Temporal heterogeneity of cold acclimation phenotypes in *Arabidopsis* leaves

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ABSTRACT

To predict the effects of temperature changes on plant growth and performance, it is crucial to understand the impact of thermal history on leaf morphology, anatomy and physiology. Here, we document a comprehensive range of leaf phenotypes in 25/20 °C-grown *Arabidopsis thaliana* plants that were shifted to 5 °C for up to 2 months. When warm-grown, pre-existing (PE) leaves were exposed to cold, leaf thickness increased due to an increase in mesophyll cell size. Leaves that were entirely cold-developed (CD) were twice as thick (eight cell layers) as their warm-developed (WD) counterparts (six layers), and also had higher epidermal and stomatal cell densities. After 4 d of cold, PE leaves accumulated high levels of total non-structural carbohydrates (TNC). However, glucose and starch levels declined thereafter, and after 45 d in the cold, PE leaves exhibited similar TNC to CD leaves. A similar phenomenon was observed in $\delta^{13}C$ and a range of photosynthetic parameters. In cold-treated PE leaves, an increase in respiration (R_{dark}) with cold exposure time was evident when measured at 25 °C but not 5 °C. Cold acclimation was associated with a large increase in the ratio of leaf R_{dark} to photosynthesis. The data highlight the importance of understanding developmental thermal history in determining individual phenotypic traits.

Key-words: *Arabidopsis*; anatomy; carbohydrates; phenotypic plasticity; photosynthesis; respiration; stomata; temperature.

INTRODUCTION

Plant tissues can be profoundly affected by a drop in temperature below their thermal optimum. If the cold stimulus is sustained, organ expansion and development may be severely restricted as essential biochemical, physical and physiological processes are inhibited. Subsequent compensatory mechanisms (collectively known as cold acclimation)

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and their resulting phenotypes have been intensively studied (e.g. Thomashow 2001; Stitt & Hurry 2002; Atkin *et al.* 2006a). Particular emphasis has been placed on contrasting the phenotypes exhibited by warm-developed (WD) leaves, with those of WD pre-existing (PE) leaves that shifted to the cold for fixed periods (often 7, 10 or 21 d), and cold-developed (CD) leaves that subsequently form (Strand *et al.* 1997; Armstrong, Logan & Atkin 2006a). CD leaves are often thicker (Boese & Huner 1990; Equiza & Tognetti 2002; Atkin *et al.* 2006a; Koeda *et al.* 2009), and they exhibit enhanced metabolic rates and altered sugar concentrations (Strand *et al.* 1997; Hurry *et al.* 2000; Armstrong *et al.* 2006b) compared with their WD and cold-treated PE counterparts. However, despite the growing understanding of the differences in leaf phenotypes exhibited by warm and cold-treated plants, we still lack a comprehensive understanding of the speed with which phenotypic changes occur in PE leaves, following shifting from warm to cold. Moreover, we lack a detailed account of the extent to which PE leaves are able to undergo further phenotypic changes following extended cold treatment (i.e. >21 d), or the anatomical changes underpinning cold-induced changes in leaf thickness in CD leaves.

Several studies have reported that leaf development in the cold is associated with an increase in palisade mesophyll thickness (Huner *et al.* 1981; Boese & Huner 1990); in spinach, increases in leaf thickness are associated with an increase in the number of component cell layers (Boese & Huner 1990). Whether changes in leaf thickness are consistently associated with other changes (e.g. increases in epidermal cell layer thickness, airspaces and/or vascular tissue) is, however, not known. The impact of low growth temperatures on cell densities in the epidermal layer also remains unclear, with little consensus among the few relevant studies (Miskin & Rasmussen 1970; Boese & Huner 1990; Beerling & Chaloner 1993; Equiza, Miravé & Tognetti 2001). Temperature-mediated changes in epidermal cell layers could potentially complicate interpretation of palaeoecological studies employing stomatal density (SD) and stomatal index (SI) in the reconstruction of past climate from fossil leaves, especially with reference to atmospheric CO_2 concentration (McElwain & Chaloner 1995; Royer 2001).

A number of studies have shown that sustained exposure of warm-grown, fully expanded PE leaves to cold (for

periods of several days up to 3 weeks) results in dynamic changes in the metabolome. For example, sugars quickly accumulate beyond levels found in their warm-maintained counterparts (Ristic & Ashworth 1993; Kaplan *et al.* 2004), with the responses of glucose, sucrose and fructose being particularly dramatic (Cook *et al.* 2004). Sugars reach an early peak after a few days of exposure, but remain elevated in comparison to warm-grown leaves (Koster & Lynch 1992; Ristic & Ashworth 1993; Strand *et al.* 1997) due to increases in the abundance and activity of synthetic enzymes (Guy, Huber & Huber 1992; Holaday *et al.* 1992; Sasaki *et al.* 2001). Carbohydrate accumulation partly explains the disproportionate increase in leaf dry mass (DM) to either area or fresh mass (FM) (Stuiver *et al.* 1995; Strand *et al.* 1999; Hurry *et al.* 2000; Armstrong *et al.* 2006a). Although it is less clear whether such changes are maintained in PE leaves following more extended cold treatment (e.g. >30 d), the pattern and extent of metabolite accumulation is known to undergo further changes in CD leaves (Gray & Heath 2005). In CD leaves, sugar levels are often intermediate between 7–21 d of cold-treated PE and warm control levels, and are consistent with a relative shift from starch to sucrose synthesis (Strand *et al.* 1997, 1999, 2003). There is thus considerable temporal heterogeneity in the concentration of key metabolites, following sustained exposure to cold.

Coupled with the low temperature metabolomic responses are dynamic changes in rates of net photosynthetic CO_2 assimilation (A) and respiratory CO_2 release in darkness (R_{dark}). Initial exposure of warm-grown, fully expanded PE leaves to cold often results in a rapid reduction in the rate of A (Berry & Björkman 1980; Strand *et al.* 1997) and R_{dark} (Blackman & Matthaei 1905; Matthaei 1905). However, both A and R_{dark} can subsequently cold acclimate (thus increasing the rate of metabolism in the cold) – often rapidly, e.g. in 1–3 d in PE leaves (Billings *et al.* 1971; Slatyer & Ferrar 1977; Atkin, Holly & Ball 2000; Bolstad, Reich & Lee 2003). Further, more substantial recovery of A and R_{dark} occurs following the formation of newly developed (ND) leaves in the cold (Strand *et al.* 1997, 2003; Savitch *et al.* 2001; Loveys *et al.* 2003; Armstrong *et al.* 2006a; Armstrong *et al.* 2008). Based on such findings, many studies now assume that the potential extent of cold acclimation of A and R_{dark} in PE leaves is lower than that in ND leaves that form in the cold. Such an assumption has important implications for how acclimation of metabolism might be accounted for in large scale, predictive vegetation-climate models. However, as noted above, few studies have investigated the impacts of cold treatments >21 d on PE leaves. One notable exception was a recent study using long-lived PE leaves of *Quercus ilex*, which exhibited a high degree of thermal acclimation of R_{dark} when cold treated for 60 d (Zaragoza-Castells *et al.* 2007). This raises the possibility that assumptions of limited cold acclimation potential of PE leaves might be incorrect, and that recovery of metabolism is possible given sufficient time.

The many structural and metabolic changes associated with cold acclimation are likely to affect the fractionation of stable carbon isotopes (e.g. ^{13}C). Discrimination against $^{13}CO_2$ occurs during transfer across stomata into the intercellular airspace, during movement from the airspace into the symplasm of mesophyll cells, and during fixation by Rubisco (Farquhar, Ehleringer & Hubick 1989). Past studies have reported that discrimination against ^{13}C is lower (i.e. $\delta^{13}C$ is higher) in tissues that develop at low temperature (Smith, Herath & Chase 1973; Smith, Oliver & McMillan 1976; Körner, Farquhar & Wong 1991; Yamori *et al.* 2006). Yamori *et al.* (2006) suggested that this is primarily a result of decreasing internal conductance (g_i) in CD leaves. However, it is not known whether discrimination against ^{13}C is also lower in cold-acclimating PE leaves, or whether the degree of discrimination varies through time as PE leaves experience increasing periods of cold.

