}), CO_2 concentration (Pa), and vapor pressure deficit (VPD; kPa). Because VPD response phases are defined by changes in g_s, standard and maximum g_s VPD responses are the same.

correlation tests (Pearson correlation on untransformed data, Pearson correlation on log-transformed data, and Spearman correlation on ranked data; see later, Table S5). We focused on testing only previously hypothesized relationships, and thus did not use correction for multiple correlation tests, as that would have reduced the power to test *a priori* hypotheses. Although for interest we present a correlation matrix of all variables to show the structure of interrelationships among measurements, we recommend statistical correction before 'mining' of unhypothesized correlations given the risk of inflated type I error (Garcia, 2003; Moran, 2003; Givnish *et al.*, 2004; Edwards, 2006; Waite & Sack, 2009).

Results

Variation in fern stomatal sensitivity to different environmental factors

A novel finding of this study was a substantial stomatal opening in fern species under very low irradiance, high CO_2 and high VPD, rather than the strong closure expected from previous studies of angiosperms. For the majority of species tested, stomata remained substantially open at 0 μmol m^{-2} s^{-1} PAR, 200 Pa of CO_2 and 2 kPa of VPD, respectively (Figs 1–3). The stomatal opening observed at low irradiance and high CO_2 was found despite adequate equilibration times at these levels (see Methods S1).

Despite this lack of a total closure response, we found significant short-term responsiveness of fern stomata to irradiance, CO_2, and VPD (Figs 1–3) based on the comparison of standardized minimum and maximum stomatal conductance (g_s) values. Twelve of 13 species showed significant decline in g_s with lower irradiance (by 15–48% in maximum relative responsiveness), nine of 10 measured species showed a significant decline in g_s in response to a 1-kPa increase in VPD (by 7–39%), and five of 10 measured species showed a significant change in g_s over the 200-Pa increase in CO_2 (by 3–16%), with stomata opening in two species and stomata closing in three species ($P \leq 0.05$; Fig. 2, Table S1). Thus, in general, when flux traits responded to light and VPD for a given species, they did so in the expected directions (Figs 1–3, Tables S2, S3). However, for the short-term response of g_s to CO_2, species differed in direction. For three of the five species that responded significantly to CO_2, g_s declined as expected at higher CO_2. However, *Ophioglossum nudicaule* and *Pityrogramma calomelanos* significantly increased in g_s at high CO_2 (Fig. 2).

As found in previous studies of stomatal responses, there was very strong variance within species (i.e. differences among individuals of given species in given treatments; the unexplained 'error' term in the ANOVA). This intraspecific variance was generally larger than the variance explained by species, habitat, and growth irradiance treatment (see Tables S2–S4 for ANOVA results).

Fern stomatal responses vary with native habitat and growth irradiance

The magnitude of short-term g_s responses varied across species with native irradiance habitat and within given species according to the growth irradiance. Species from open habitats tended to show stronger g_s responses to irradiance and CO_2, while shade habitat species tended to show stronger g_s responses to VPD (Figs 1–4, see Tables S2–S4 for ANOVA results). Open habitat species showed greater standard absolute responses of g_s to irradiance, greater maximum absolute and relative responses of g_s to CO_2, and weaker standard and maximum relative responses to VPD compared with shade habitat species (Fig. 4, Table S2).

Plastic differences in the responsiveness of g_s to growth irradiance were simpler, in that plants of given species grown under the high-irradiance growth treatment exhibited stronger g_s responses to all three environmental stimuli than those grown under low irradiance. High-irradiance-grown plants showed significantly greater absolute and relative responses of g_s to irradiance and VPD, and a greater standard absolute response of g_s to CO_2 (Figs 1–4; see also Figs S1, S2, and Tables S2–S4 for ANOVA results).