Here, we document a comprehensive range of physiological, structural and chemical composition phenotypes exhibited by *Arabidopsis* leaves following shifting of warm-grown plants to the cold for extended periods. We address the following questions: (1) what underlying anatomical changes are responsible for cold-induced increases in leaf thickness; (2) can warm-grown PE leaves ultimately exhibit rates of carbon metabolism and carbohydrate profiles similar to those exhibited by CD leaves, when exposed to cold for periods >21 d; and, (3) what impact does increasing duration of cold treatment have on the $\delta^{13}C$ content of PE (and CD) *Arabidopsis* leaves? In addition to identifying the underlying anatomical changes responsible for cold-mediated changes in leaf thickness, our results demonstrate for the first time that many aspects of the CD leaf phenotype are very similar to that of PE leaves exposed to long-term cold; this finding has important implications for our understanding of the extent to which leaf metabolism of fully formed PE leaves can adjust to prolonged changes in growth temperature.

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* (Col-0) were sown in peat-based compost (Levingtons, F2) in a growth chamber (Snijders Microclima 1750, Snijders Scientific BV, Tilburg, Netherlands). An 8-h day/16-h night temperature regime of 25 °C/20 °C was imposed, with 60–70% relative humidity (RH) throughout and 150 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent tubes. When approximately 20 leaf insertions had emerged from the apical bud (visible at close inspection), plants were transferred to 17 litre hydroponics tanks containing fully-aerated modified Hoagland's nutrient solution (Poorter & Remkes 1990), maintained at pH 5.8. Plants were held on foam discs in pairs from this point. When 10 further insertions had emerged, experiments were initiated ('day zero' of the temperature treatments), and a subset of the warm-grown plants were shifted to an identical cabinet, except that the temperature was set to a constant 5 °C.

After a pre-determined cold treatment period (0–45 d), rates of CO_2 exchange (photosynthesis and respiration) and

a range of structural and chemical composition traits were quantified for PE leaves that had previously fully expanded in the warm cabinet. Other plants were used to examine the leaf traits of ND leaves that fully expanded in either sustained warm or cold temperatures. Separate plants were used for the CO_2 exchange and structural/chemical composition measurements (see below). Supporting Information Fig. S1 shows the physical appearance of warm-grown plants and shows plants after 6, 28 and 56 d in the cold. To ensure that ND leaves were harvested at a comparable developmental stage, we monitored leaf expansion (Supporting Information Fig. S2) and sampled leaves when the leaf area (a) of a reference leaf (the first leaf to visibly emerge from the apical bud after day zero, hereon called '$Leaf_{ref}$') was near maximal, i.e. its expansion rate was slowing. The a (in cm^2) was calculated from measurements of leaf width (w, cm) and length (l, cm) using:

$$a = (0.7475l * w) + 0.12 \qquad (1)$$

In preliminary experiments, this relationship between ($l*w$) and a (measured using an area meter, Li-3000, Li-Cor Inc., Lincoln, NE, USA) was found to be very strong ($R^2 = 0.97$, $n = 107$), linear across a wide range of a values, and independent of cold treatment and age in a range of insertions. Typically, a WD $Leaf_{ref}$ took 13–18 d to exhibit near-maximal a values and a CD $Leaf_{ref}$ around 60 d (Supporting Information Fig. S2). Plants were harvested in foam disc pairs 2–5 h after the commencement of the photoperiod. To enable comparison of leaf phenotypes of a common developmental stage, the same insertions (PE leaves) or insertion numbers relative to $Leaf_{ref}$ (for ND leaves) were used for each parameter measured.

Leaf gas exchange measurements

For PE leaves and ND leaves, CO_2 exchange measurements were carried out *in situ* using a Li-6400 Portable Photosynthesis System (Li-Cor Inc.) over 2 d. Readings on the first day were at the temperature immediately preceding harvest (i.e. 25 °C for warm-grown plants and 5 °C for cold-treated plants); on the following day, readings were taken at the other temperature. Initially, the leaf chamber was set to the intended temperature, incoming air with a 40% RH (higher humidity caused condensation in the system), 150 μmol photons m^{-2} s^{-1} and a CO_2 concentration of 400 μmol mol^{-1}, to give the assimilation rate at ambient conditions (A_{400}), during which the intercellular CO_2 concentration (C_i), stomatal conductance (g_s), the rate of transpiration and leaf vapour pressure deficit (VPD_L) were also measured. Instantaneous water-use efficiency (iWUE) was calculated from transpiration and A_{400} data. After steady-state assimilation was reached, the irradiance was increased to 500 μmol photons m^{-2} s^{-1} to enable measurements of the light-saturated rate (A_{sat}), and then the CO_2 level was increased to 1000 μmol mol^{-1} to give a CO_2-saturated/light-saturated rate (A_{max}). Finally, the plant was darkened for 30 min under a CO_2 concentration of 400 μmol mol^{-1} for dark respiration

(R_{dark}) readings. Data were corrected for CO_2 diffusion across the gasket (Bruhn, Mikkelsen & Atkin 2002). Leaves were then harvested and the FM and area of leaf parts contained in the photosynthesis system quantified with a LI-3000A leaf area meter (Li-Cor Inc.). The DM was recorded following freeze-drying under vacuum (Edwards Modulyo Freeze Drier, York, UK).

Leaf structural characteristics

The parameters described below were measured on separate plants to those used for gas exchange analysis. Leaf dry mass per unit area (LDMA, area and DM measured as above), fresh mass per unit leaf area (LFMA) and dry matter content (DMC, the ratio of DM to FM) were calculated. To investigate internal anatomy, proximal halves of individual leaves were fixed in 0.3 mg mL^{-1} paraformaldehyde, 5% ethanoic acid and 50% ethanol. Samples were dehydrated in ethanol, and infiltrated and embedded in L.R. white resin (London Resin Company, London, UK). One micrometre sections were microtome cut (Ultracut UCT, Leica Microsystems, Welzlar, Germany), stained with toluidine blue, and imaged with a microscope and imaging system (Optiphot 2 with DS-L1, Nikon, Tokyo, Japan). The cut surface was mid-way between midrib and margin, near the widest point of the leaf. Enhanced images were analysed with ImageJ (v. 1.38, NIH, Betheseda, MD, USA). Epidermal peels of the *distal* halves of the same leaves were made using the method of Kagan, Novoplansky & Sachs (1992). Epidermal cells and stomata were counted at 25× magnification, with (where possible) five counts per leaf from a 0.43 mm × 0.43 mm grid. Counts were made at least 0.9 mm from midrib, leaf edge or any other count, and 2.6 mm from the leaf tip. Veins were avoided. SI and stomatal ratio (SR) were calculated according to:

$$SI = 100 (SD/(ECD + SD)) \qquad (2)$$

$$SR = SD_{Ab}/SD_{Ad} \qquad (3)$$

where ECD stands for epidermal cell density, Ab for abaxial (lower) surface and Ad for adaxial surface.

Non-structural carbohydrates and stable carbon isotope analysis

The concentrations of sucrose, fructose, glucose and starch were analysed according to Loveys *et al.* (2003) and Campbell *et al.* (2007). Sugars were extracted from freeze-dried, ground leaves into ethanol supernatant, which was dried and then re-suspended in water. A sugar assay kit (FA-20, Sigma, St. Louis, MO, USA) was used, with absorbance at 340 nm associated with glucose, and enzymes catalysing the breakdown of fructose and sucrose with this sugar. Plates were measured using a reader (POLARstar OPTIMA, BMG Labtech, Offenburg, Germany). Small PE leaves were pooled from day 0 (into one sample), 4 d and 6 d (two each).

Total starch was analysed on the sugar analysis pellet using an assay kit (Megazyme International Ireland Ltd., Bray, Ireland). Samples were re-suspended in ethanol and 3-(N-morpholino) propane sulfonic acid (MOPS) with α-amylase before incubation at 100 °C. Sodium acetate buffer and amyloglucosidase were added, and each tube was incubated at 50 °C for 30 min. Samples were spun, and 10 μL supernatant of each was added to a 96-well plate, along with 30 μL water and 300 μL glucose oxidase/peroxidase. The plate was incubated at 55 °C for 20 min and read at 515 nm, with glucose standards alongside.

To determine carbon concentration and carbon isotope ratio (δ^{13}C), one freeze-dried leaf per plant was ground, with 2.5 mg subjected to continuous flow gas chromatograph isotope ratio mass spectrometry (Provac Services Ltd., Crewe, UK). Day 0, 4- and 6-d cold PE leaves were pooled into one sample for each treatment.

Statistical analysis

For PE leaves where analyses of covariance (ANCOVA) are described, data were from a series of experimental runs (treated as a factor) and the reported statistics refer to cold treatment duration as the covariate. Regressions were performed where all data pertain to one experimental run or if control means were comparable among runs. For ND leaves, two-way analyses of variance (ANOVA) were carried out with the experimental run as a factor, or t-tests if all data were from one run or if means were comparable. Further tests are described in the text. Proportions were arcsine transformed before analysis.