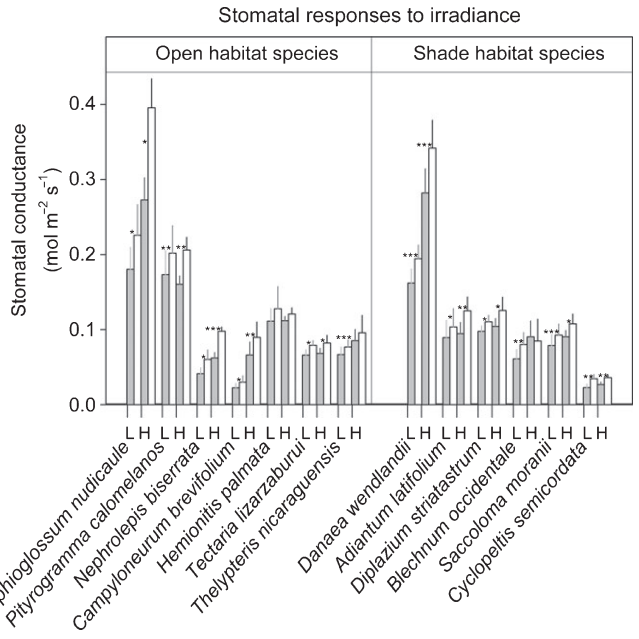

Fig. 1 Mean species stomatal conductance (g_s) at low versus high photosynthetically active radiation (0 versus 1500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD); gray and white bars, respectively) for plants grown under the low-irradiance treatment (L) versus the high-irradiance treatment (H); open habitat species and shade habitat species are ordered from left to right by greatest to weakest maximum responsiveness of g_s, that is, by the greatest difference between minimum and maximum g_s values in response to an increase in irradiance. Greater short-term responsiveness was observed for open habitat species, for species with higher flux rates, and for plants grown under the high-irradiance growth treatment. For example, leaves of *Ophioglossum nudicaule* showed the fastest flux rates as well as greater g_s responsiveness to irradiance than other open habitat species and shade habitat species, and exhibited greater g_s responsiveness for its high-irradiance grown plants compared with the low-irradiance grown plants. Significant differences between minimum and maximum g_s values were determined by paired *t*-test: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; error bars, + SE. For relative responses of g_s to irradiance, see Supporting Information Table S6.

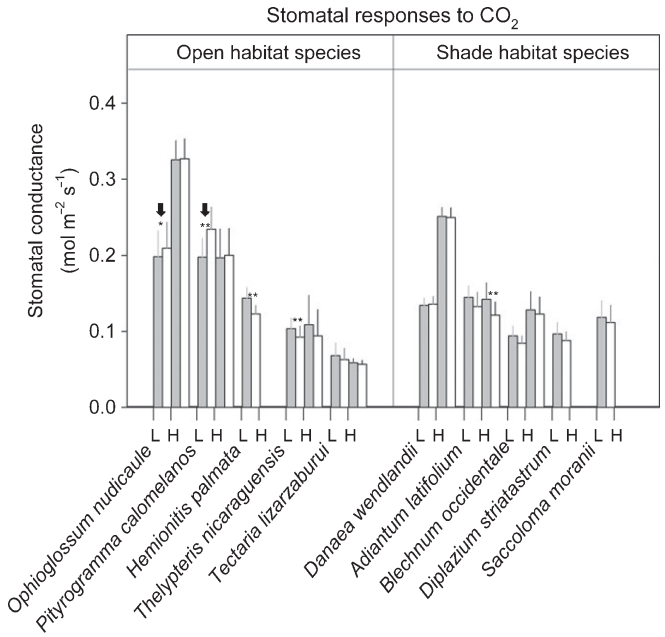

Fig. 2 Mean species stomatal conductance (g_s) at low versus high CO_2 concentration (0 versus 200 Pa; gray and white bars, respectively) for plants grown under the low-irradiance (L) versus the high-irradiance treatment (H); open habitat species and shade habitat species are ordered from left to right by greatest to weakest maximum responsiveness of g_s, that is, by the greatest difference between minimum and maximum g_s values in response to an increase in CO_2. Notably, *Adiantum latifolium*, *Hemionitis palmata*, and *Thelypteris nicaraguensis* significantly reduced g_s as expected to conserve water under high CO_2, while *Ophioglossum nudicaule* and *Pityrogramma calomelanos* showed the opposite pattern of significantly increased g_s with CO_2 (denoted by arrows). Significant differences between minimum and maximum g_s values were determined by paired *t*-test: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; error bars, + SE. For relative responses of g_s to CO_2, see Table S6.

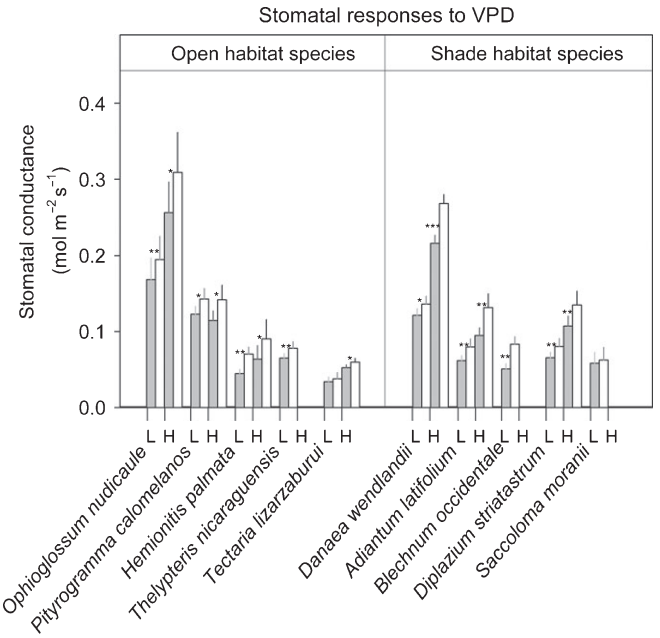

Fig. 3 Mean species differences in stomatal conductance (g_s) measured under dry air versus moist air (2 kPa vapor pressure deficit (VPD) versus 1 kPa VPD; gray and white bars, respectively) for plants grown under the low-irradiance (L) versus the high-irradiance treatment (H); open habitat species and shade habitat species are ordered from left to right by greatest to weakest maximum responsiveness of g_s, that is, by the greatest difference between minimum and maximum g_s values from 1 to 2 kPa VPD. Greater short-term responsiveness was observed for open habitat species, for species with higher flux rates, and for plants grown under the high-irradiance growth treatment. Significant differences between minimum and maximum g_s values were determined by paired *t*-test: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; error bars, + SE. For relative responses of g_s to VPD, see Table S6.

Coordination of fern stomatal responses to irradiance, CO_2 and VPD

Across fern species, the g_s responses to environmental variables were partially inter-correlated. The rankings of species in their maximum stomatal responses to irradiance and VPD were similar, with a tendency toward greater responsiveness in species with higher flux rates (Figs 1–3, 5a–c). The absolute maximum responses of g_s to irradiance and VPD were positively correlated (Fig. 5c), although the species' relative percentage responses of g_s to irradiance and VPD were uncorrelated. By contrast, responses to CO_2 were independent of those to irradiance or VPD: there were no significant correlations between absolute and relative responses of g_s to irradiance or VPD with those responses to CO_2 (see correlation matrix in Table S5).

Additionally, across species, g_s responses were positively correlated with maximum g_s and photosynthetic rates. Averaged across growth irradiances, species with the highest light-saturated g_s showed the greatest absolute magnitude responses of g_s to irradiance and VPD (Fig. 5a,b; Table S5). We considered carefully and rejected the possibility that this correlation would have been driven automatically if, for example, our measurement of light-saturated g_s did not allow enough response time; in that case, species with greater responsiveness would also seem to have highest light-saturated values. The light-saturated g_s values from the light response curve were attained after complete stabilization at irradiances $\geq 1000\ \mu mol\ m^{-2}\ s^{-1}$ PAR, achieved after 15–30 min, and they did not differ significantly from those measured after even longer equilibration for the VPD response, that is, at 1 kPa VPD and high irradiance for 50 min on average across species (paired *t*-test; $P = 0.33$). The two measures of light-saturated g_s were also tightly correlated ($r = 0.94$; $P < 0.001$). We concluded, as hypothesized, that the correlation of maximum g_s responsiveness to light and to the step-change in VPD reflected a greater true responsiveness in the species with higher maximum flux rates. By contrast with responsiveness of g_s to irradiance and VPD, the responsiveness of g_s to CO_2 was not significantly correlated with light-saturated g_s (Table S5).

Coordination of stomatal responsiveness with other flux traits

Across species, the relative responses of g_s and A to VPD were positively correlated ($r = 0.88$; $P < 0.001$; Table 3; see correlation matrix in Table S5), and the absolute and relative responsivenesses of g_s to irradiance and VPD were significantly correlated with concomitant changes in c_i ($r > 0.65$; $P \leq 0.05$; Table 3). However, absolute and relative changes in the magnitude of g_s in response to irradiance and CO_2 were not significantly correlated with the responsiveness variables for A, or with R_d (Table 3).