RESULTS

Leaf structure

Figure 1 shows the effect of extended cold treatment on leaf area and mass relationships of warm-grown, PE leaves that were fully expanded prior to the shift to 5 °C. LDMA, LFMA and DMC values (expressed as a proportion of the day 0 value) increased with cold treatment duration (Fig. 1a–c). Subsequently, CD leaves exhibited higher LDMA, LFMA and DMC values at maturity compared with WD controls (Table 1).

Cross sections of cold-exposed PE leaves revealed a positive relationship between leaf thickness and treatment duration (Fig. 2a). The adaxial (upper) epidermis contributed to this (Fig. 2a), but palisade and spongy mesophyll layer changes were predominant (Fig. 2a), with the percentage of leaf that was palisade layer also increasing ($F_{1,14} = 6.7$, $P < 0.05$; Fig. 3a). The proportion of total leaf cross section area that was airspace decreased (Fig. 3a). There were fewer mesophyll cells per unit cross section area with increasing treatment duration ($F_{1,14} = 16.4$, $P < 0.005$), underpinned by decreases in both palisade ($F_{1,14} = 10.1$, $P < 0.01$) and spongy ($F_{1,14} = 18.3$, $P < 0.001$) cell densities within their respective layers (data not shown); however, when the thickness of each layer was added as a covariate in
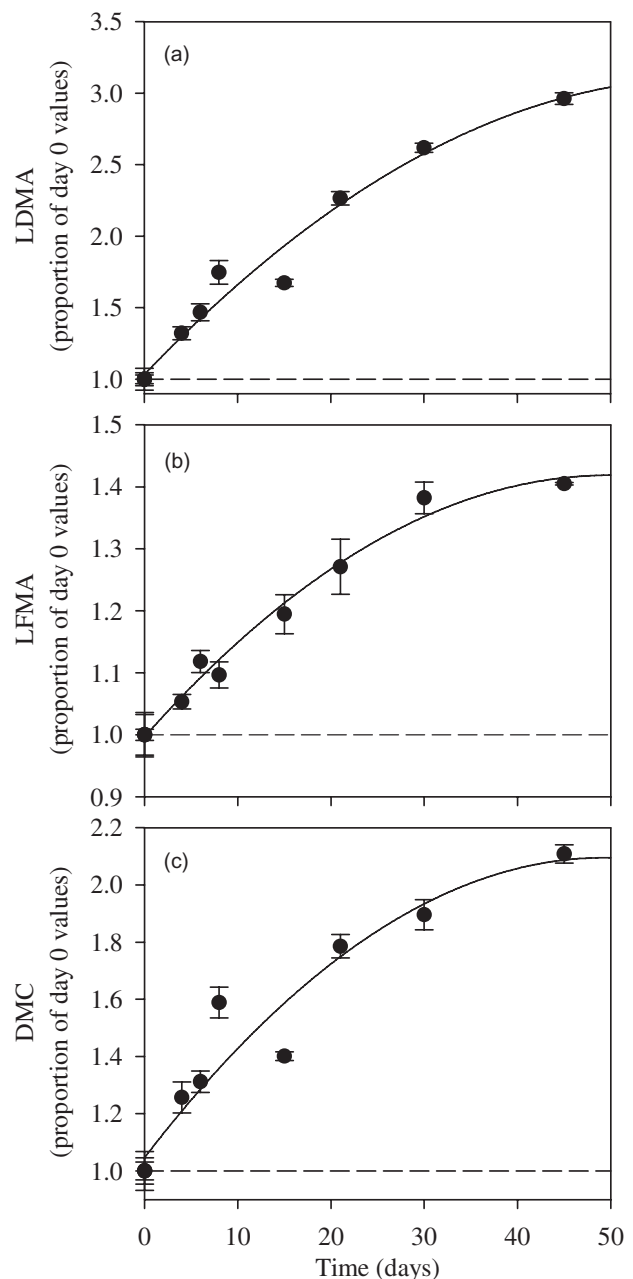

Figure 1. Impacts of cold exposure duration on area and mass relationships of cold-exposed, pre-existing (PE) leaves that had previously developed in the warm ($\pm$SE). Data for each time point are shown relative to respective day zero means for each experiment, with data from three independent experiments in total. FM, fresh mass; DM, dry mass; LDMA, leaf dry mass per unit area; LFMA, leaf fresh mass per unit area; DMC, dry matter content (ratio of dry to fresh mass). Mean ($\pm$SE) absolute values for 0 d plants: LDMA, 12.7 g_{DM} m^{-2} ($\pm$0.4); LFMA, 139 g_{FM} m^{-2} ($\pm$5); DMC, 0.091 ($\pm$0.002).

a multiple regression, only spongy mesophyll remained significantly related to cold exposure ($F_{1,13} = 6.0$, $P < 0.05$). The number of cell layers present within PE leaves was near six, regardless of the treatment (Fig. 3b).

We then compared cross sections of WD and CD leaves at maturity. CD leaves were much thicker than their WD

Table 1. Mean warm-developed (WD) and cold-developed (CD) values for leaf morphological, and chemical composition parameters ($\pm$SE. $n \geq 4$)

Leaf type	LDMA (g_{DM} m^{-2})	LFMA (g_{FM} m^{-2})	DMC (% FM)	C-conc (%)	$\delta^{13}C$ (‰)
	Leaf trait				
WD	15.5 ± 2.0	194.0 ± 0.2	8.0 ± 0.1	36.1 ± 0.3	-29.7 ± 0.2
CD	44.1 ± 3.0	297.0 ± 1.2	14.9 ± 0.4	38.5 ± 0.3	-28.4 ± 0.1
P	***	***	***	***	***

Significance values: ***$P < 0.001$.
See Tables 3 and 4 and text for statistical analyses.
DM, dry mass; FM, fresh mass; LDMA, leaf dry mass per unit area; LFMA, leaf fresh mass per unit area; DMC, dry matter content (ratio of dry to fresh mass; expressed on a % basis); C-conc, carbon concentration (% dry mass).

counterparts, although this thickening varied considerably between two independent experiments: 60% (not shown) and 96% (Fig. 2b). Thickness increases also occurred in the abaxial (but not adaxial), epidermis (Fig. 2b), the vascular tissue 'thickness' (as if spread in an even layer across the section), and both palisade and spongy mesophyll layers (Fig. 2b) – the palisade layer thickness nearly trebled, also increasing as a proportion of the total leaf thickness ($F_{1,13} = 28.4$, $P < 0.001$; Fig. 3c). The section proportion that was airspace responded in one experiment but was not significantly lower in CD leaves overall ($P = 0.082$). Cell densities of both mesophyll types were lower in CD than

WD leaves, but significance was annulled when the layer thickness was added to the model as a covariate (data not shown). Overall, WD leaves exhibited six cell layers, whereas there were more than eight cell layers in CD leaves (Fig. 3d; $F_{1,13} = 111$, $P < 0.001$).

Epidermal surface cell characteristics

ECD and SD decreased with cold treatment duration of PE leaves (ANCOVAs; data not shown), but adding leaf area to each model as a covariate rendered these relationships insignificant. SI increased marginally with exposure time on
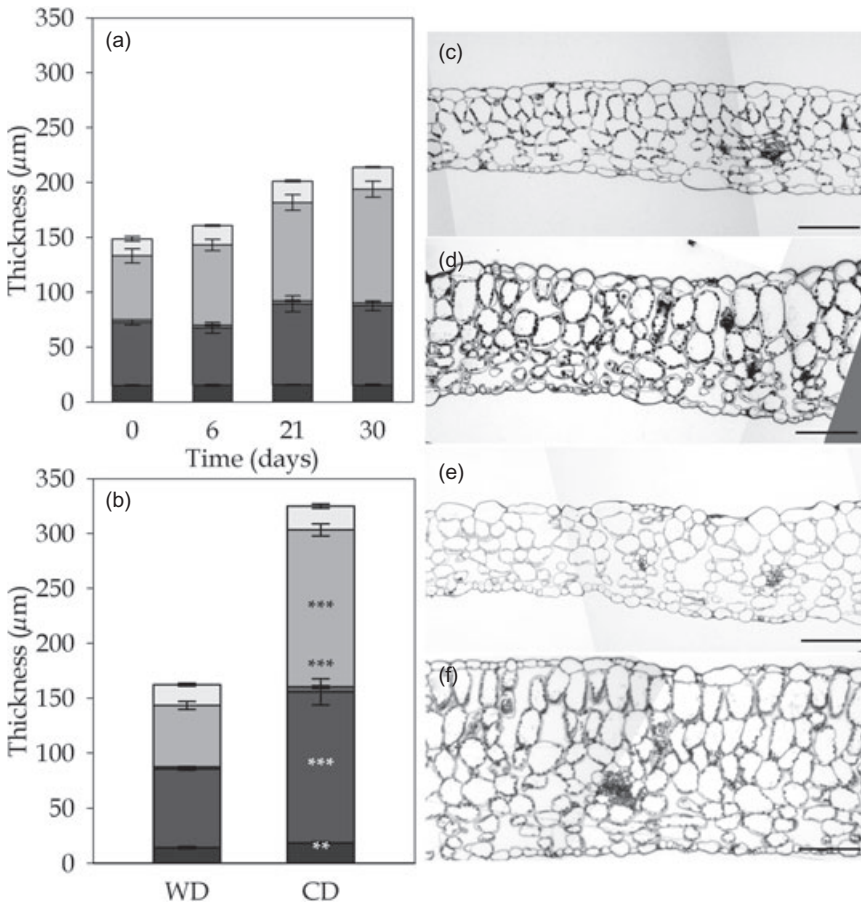