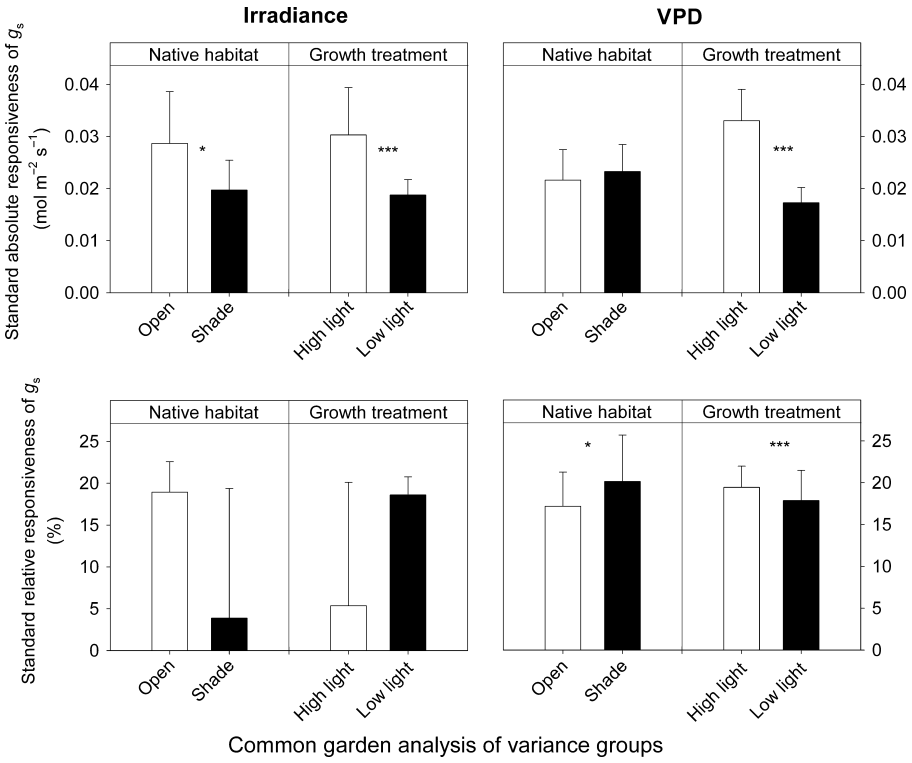

Fig. 4 Standard responses of stomatal conductance (g_s) to an increase in irradiance from 0 to 1500 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (left), and to an increase in vapor pressure deficit (VPD) from 1 to 2 kPa (right), for leaves grouped by species native habitat (open versus shade), and by irradiance growth treatment (high 20% PAR versus low 2% PAR); bars are mean values + SE. Absolute responses (top) represent the difference between maximum and minimum values of g_s determined from the respective ends of the response curve; relative responses were calculated as the absolute response divided by maximum g_s and multiplied by 100% (see Table 2). Species adapted in or grown under high irradiance generally showed greater responsiveness, except for a greater VPD responsiveness in shade habitat fern species. Significant differences established by nested analyses of variance (see the Materials and Methods section): ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

Discussion

We found significant fern stomatal responses to irradiance, CO_2 and VPD, which reveal greater variability in sensitivity than ever previously reported. These fern species showed a typically lower but wider range of responses than previously reported for angiosperms (e.g. Aasamaa & Sõber, 2011b; Buckley *et al.*, 2011; Brodribb & McAdam, 2013; see below), and high variation in stomatal responses within species, as has been found for angiosperms (Buckley *et al.*, 2011), indicating the importance of plant- and leaf-specific processes in modulating stomatal behavior. The correlation between stomatal responses to light and VPD is a major novel finding and suggests coordination of fern response pathways. The striking variation in sensitivity of fern stomata to different environmental stimuli and in the magnitude of their responses within and across species and habitats also demonstrates a capacity for diverse optimization of hydraulic and metabolic needs at growth and evolutionary timescales. These results provide a new framework for understanding the role of stomatal responsiveness in fern habitat differentiation within the forest community. Different directionality of CO_2 responses across fern species highlights the need for further investigation into their stomatal control mechanisms.

Fern species can respond strongly to irradiance and VPD as for angiosperms, but show weaker and more variable responses to CO_2

As predicted, the fern species typically increased g_s under higher irradiance and lower VPD – which would contribute to a greater photosynthetic rate – and decreased g_s under low irradiance and high VPD, as would be optimal to conserve water. However, not all species showed the expected closure response under increasing CO_2 to reduce water loss. The sensitivity to stimuli varied strongly across species, with more species showing significant responses of g_s to irradiance and VPD than to CO_2 (12 of 13 and nine of 10 versus five of 10 tested species, respectively).

We found that the response of g_s to irradiance for ferns (15–48% increase of g_s with irradiance) was generally strong and substantial, as has been shown for angiosperms, and that the fern species in this study exhibited g_s responsivenesses to VPD similar in magnitude to those of nonwoody angiosperms (Buckley *et al.*, 2011). This suggests that proposed hydropassive stomatal control given ABA insensitivity (Brodribb & McAdam, 2011; McAdam & Brodribb, 2013) is not necessarily a hindrance to ferns. In angiosperms, stomatal responses include active osmotic changes in guard cell potassium (Losch & Schenk, 1978) and changes in hydration suggesting a vapor signal (Shope *et al.*, 2008; Sibbernsen & Mott, 2010). Detailed studies on cellular-level changes in solute concentrations and turgor during fern VPD responses are needed to further explore the strong stomatal control of dehydrating leaves across a wide range of fern species.

Our findings for CO_2 sensitivity in ferns also extended those of previous studies, which found low responsiveness relative to angiosperms. Previous studies showed that g_s declined under higher CO_2 by 1–11% for one to five fern species (Brodribb *et al.*, 2009; Franks *et al.*, 2012). Brodribb & McAdam (2013) reported that stomata of 10 fern species did not open in response to low CO_2 in the dark, and did not close at higher than ambient CO_2 concentration in the light. They proposed that an absence of the Ca^{2+}-dependent signaling pathway mediating stomatal closure in response to elevated CO_2 may help to explain the reduced CO_2 sensitivity in ferns compared with responses to irradiance

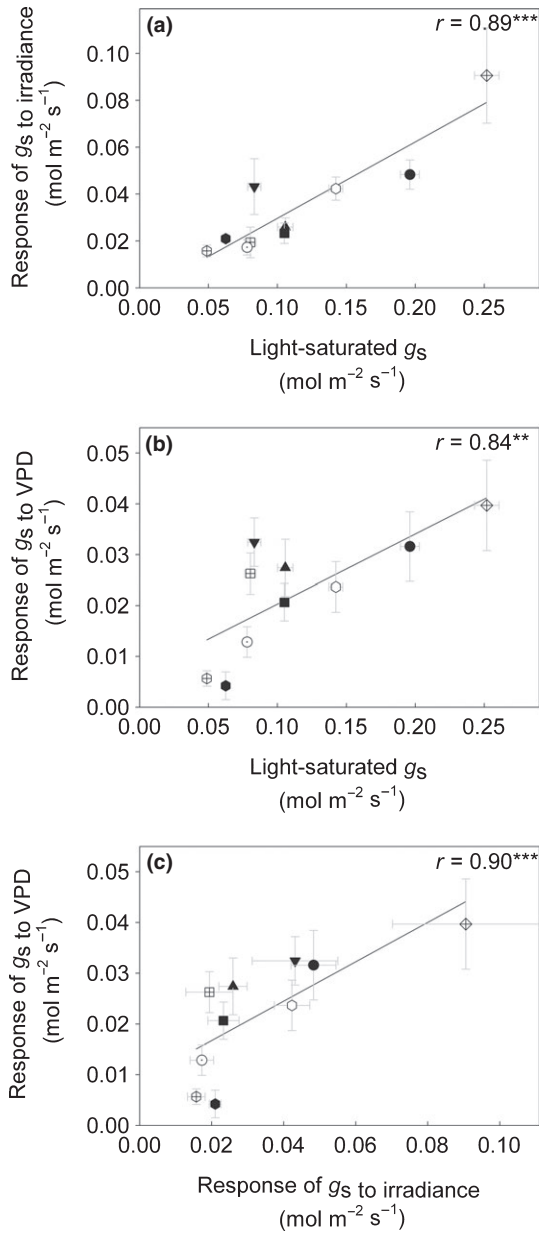

Fig. 5 Correlations of maximum responsiveness of stomatal conductance (g_s) to irradiance and vapor pressure deficit (VPD) with light-saturated stomatal conductance (g_s) for different species (open symbols, open habitat species; closed symbols, shade habitat species) with means calculated across light treatments: (a) the maximum absolute response of g_s to irradiance and (b) the maximum absolute response of g_s to VPD were correlated across species with light-saturated g_s at 1 kPa VPD (measured after an average 50-min acclimation period); and (c) greater maximum absolute responses of g_s to irradiance and VPD were positively correlated: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; error bars, + SE.

and VPD. Our study also extends in novel ways the range and complexity of the stomatal CO_2 response observed in ferns: we found responses ranging up to 16% stomatal closure for *Thelypteris nicaraguensis*, and a directionality depending on species. Two species showed an unexpected stomatal opening in response to high CO_2, highlighting extraordinary diversity of stomatal behavior in the ferns. The signaling pathway for the opening response reported here requires further investigation, and possible processes involved might include increasing photosynthesis in the guard cells and/or mesophyll, or c_a or c_i (von Caemmerer *et al.*, 2004; Lawson *et al.*, 2011). Notably, the CO_2 responses of ferns may be even stronger than those observed in our study. Even species with a significant CO_2 response did not show saturation of this CO_2 response at the highest c_a (200 Pa) provided by the Li-Cor 6400 using the CO_2 injector (Fig. S2). Because the fern lineage originated in the Devonian under a CO_2 concentration that may have been four times higher than this level, that is, 10–20 times higher than ambient levels today (Berner, 1993; Royer, 2006), new approaches are needed to determine whether ferns would exhibit even greater magnitude depressions of g_s under substantially higher CO_2 concentrations. Fern species with an evolutionary history that includes periods with greater or longer duration exposure to high CO_2 may have a g_s response mechanism similar to that of angiosperms, but might respond to a wider range of CO_2 for decreasing g_s. The CO_2-induced opening of stomata in *Ophioglossum nudicaule* and *Pityrogramma calomelanos* might be explained by these species having been adapted in ancient environments with much higher concentrations of CO_2 (Smith *et al.*, 2006), thus requiring a higher CO_2 threshold to reduce stomatal apertures for water conservation. Alternatively, because these rainforest species adapted to competitive habitats with abundant water supply, all else being equal, strong selection for optimization of carbon fixation at the expense of water conservation may have overcome the stomatal closure response to high CO_2. Studies examining responses to simultaneous changes in CO_2, irradiance, and humidity are the next step toward clarifying separate optimization and coordination of stimulus detection and response pathways (e.g. Aasamaa & Sõber, 2011a; Merilo *et al.*, 2014).