Figure 2. Thickness of each cell type layer (a–b) in leaf sections (c–f) of pre-existing (PE) and newly developed leaves in representative runs. (a) PE leaf measurements taken from leaf sections made following cold exposure times of between 0 d (c) to 30 d (d). (b) Measurements from sections of leaves that fully developed in the warm (e) or cold (f). Reading from the top to the bottom of a and b are representations of a leaf showing thicknesses of adaxial epidermis (light grey), palisade mesophyll layer (mid-grey), vascular tissue (grey), spongy mesophyll layer (dark grey) and abaxial epidermis (near-black). Mesophyll thickness measurements include intercellular airspaces. Asterisks denote significant two-way analysis of variance (ANOVA) developmental temperature treatment effects (placed above or inside the bar of the treatment with the higher mean): *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Bars in (c–f) represent 100 μm. Bars refer to standard errors. WD, warm-developed; CD, cold-developed.
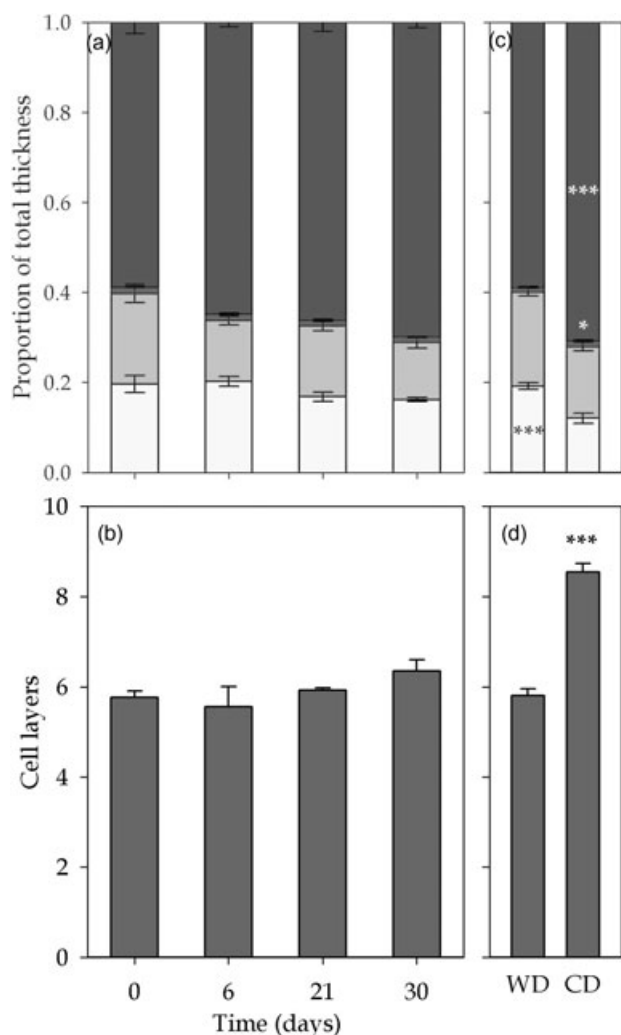
Figure 3. Cell components of leaf thickness. Graphs show the proportions of leaf thickness in (a) pre-existing (PE) and (c) newly developed leaves, and the number of cell layers within (b) PE and (d) newly developed leaves. In b and d, asterisks denote significant two-way analysis of variance (ANOVA) developmental temperature treatment effects (placed above or inside the bar of the treatment with the higher mean): *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Reading from the top to the bottom of a and c dark grey, mesophyll cell proportion; grey, vascular cell proportion; light-grey, airspace proportion; white, epidermal cell proportion; WD, warm-developed; CD, cold-developed. Bars refer to standard errors.

both surfaces [abaxial (lower) surface $F_{1,19} = 3.5$, $P = 0.077$; adaxial (upper) $F_{1,19} = 3.6$, $P = 0.072$], but SR was unaffected.

ECD was higher in CD than WD leaves on both surfaces (Table 2), even after the inclusion of leaf area as a covariate (abaxial $F_{1,12} = 13.3$, $P < 0.005$; adaxial $F_{1,12} = 22.1$, $P < 0.001$). SD responded similarly (Table 2; abaxial $F_{1,12} = 11.5$, $P < 0.01$; adaxial $F_{1,12} = 48.0$, $P < 0.001$). Cold development increased adaxial (but not abaxial, $P = 0.95$) SI, due to an effect in one of the two runs analysed (treatment/run interaction $P < 0.05$). In WD leaves, abaxial

stomata were more abundant than adaxial (SR > 1; Table 2), but they were at parity in CD leaves.

Sugar and starch concentration

Levels of all three measured sugars increased sharply and rapidly following cold exposure of PE leaves (Fig. 4a; day 0 values were pooled and therefore this could not be statistically analysed). Glucose levels declined steadily from 4 d of exposure (linear regression $F_{1,12} = 12.6$, $P < 0.005$), whereas fructose and sucrose, and the total of all three sugars, did not change. As a proportion of the total sugars, glucose decreased with increasing exposure time (regression $F_{1,13} = 15.6$, $P < 0.001$) in contrast to both fructose and sucrose. Starch levels (Fig. 4a) also decreased, with a linear regression significant on a DM basis ($F_{1,14} = 6.5$, $P < 0.05$), although the data suggest a peak at 6–21 d. Total non-structural carbohydrates (TNCs) responded non-linearly, due to the influence of starch and glucose. From 4 d, TNCs per unit DM decreased with time (regression $F_{1,12} = 5.7$, $P < 0.05$).

Cold development increased total sugar levels relative to warm controls (two-way ANOVAs; Fig. 4b), although to less than half of the cold-exposed, PE maximum. All three sugars responded (Fig. 4b) – especially fructose and sucrose – as a proportion of total sugars, glucose decreased ($F_{1,12} = 19.2$, $P < 0.001$), whereas fructose increased marginally ($F_{1,12} = 4.7$, $P = 0.052$) and sucrose significantly ($F_{1,12} = 11.4$, $P < 0.01$). Starch (Fig. 4b) and TNC did not differ between treatments. See Table 3 for statistics of all leaf structural and metabolic analyses.

Photosynthesis

Statistics for all gas exchange data are shown in Table 4. Photosynthesis rates per unit area and DM are shown in Fig. 5. When measured at 5 °C on an area basis, rates of A in PE leaves did not change with cold exposure time. In contrast, expressed per unit DM, 5 °C-measured rates of PE A_{400}, A_{sat} and A_{max} decreased with increasing cold exposure (Fig. 5g–i). In most cases, rates of A in PE leaves were higher at 25 °C than at 5 °C (ANCOVA measurement temperature factor effects, cold duration as a covariate); such differences were subtle in comparisons of A_{400}, but were more apparent in A_{sat} and A_{max} (Fig. 5c,i). Only A_{max} per unit DM exhibited a significant measurement temperature × cold duration interaction, with the temperature sensitivity of A_{max} decreasing with increasing cold exposure (Fig. 5i). At 25 °C, regression showed all measures of photosynthesis to decrease with increasing exposure apart from A_{max} per unit area (Fig. 5).