The magnitude of stomatal responses varies significantly with irradiance habitat and growth environment

Our first main hypothesis was that ferns adapted and grown in high-irradiance environments would have greater responses to all stimuli given higher flux rates and to better cope with extreme conditions. This hypothesis was largely supported, although stomatal responses to VPD were a special case of plasticity and adaptation tending in different directions.

A major novel finding of our study was that ferns native to high-irradiance habitat showed greater short-term responses of g_s to irradiance and CO_2, but weaker responses to VPD than ferns native to shade. Adaptation to open habitats would have selected for a greater capacity to maximize photosynthesis, even under high VPD, to take advantage of high irradiance and CO_2. By contrast, adaptation to shade may render leaves more vulnerable to drying air, such that a stronger reduction in g_s under high VPD is required. Future work is needed to determine whether open habitat fern species rely on other leaf traits to buffer against high VPD, such as a high leaf hydraulic conductance relative to their g_s (Brodribb & Jordan, 2008), compared with shade species, which may rely more strongly on stomatal control to reduce the impact of atmospheric drought.

New Phytologist

Table 3 Correlations of stomatal conductance (g_s) responsiveness to increasing irradiance, CO_2, and vapor pressure deficit (VPD) with magnitude changes in photosynthesis (A), intercellular CO_2 concentration (c_i), and instantaneous dark respiration (R_d; see the Materials and Methods section)

	$StdResp_{abs}$		$StdResp_{rel}$		$MaxResp_{abs}$		$MaxResp_{rel}$		Instantaneous
Flux trait responsiveness	A	c_i	A	c_i	A	c_i	A	c_i	R_d
Irradiance									
g_s $StdResp_{abs}$	–	0.65*	–	0.60*	–	−0.56*	–	−0.56*	–
g_s $StdResp_{rel}$	–	–	−0.72**	–	–	–	–	–	–
g_s $MaxResp_{abs}$	–	–	–	–	–	–	–	–	–
g_s $MaxResp_{rel}$	–	−0.61*	–	−0.61*	–	0.80***	–	0.81***	–
CO_2									
g_s $StdResp_{abs}$	–	–	–	–	–	–	–	–	–
g_s $StdResp_{rel}$	–	–	–	–	–	–	–	–	–
g_s $MaxResp_{abs}$	–	–	–	–	–	–	–	–	–
g_s $MaxResp_{rel}$	–	–	–	–	–	–	–	–	–
VPD									
g_s $StdResp_{abs}$	–	0.67*	–	0.67*	–	0.76*	–	–	–
g_s $StdResp_{rel}$	0.94***	–	0.88***	–	0.93***	–	0.88***	0.67*	–
g_s $MaxResp_{abs}$	–	0.67*	–	0.67*	–	0.76*	–	–	–
g_s $MaxResp_{rel}$	0.94***	–	0.88***	–	0.93***	–	0.88***	0.67*	–

Responsiveness traits include standard absolute ($StdResp_{abs}$), standard relative ($StdResp_{rel}$), maximum absolute ($MaxResp_{abs}$), and maximum relative ($MaxResp_{rel}$) responses (see Table 2 for calculation of these traits).
***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; –, nonsignificant relationships.

Another major finding of our study was that high-irradiance-grown plants showed stomatal responses of greater magnitude to irradiance and VPD, and to a subtler degree, to CO_2. To our knowledge, our study is the first to show plasticity of stomatal responses to irradiance in ferns. High-irradiance-grown plants showed greater responses of g_s to CO_2, irradiance and VPD compared with low-irradiance-grown plants. The VPD responsiveness of g_s in ferns across irradiances is a novel case of adaptation and acclimation tending in opposite directions: high-irradiance-grown plants had stronger VPD responses than shade-grown plants, whereas shade habitat species had stronger VPD responses than high-irradiance habitat species. These contrary tendencies parallel those of leaf mass per area (LMA) across irradiances in angiosperms, where higher LMA is found in high-irradiance-grown plants of given species, but shade-adapted evergreen plants have higher LMA than high-light-adapted species (Walters & Reich, 1999; Lusk *et al.*, 2008). An explanation for the apparent contradiction in fern stomatal responsiveness is that a greater VPD response can help a plant to acclimate to higher irradiance by compensating for greater water loss, but over long-term adaptation to high irradiance, species can achieve greater tolerance of high VPD via other mechanisms that result in relative insensitivity by comparison with shade-adapted fern species. Thus we expect the specific compromise between long-term adaptation and short-term acclimation to atmospheric drought to strongly influence the performance of fern species across their habitat distributions.