In CD and WD leaves, rates of A_{400} were comparable within both growth (i.e. 5 and 25 °C, respectively) and non-growth temperature mean pairs (Fig. 5d, see annotations for one-way ANOVA least significant difference (LSD) post hoc groupings). WD plants exhibited a relatively greater decrease in A_{sat} than in A_{400} when measured at 5 °C (Fig. 5d,e). In contrast, CD leaf 25 °C rates were increased

Table 2. Mean warm-developed (WD) and cold-developed (CD) values for leaf epidermal and stomatal parameters ($\pm$SE. $n \geq 4$)

| Leaf surface | Leaf type | Epidermal trait | | | | | |
		ECD (mm^{-2})	SD (mm^{-2})	SI (%)	ECs $\times 10^3$ (leaf^{-1})	Stomata $\times 10^3$ (leaf^{-1})	SR (Ab/Ad)
Ab	WD	363 ± 23	132 ± 8	26.8 ± 0.5	218 ± 19	80 ± 7	1.27 ± 0.06
	CD	657 ± 31	241 ± 16	26.8 ± 1.2	244 ± 10	89 ± 6	1.06 ± 0.04
	P	***	***	NS	NS	NS	***
Ad	WD	259 ± 18	108 ± 7	29.5 ± 0.3	156 ± 14	65 ± 6	
	CD	515 ± 21	229 ± 11	30.7 ± 0.7	193 ± 10	85 ± 5	
	P	***	***	*	NS	NS	

Significance values: *$P < 0.05$, ***$P < 0.001$.
See Tables 3 and 4 and text for statistical analyses.
Ab, abaxial (lower) epidermis; Ad, adaxial epidermis; ECD, epidermal cell density; SD, stomatal density; SI, stomatal index; ECs, epidermal cells; SR, stomatal ratio (abaxial/adaxial).

to 5 °C levels by the increase in light, thus abolishing temperature sensitivity. As in A_{400}, ND leaf A_{sat} was comparable between treatments when measured per unit area at the development temperature (Fig. 5e). A_{max} was always higher at 25 °C than 5 °C. On an area basis (Fig. 5f), 25 °C A_{max} did not differ between treatments, and WD A_{max} was significantly lower in the cold than in CD leaves at 5 °C ($P < 0.005$). On a DM basis, 5 °C rates were similar, whilst at 25 °C, warm control rates were nearly double those of CD leaves ($P < 0.001$; Fig. 5l).

Respiration and R_{dark} : A_{400} ratios

Respiration (R_{dark}) in PE leaves increased significantly with cold exposure duration when measured at 25 °C, but not 5 °C (Fig. 6a,c), and the ratio of R_{dark}-to-net photosynthesis (R_{dark} : A_{400}) responded likewise (Fig. 6e). At the warmer measuring temperature, respiration exceeded photosynthesis following 30 d in the cold.

On an area basis, LSD tests showed that CD leaves had higher rates of R_{dark} than WD controls when measured at 5 °C ($P < 0.01$) and 25 °C ($P < 0.001$). On a DM basis, this was only true at 25 °C ($P < 0.05$). Rates increased with mea-

surement temperature in both treatments (Fig. 6b,d). Measured at 5 °C, CD rates were comparable with WD controls measured at 25 °C, on an area basis (Fig. 6b), but on a DM basis (Fig. 6d) the measurement temperature effect predominated. The R_{dark} : A_{400} was higher at 25 °C than 5 °C in leaves that were allowed to fully develop at either temperature (Fig. 6f), although CD leaves responded more strongly than WD leaves. When CD leaves were measured at 5 °C and when WD leaves were measured at 25 °C, R_{dark} : A_{400} was comparable (Fig. 6f).

Other gas exchange parameters

None of the gas exchange parameters measured simultaneously to A_{400} varied with PE leaf cold exposure duration, at either measurement temperature (Supporting Information Fig. S3). Increasing the measurement temperature had an overall effect (ANCOVA) on all parameters apart from stomatal conductance (g_s). Internal CO_2 concentration (C_i) of CD leaves measured at 5 °C was lower than other ND leaf readings (Supporting Information Fig. S3f). Measurement temperature had a strong effect on transpiration in these leaves (Supporting Information Fig. S3h; two-way
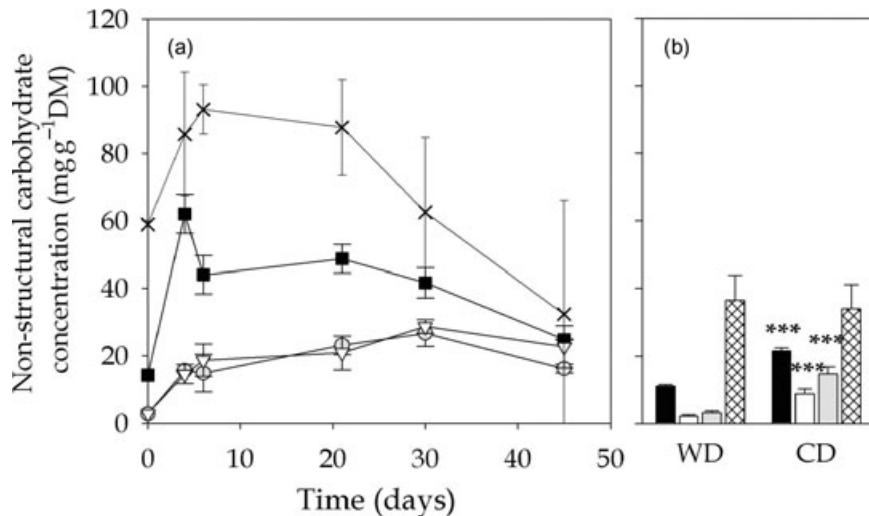

Figure 4. Sugar and starch content per unit dry mass (DM) of experimental leaves. (a) Pre-existing, cold-exposed leaves, (b) Newly warm-developed (WD) or cold-developed (CD) leaves. Filled squares/bars: glucose, open triangles/bars: fructose, grey circles/bars: sucrose, crosses/hatched bars: starch. *** (placed above the bar of the treatment with the higher mean for each sugar) denotes significant ($P < 0.001$) two-way analysis of variance (ANOVA) developmental temperature treatment effects. Black (squares), glucose; grey (circles), fructose; open (triangles), sucrose; crosses/cross-hatching, starch. Bars refer to standard errors.

Table 3. Cold treatment effect statistics for structural and metabolic analyses

Variable			PE leaves: cold time effect				WD versus CD comparison			
			F	P	↑/↓	Location	F/t	P	↑/↓	Location
Area, FM and DM relationships		LDMA	348	***	↑	Fig. 1	24	***	↑	Table 1
		LFMA	157	***	↑		31	***	↑	
		DMC	179	***	↑		16	***	↑	
Absolute thickness		Whole leaf	27	*	↑	Fig. 2	161	***	↑	Fig. 2
		Ad epidermis	7.4	*	↑		–	NS	–	
		Palisade mes.	31	***	↑		156	***	↑	
		Vascular tissue	–	NS	–		19.0	***	↑	
		Spongy mes.	9.4	**	↑		22	***	↑	
		Ab epidermis	–	NS	–		12	**	↑	
Proportion of total cross-sectional area		Mesophyll cell	16	***	↑	Fig. 3	37	***	↑	Fig. 2
		Airspace	5.0	*	↓		–	NS	–	
		Vascular	–	NS	–		8.3	*	↑	
		Epidermal cell	7.7	*	↓		46.0	***	↓	
Epidermal data	Ab	ECD	7.6	*	↓	–	39	***	↑	Table 2
		SD	–	NS	–		34	***	↑	
		SI	–	NS	–		–	NS	–	
	Ad	ECD	11	**	↓	–	56	***	↑	Table 2
		SD	7.8	*	↓		108	***	↑	
		SI	–	NS	–		4.9	*	↑	
		SR (Ab/Ad)	–	NS	–	–	13	**	↓	Table 2
Carbohydrates (DM basis)		Glucose				Fig. 3a	94	***	↑	Fig. 3b
		Fructose	See text for details				62	***	↑	
		Sucrose					38	***	↑	
		Sugars					66	***	↑	
		Starch					–	NS	–	
		Carbohydrates					–	NS	–	
Mass spectrometry		% Carbon	–	NS	–	–	41	***	↑	Table 1
		$\delta^{13}C$	15	**	↑	Fig. 7	40	***	↑	

Analysis of covariance (ANCOVA) (plant batch as a factor) results are shown for pre-existing (PE) leaf epidermal data, regressions for all other parameters. Warm-developed (WD) versus cold-developed (CD) leaf comparisons were *t*-tests for area and mass relationships, two-way analysis of variance (ANOVA) (using plant batch) for all others. Further tests are described in the text. Area, fresh matter (FM) and dry matter (DM) relationship data were log-log transformed for analysis. Arrows denote trends as cold exposure time increased, or in CD compared with WD leaves. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Degrees of freedom (d.f.) for PE/newly developed leaf tests (respectively) are as follows: area, FM and DM data d.f. = 1,36/12 (mean); thicknesses and cell layers d.f. = 1,14/1,13; epidermis d.f. = 1,19/1,13; mass spectrometry d.f. = 1,11/1,13. WD and CD leaf carbohydrate d.f. = 1,12. Ab, abaxial (lower) epidermis; Ad, adaxial epidermis; ECD, epidermal cell density; SD, stomatal density; SI, stomatal index; ECs, epidermal cells; SR, stomatal ratio (abaxial/adaxial); LDMA, leaf dry mass per unit area; LFMA, leaf fresh mass per unit area; mes., mesophyll.