Stomatal responses are variably correlated with leaf flux traits

We predicted correlations of flux trait responsiveness across species because A and c_i are influenced by g_s (Meinzer, 2002; Mott *et al.*, 2008; Brodribb *et al.*, 2009; Nobel, 2009; Sibbernsen & Mott, 2010), and predicted that species with faster light-saturated carbon assimilation (A) and dark respiration rates (R_d) under low VPD and ambient CO_2 might achieve greater magnitude stomatal responses as a result of their more rapid metabolism. However, there was no cross-species correlation of magnitude changes in g_s in response to the three environmental stimuli with maximum A or R_d, indicating that species with a slower metabolism are not limited in their capacity to adjust g_s to optimize gas exchange. As predicted, magnitude responses of c_i were significantly correlated with changes in g_s under increasing VPD and irradiance, suggesting that c_i may be an important signal for guard cell aperture modulation (Roelfsema *et al.*, 2002; Messinger *et al.*, 2006; Mott *et al.*, 2008). Under increasing irradiance, species that experienced a greater depression of c_i and higher A showed a greater compensatory increase in g_s to improve carbon supply rate, and thus a depression in c_i may have further stimulated stomatal opening at high irradiance. At this leaf-level scale of measurement, changes in c_i appear to concomitantly track changes in g_s. Therefore, the degree to which responses of g_s to irradiance and VPD may be driven by c_i merits further investigation at the tissue level.

Coordination of stomatal responses to different stimuli

Our second main hypothesis was that species' responsiveness to different factors might be correlated because of the simplicity of fern stomatal control (Brodribb & McAdam, 2011, 2013). We also expected that if stomatal responses for a given species were constrained only by anatomy and guard cell mechanics (Franks & Farquhar, 2007; Dow *et al.*, 2014), we might expect correlations of g_s responses to all stimuli because a species with highly responsive stomata ought to be highly responsive to all stimuli,

all else being equal. Across species, the absolute magnitude increases in g_s under high irradiance were correlated with the absolute magnitude decreases in g_s under high VPD. Thus, ferns with higher maximum flux rates are geared toward higher absolute responsiveness, with control of both carbon and water fluxes proportionately to their maximum transport capacity, and this scaling allows high-irradiance species in particular to benefit from strong responsiveness to optimize g_s. However, the responses of g_s to CO_2 were not significantly correlated with those to irradiance or VPD. The coordination of light and VPD responsivenesses and independence of CO_2 responsiveness could not arise simply from constraints of guard cell mechanics. Therefore, the independence of CO_2 stomatal responsiveness points to a different stimulus detection and response mechanism to manage stomatal apertures, which may depend on a signal other than cytosolic Ca^{2+} for CO_2 responses (Young *et al.*, 2006; Brodribb & McAdam, 2013), and suggests a capacity for separate optimization of stimulus signaling pathways. This raises new questions for future research into fern stomatal behavior, such as how partial coordination of stimulus response signaling pathways may be reconciled with existing models of fern stomatal control.

A major novel finding of this study is the coordination of light and VPD stomatal responses, which are thought to involve different signaling networks. Fern stomatal responses to VPD are thought to be directly and passively related to the water status of leaf cells (Brodribb & McAdam, 2011; McAdam & Brodribb, 2013), while fern irradiance responses are probably stimulated by red light absorption (Doi *et al.*, 2006) by guard cells and/or mesophyll cells in addition to water balance and/or c_i mediated changes in guard cell apertures that depend on ion pumping (Roelfsema *et al.*, 2002; Messinger *et al.*, 2006; Mott *et al.*, 2008). Mechanical constraints of leaf anatomy would not explain this coordination of light and VPD stomatal responsivenesses given the independence of CO_2 responsiveness. Three possible explanations include: light and VPD stimulus detection and response pathways are shared, such that a species with capacity for large responsiveness to one stimulus would also have large responsiveness to the other stimulus; light and VPD signaling pathways are entirely distinct and the correlation may arise from selection on these two different pathways for large responsiveness; or there is coordination at certain points in the light and VPD signaling pathways, such that a large responsiveness at these points of intersection would support a large responsiveness to the two different stimuli. Aasamaa & Sõber (2011a) posit that, for angiosperm trees, communication between different stomatal signaling pathways is weak. However, our results suggest that, while there is a capacity for separate optimization of signaling paths, there is also potential for 'crosstalk' between the photosynthetic and hydraulic signaling networks in ferns. This 'crosstalk' has been suggested for herbaceous monocots and dicots to explain species-specific responses to concomitant changes in two-factor combinations of light, CO_2, and VPD (Merilo *et al.*, 2014). The partial coordination of stomatal responses to different stimuli we report here is consistent with a scale-free network of stomatal signaling (Hetherington & Woodward, 2003), whereby highly connected nodes (signaling molecules) may coordinate and respond to multiple stimuli, and more sparsely connected nodes allow for distinct responses. Merilo *et al.* (2014) point to H^+-ATPases, anion and K^+ channels, and their regulatory kinases/phosphatases as potential intersections for 'crosstalk' (Jacob *et al.*, 1999; Marten *et al.*, 2007; Merilo *et al.*, 2013), though 'crosstalk' points in fern stomatal signaling may differ because ferns as a group appear to lack the phototropin-dependent blue light response and stomatal ABA sensitivity of angiosperms (Doi *et al.*, 2006; Brodribb & McAdam, 2011, 2013). Future work manipulating components of the signaling pathways is needed to identify the independent and interconnected transduction of signals to guard cells in response to different stimuli.