ANOVA $F_{1,10} = 84.6$, $P < 0.001$), but treatment did not. The iWUE was highest at 5 °C in CD leaves (LSD $P < 0.01$; Supporting Information Fig. S3j). Here, a two-way ANOVA showed significance in both measurement temperature ($F_{1,10} = 14.7$, $P < 0.005$) and development/measurement temperature interaction term ($F_{1,10} = 6.0$, $P < 0.05$).

$\delta^{13}C$ content

As PE leaf cold exposure time increased, $\delta^{13}C$ increased (i.e. discrimination against ^{13}C decreased; Fig. 7), but their total carbon content (% C) was unaffected (data not shown). CD leaves exhibited a higher % C and $\delta^{13}C$ than their WD counterparts (Table 1).

DISCUSSION

Our study sought to quantify the impact of growth temperature and development on a wide range of leaf phenotypic traits crucial to growth in cold habitats. By integrating structural, physiological and chemical composition data, and simultaneously employing both time courses in pre-existing leaves and comparisons of CD and WD leaves, our study represents arguably the most comprehensive single investigation of leaf phenotypic acclimation to a controlled, low-temperature environment yet published. The traits studied ranged from the anatomy that provides the framework for light interception and carbon exchange, through to CO_2 fixation/release and resultant carbohydrate pools. Collectively, our results highlight the importance of thermal history in determining the extent of the acclimation phenotype.

Large increases in leaf thickness only occurred following the development of new leaves

Existing studies have demonstrated that the ratio of leaf area to leaf mass is temperature dependent (Loveys *et al.*

Table 4. Results of statistical tests on gas exchange characteristics of cold-exposed, pre-existing (PE) leaves

Variable		Meas. T (°C)	PE cold duration effect			PE ANCOVAS					Location
						Meas. T effect			Interaction		
			F	P	↑/↓	F	P	↑/↓	F	P	
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	A_{400}	5	–	NS	–	10.0	**	↑	–	NS	Fig. 5
		25	5.8	*	↓						
	A_{sat}	5	–	NS	–	22	***	↑	–	NS	
		25	4.8	*	↓						
	A_{max}	5	–	NS	–	63	***	↑	–	NS	
		25	–	NS	–						
Photosynthesis (μmol CO_2 g$_{DM}^{-1}$ s^{-1})	A_{400}	5	11.2	**	↓	10.9	**	↑	–	NS	Fig. 5
		25	19	***	↓						
	A_{sat}	5	8.7	**	↓	19	***	↑	–	NS	
		25	14.2	***	↓						
	A_{max}	5	4.4	*	↓	71	***	↑	6.0	*	
		25	12.1	**	↓						
Respiration (μmol CO_2 m^{-2} s^{-1})		5	–	NS	–	28	***	↑	12	***	Fig. 6
		25	49.5	***	↑						
Respiration (μmol CO_2 g$_{DM}^{-1}$ s^{-1})		5	–	NS	–	44	***	↑	–	NS	Fig. 6
		25	–	NS	–						
$R_{dark} : A_{400}$		5	–	NS	–	–	NS	–	9.2	**	Fig. 6
		25	16.7	***	↑						
C_i (mmol mol^{-1})		5	–	NS	–	6.7	*	↓	–	NS	Fig. S3
		25	–	NS	–						
Stomatal conductance (mol H_2O m^{-2} s^{-1})		5	–	NS	–	–	NS	–	–	NS	Fig. S3
		25	–	NS	–						
Transpiration (mmol H_2O m^{-2} s^{-1})		5	–	NS	–	21	***	↑	–	NS	Fig. S3
		25	–	NS	–						
VPD$_L$ (kPa)		5	–	NS	–	70	***	↑	–	NS	Fig. S3
		25	–	NS	–						
iWUE (mmol CO_2 mmol H_2O^{-1})		5	–	NS	–	19	***	↓	–	NS	Fig. S3
		25	–	NS	–						

Asterisks denote significance levels: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Degrees of freedom for PE cold exposure regressions were 1.29 to 1.31, for analysis of covariance (ANCOVAS) they were 1.57–1.62. Cold exposure time effect refers to linear regression analysis. ANCOVAS tested the effect of an increase in measurement temperature from 5 °C to 25 °C on each parameter. Arrows denote the direction of observed changes as cold exposure time or measurement temperature increased. ANCOVA measurement temperature by cold exposure duration interaction terms is also shown.

See text for explanation of photosynthesis abbreviations: NS, not significant; meas. T, measurement temperature; DM, dry mass; $R_{dark} : A_{400}$, ratio of respiration to photosynthesis; g_s, stomatal conductance; C_i, internal CO_2 concentration; VPD$_L$, leaf vapour pressure deficit; iWUE, instantaneous water-use efficiency.

2003; Campbell *et al.* 2007; Poorter *et al.* 2009) and that CD leaves are often thicker than their WD counterparts (Boese & Huner 1990; Equiza & Tognetti 2002; Atkin *et al.* 2006a; Koeda *et al.* 2009). Moreover, previous studies have quantified differences in anatomy exhibited by plants that adapted to contrasting thermal regimes (Körner *et al.* 1989). There is also evidence that in WD PE leaves of an evergreen broadleaved species, leaves formed in summer are thick enough to accommodate increases in mesophyll chloroplast surface area during winter (Muller *et al.* 2009). However, to our knowledge, no study has previously considered the impacts of chilling growth temperatures *per se* on the cross-sectional anatomy of *Arabidopsis* leaves. Here, we found that CD leaves exhibited considerably greater thickness (Fig. 2) and LFMA (Fig. 1a) than their WD counterparts. By contrast, extended exposure of PE leaves to cold had relatively little impact on thickness of PE leaves.

Although an age effect could not be ruled out, the two batches of WD leaves analysed had mean thicknesses comparable to both day 0 and 30 d cold PE leaves, strongly suggesting that little change in leaf anatomy occurred with time *per se*. The PE leaf anatomical change that did occur in response to cold treatment appeared mainly to be driven by palisade and spongy mesophyll cell expansion, both outwards into airspace (Fig. 3a) and vertically (Fig. 2a), as their number per unit cross sectional area showed no change beyond passive spacing and the number of layers did not increase. The much greater increase in thickness associated with cold development (Fig. 2b) was underpinned both by larger increases in palisade and spongy mesophyll thickness (Fig. 2d) and a proliferation of mesophyll cell layers (data not shown). This dual response has previously been observed in CD spinach leaves (Boese & Huner 1990), and consistency with unmeasured *Arabidopsis* leaf section
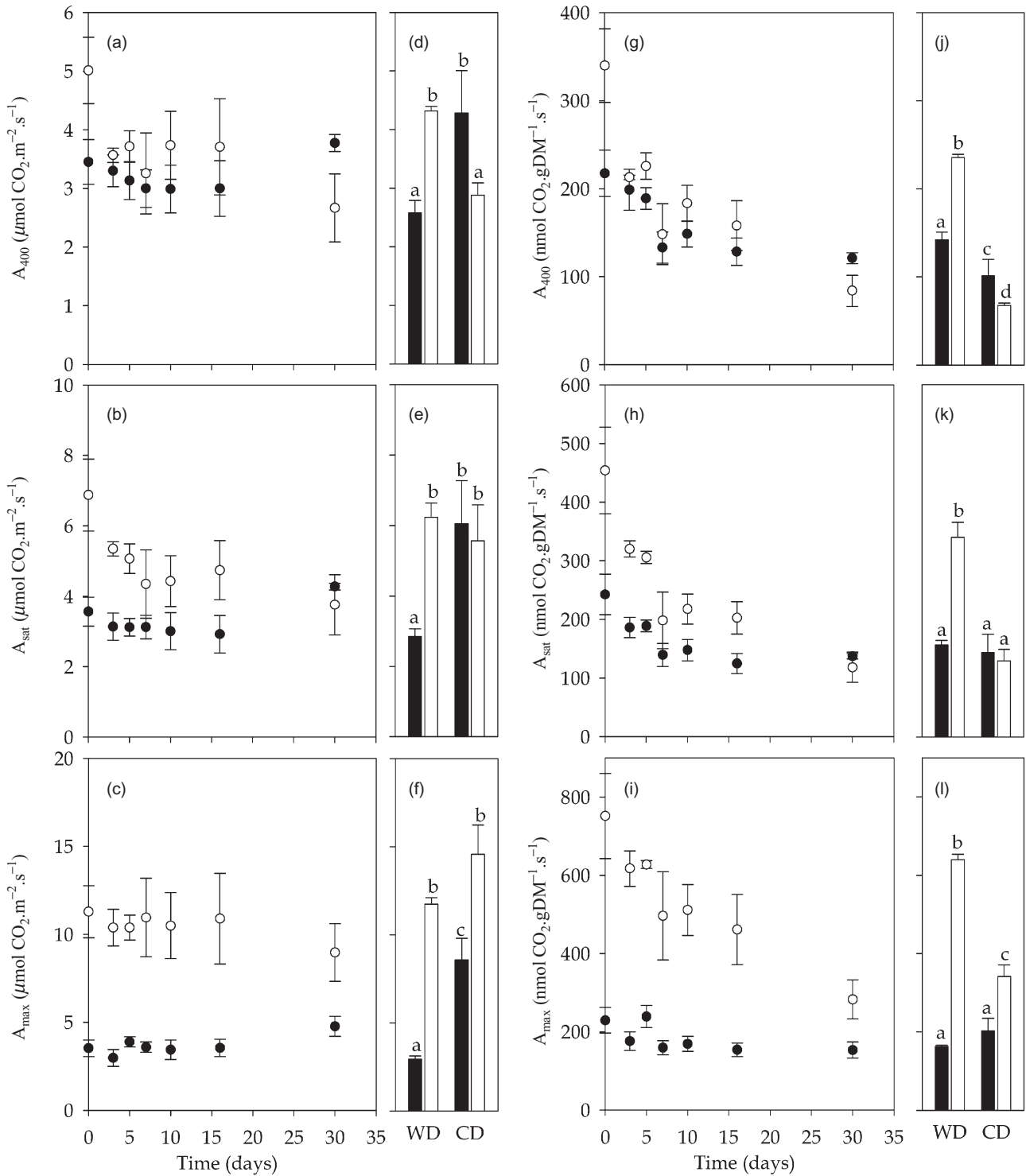
Figure 5. Photosynthesis (±SE) at growth levels of CO_2 and irradiance (A_{400}, top row), saturating irradiance (A_{sat}, middle row), and saturating irradiance and CO_2 (A_{max}, bottom row). Data are expressed per unit area (a–f, left-hand side) and dry mass (DM, g–l, right-hand side). Readings were carried out at 25 °C (open symbols) and 5 °C (filled symbols). (a–c, g–i) cold-exposed, pre-existing leaves; (d–f, j–l) leaves that developed fully in warm (WD) or cold (CD). Lowercase letters denote groups significantly differentiated via a least significant difference (LSD) post hoc test performed as part of a one-way analysis of variance (ANOVA).
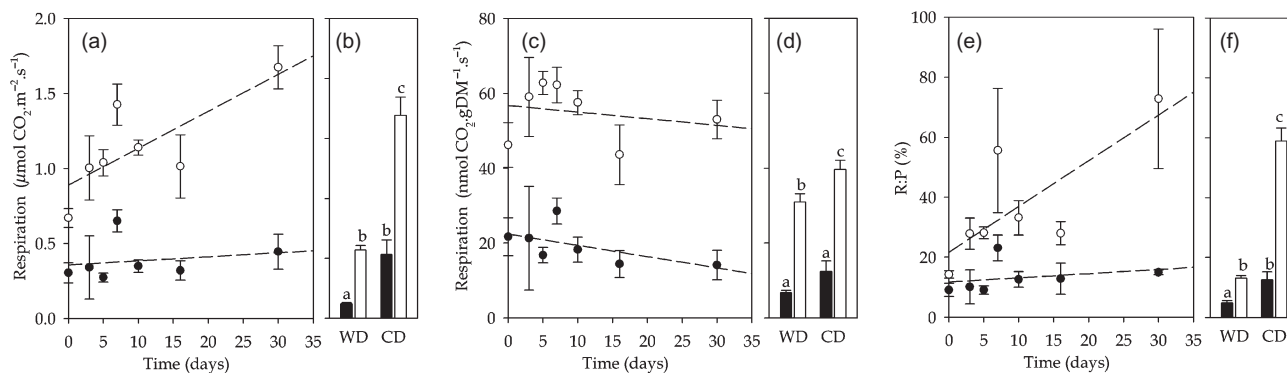
Figure 6. Dark respiration at ambient CO_2 (R_{dark}) per unit (a–b) area and (c–d) dry mass (DM), and (e–f) R_{dark} to photosynthesis ratio ($R_{dark} : A_{400}$). a, c and e refer to cold-exposed, pre-existing leaves; b, d and f to leaves that developed fully in warm (WD) or cold (CD). Readings were carried out at 25 °C (open symbols) and 5 °C (filled symbols). Lowercase letters denote significantly differing groups as determined using LSD post hoc tests. Bars refer to standard errors.

images provides further support (Armstrong *et al.* 2006b). The distinctions between PE and CD leaf thickness data are reminiscent of a study of sun-shifted, shade-grown plants by Sims & Pearcy (1992). The reason for the observed increase in vascular tissue as a proportion of the total (Fig. 3c) is unclear. Overall, the data suggest that conditions during the main leaf expansion period are crucial in determining thickness, and that pre-expanded leaves cannot form new mesophyll cell layers.

Leaf thickness trends underpin the acclimation of area-based gas exchange

The proliferation of mesophyll layers in CD leaves was consistent with their area-based photosynthetic phenotype in the current investigation (Fig. 5), which largely supported observations from published works (e.g. Hurry & Huner 1991; Atkin, Scheurwater & Pons 2006b). When
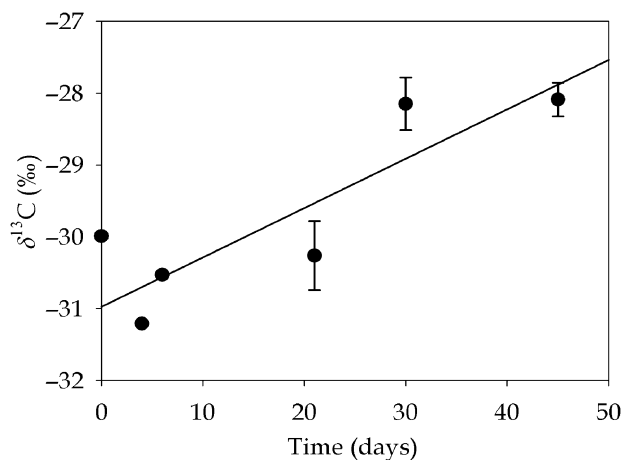

Figure 7. Mass spectrometry analysis (±SE) of stable carbon isotope composition of pre-existing leaves during cold exposure. 0-, 3- and 5-d readings consisted of single, pooled samples, for other means $n = 4$.

measured per unit leaf area at their respective growth temperatures, CD and WD leaf photosynthetic rates were comparable (Fig. 5b), due to the increased depth of assimilatory tissue in the former. In PE leaves, some acclimation did take place by 30 d when A_{400} rates were comparable to CD leaves at both temperatures (and net assimilation was lower at 25 °C than 5 °C), but low A_{sat} and A_{max} in 30 d PE leaves was an important distinction – CD leaves were better able to utilize additional light and CO_2, presumably due to a lower inorganic phosphate limitation. In contrast, increased deposition of carbohydrates (Fig. 3b) and structural matter in cold-acclimated leaves skewed DM basis data (Fig. 5j), which suggested that little acclimation had taken place.

The increased thickness of CD leaves would have contributed to the area-based respiratory data (Fig. 6a,b), which matched previous studies well (e.g. Campbell *et al.* 2007). As with photosynthesis, there was homeostasis of R_{dark} between CD and WD leaves per unit area, but not DM (Fig. 6c,d). Importantly, $R_{dark} : A_{400}$ remained homeostatic when measured at each respective growth temperature, as previously reported in some species (Gifford 1995; Dewar, Medlyn & McMurtrie 1999; Loveys *et al.* 2003) but not others (Atkin *et al.* 2006b; Campbell *et al.* 2007). PE leaves showed increasing R_{dark} with exposure duration when measured at 25 °C, and a relatively small increase at 5 °C. This supports and builds on a study of the longest previously examined duration of 10 d (Armstrong *et al.* 2008).