The capacity for diverse and finely tuned optimization of fern stomatal behavior at growth and evolutionary time-scales helps to explain the persistence of ferns in angiosperm-dominated communities (Watkins & Cardelús, 2012).

Conclusions

We emphasize three novel findings with implications for underlying mechanisms.

(1) We found a coordination of light and VPD stomatal responses – which are thought to involve different signaling networks – that cannot be explained by mechanical constraints of leaf anatomy, given the independence of CO_2 responses. We believe this coordination could be explained by one of the following three mechanisms. The light and VPD stimulus detection and response pathways may be shared, such that a species with capacity for large responsiveness to one stimulus would also have large responsiveness to the other stimulus. Alternatively, there may be coordination at certain points in the light and VPD signaling pathways, such that a large responsiveness at these points of intersection would support a large responsiveness to the two different stimuli. Such 'crosstalk' points have been suggested for angiosperms (Merilo *et al.*, 2014), and would require discovery in ferns. Finally, light and VPD signaling pathways might be entirely distinct and the correlation may arise from selection on these two different pathways for large responsiveness.

(2) We showed that plastic and adaptive fern stomatal responses to environmental factors can tend in opposite directions. Accurate models of plant responses to climate change will need to consider how stomatal responses of species that evolved in particular habitats may be altered by plastic changes as a result of growth conditions. Cases of stomatal responsiveness tending in opposite directions at growth and evolutionary time-scales can vary by environmental stimulus, and may apply to other plant groups beyond the ferns, warranting further research.

(3) We found that species differ in the directionality of stomatal responses to CO_2, suggesting the existence of a novel signaling pathway. Further research is needed to identify the distinct pathway allowing ferns to modulate stomata in response to CO_2, to determine whether it differs between species showing positive versus negative CO_2 responses, and to establish how this variation will impact on species responses to rising CO_2.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Supplementary methods for measurements of gas exchange responses.

Fig. S1 Responses of stomatal conductance (g_s) to an increase in irradiance (from 0 to 1500 μmol m^{-2} s^{-1} photosynthetically active radiation) for representative leaves of open and shade habitat species, from plants grown under high- and low-irradiance treatments.

Fig. S2 Responses of stomatal conductance (g_s) to changes in atmospheric CO_2 concentration (c_a, 0–200 Pa) and intercellular CO_2 concentration (c_i) for representative leaves of open habitat and shade habitat species, from plants grown under high-irradiance and low-irradiance treatments.

Table S1 Paired *t*-test results showing significant differences in stomatal conductance (g_s) at minimum and maximum environmental signal strength between species irradiance growth treatments in response to increasing irradiance (0–1500 μmol m^{-2} s^{-1} PAR), CO_2 concentration (0–200 Pa), and vapor pressure deficit (1–2 kPa)

Table S2 Nested analysis of variance results for stomatal responses to vapor pressure deficit for individual species and species grouped respectively by habitat and irradiance growth treatment

Table S3 Nested analysis of variance results for stomatal responses to irradiance for individual species and species grouped respectively by habitat and irradiance growth treatment

Table S4 Nested analysis of variance results for stomatal responses to CO_2 for individual species and species grouped respectively by habitat and irradiance growth treatment

Table S5 Matrix of correlation coefficients and significance results for responses of gas exchange variables to changes in irradiance, vapor pressure deficit, and CO_2

Table S6 Summary data for maximum relative responsiveness of stomatal conductance (g_s) to irradiance, vapor pressure deficit, and CO_2

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