Pre-existing leaves can eventually achieve a CD carbohydrate phenotype

Although a cold-induced increase in leaf thickness would strongly influence LFMA (Fig. 1b), and presumably also dry matter accumulation (Kubacka-Zębalska & Kacperska 1999), the observed trends in LDMA and DMC shown in Fig. 1 and Table 1 were not dictated entirely by cell wall deposition. Analysis of PE leaves showed that pool sizes

of all measured carbohydrates peaked at 4–6 d (Fig. 4a), which is consistent with previous research (Koster & Lynch 1992; Ristic & Ashworth 1993; Strand *et al.* 1997), and correspond closely with a small peak in R_{dark} (Fig. 6a). These metabolites accounted for 8% of the dry matter at 0-d cold, but caused 63% of the increase in DMC relative to initial levels (and were associated with 17% of the dry matter *per se*) after 4–6 d in the cold. By 45 d, only 19% of the increase in DMC from initial levels was related to sugar and starch accumulation, due to a decline in glucose and starch (Fig. 4a) and an increase in dry matter (whilst FM remained constant, hence a strong rise in DMC; Fig. 1c). Carbohydrates also accounted for 19% of the increase in DMC in CD leaves relative to WD controls. Although the total cell perimeter per unit cross-sectional area (data not shown) was significantly higher within WD than CD leaves, the increased thickness of the latter implied a 32% greater cell wall length in a given leaf transect, following cold development, which would presumably have increased LDMA correspondingly. Collectively, these results highlight the dynamic nature of changes in leaf metabolites through time following cold exposure, and the potential importance of such changes for overall leaf DMC.

In the only similar study of both long-term cold-exposed and CD leaves (Strand *et al.* 1997), exposure to cold resulted in an early peak in sugar concentration, but with levels still higher at 21 d than in CD counterparts. Figure 4 matches these data well, but shows that sugars continue to decrease until at least 45 d, to CD levels. Previous studies of *Arabidopsis* have demonstrated that starch content reaches a plateau during cold acclimation (Ristic & Ashworth 1993; Strand *et al.* 1997) – one that in fact does not endure (Fig. 4a) beyond the 21-d limit used by Strand *et al.* (1997). At 45 d, starch concentrations is probably lower than initial levels (Fig. 4a), and again were comparable to that of CD leaves (Fig. 4b), suggesting that the shift of carbon metabolism from starch to sugar synthesis proposed by Strand *et al.* (1997) to be a feature of CD leaves can occur in PE leaves exposed to a prolonged low-temperature stimulus.

What factor(s) underpin the decrease in soluble and insoluble carbohydrates following extended exposure of PE leaves to the cold? Carbohydrate pool sizes reflect the balance between carbon input from photosynthesis and output by export/storage/respiration. Given that mass-based leaf R_{dark} at 5 °C did not increase over time in PE leaves (Fig. 6c), it seems likely that the decline in starch and glucose concentrations resulted from the decline in A_{400} at 5 °C (Fig. 5g) and/or increased rates of carbon export to other parts of the plant. Indeed, we have recently observed that while growth largely stops when warm-acclimated *Arabidopsis* are placed in the cold, increases in relative growth rate are observed 2 weeks later (Atkinson and Atkin, unpublished data). This suggests that there is an increase in carbon demand by developing tissues as starch and total sugar concentrations in PE leaves begin to fall (Fig. 4a).

Differential regulation of epidermal and mesophyll cell division and expansion during development in the cold

Gas exchange and leaf structure are closely associated with stomatal control of the exchange of CO_2 with the atmosphere. In our study, cold exposure effects on the PE leaf epidermis were area-related and did not imply the relative initiation of new stomata (data not shown). This strong area effect supports previous attestations that the use of SD alone in palaeoecological studies of climate reconstruction is flawed without suitable controls (McElwain & Chaloner 1995; Royer 2001). In contrast, both stomatal and epidermal cell densities were much higher in CD than WD leaves, on both surfaces (Table 1), as previously observed in pedunculate oak (Beerling & Chaloner 1993) but not other species (Miskin & Rasmussen 1970; Boese & Huner 1990; Equiza *et al.* 2001). The statistically area-independent cold effect in the current study requires further investigation involving whole leaf cell counts. Notably, the trends in SD observed in this investigation did not appear to affect photosynthesis in a consistent manner. For instance, although stomata were on average 37% more dense in CD (Table 1) than 30-d cold-exposed PE leaves, A_{400} rates at 5 °C were similar (Fig. 5), and neither treatment responded strongly to an increase in CO_2 concentration. These data, and the response of stomatal conductance (g_s; Supporting Information Fig. S3), support the notion that the predominant limitation to photosynthesis at low temperature is chloroplastic inorganic phosphate (P_i), and not CO_2 (Sage & Kubien 2007).

An increase in SI by 13% on the adaxial surface of CD leaves in one of two experimental runs raises the possibility of a developmental temperature effect, despite the apparent absence of one in previous work (Boese & Huner 1990; Beerling & Chaloner 1993) – not least as a similar adaxial-specific SI response has been described in a range of species with increasing altitude (Körner *et al.* 1989). A temperature effect on SI could affect the conclusions of previous climate reconstruction studies that assume that this does not occur (Royer 2001). Further investigation is required on species (in which similar work is absent) more relevant to palaeoecological research. As SD appeared to be area-related, but CD leaves had more internal cell layers, development at low temperatures may specifically promote periclinal cell division (with no evidence of inhibition of expansion on the same plane), whilst leaving anticlinal division relatively unaffected (but inhibiting lateral expansion). Mesophyll sections parallel to the epidermis would address this hypothesis further.

Cold acclimation, water exchange and stable carbon isotope fractionation

Stomatal development is associated not just with the temperature environment but also with water exchange; in this study, both cold and warm treatments had comparable RH, but not VPD_L (Supporting Information Fig. S3). This was partly for technical reasons –low cabinet RH could not be

maintained for long at 5 °C, and high RH (as there-fore be necessary at 25 °C to match VPD_L between treatments) would cause condensation in the photosynthesis system, rendering it inoperable, and partly, as RH equality between treatments has numerous precedents. However, the lower VPD_L at 5 °C did not significantly increase g_s as might be expected (Supporting Information Fig. S3) in 0-d cold PE, nor CD or WD leaves (*t*-tests), nor in a combined dataset of the time-points with the largest apparent temperature effect (two-way ANOVAs). C_i also did not respond to temperature, either overall (ANCOVA) or in control plants. This suggests that the effects of VPD_L were probably slight.

The exchange of carbon and water was not only measured instantaneously but also indirectly, as the carbon isotope ratio of leaf dry matter (Farquhar & Richards 1984; Farquhar *et al.* 1989). In this investigation, iWUE decreased with measurement temperature due to an inhibition of transpiration (Supporting Information Fig. S2). Accordingly, discrimination against ^{13}C was relatively low ($\delta^{13}C$ was high) in both cold-treated tissues (Fig. 7, Table 1), consistently with previous studies (Smith *et al.* 1973, 1976; Yamori *et al.* 2006), and PE leaves, whose $\delta^{13}C$ reached a similar level to CD leaves (Table 1) by 30 d of exposure. At this time, there may be an endpoint – warm-derived carbon may have been replaced with isotopically heavier, cold-derived equivalents. The timing of this change corresponds with a recovery in A_{400} (Fig. 5a), and may imply that some warm-derived enzymes have been replaced with more cold-acclimated equivalents. However, this interpretation is conjectural, as cold-exposed PE leaves could achieve a similar $\delta^{13}C$ to CD leaves via a summation of other factors.

Controls in cold acclimation experiments

Making comparisons between warm-grown, cold-shifted and CD leaves is an established means of investigating cold acclimation (Strand *et al.* 1997, 1999, 2003; Armstrong *et al.* 2006a,b, 2008). Providing a warm control for every time-point of cold-exposed PE leaves is affected by the limited validity of drawing parallels between the courses of development in warm and cold-exposed plants. Rather, we chose to compare each time-point with the trait values on the day that plants were first exposed to the cold, as in previous studies (e.g. Strand *et al.* 1997; Gray & Heath 2005). Although this means that we cannot fully rule out interactions between cold treatment and senescence, plotting traits (e.g. LDMA) of many leaves against their respective DM (using data from partially expanded and fully expanded leaves) revealed that LDMA was largely constant, and independent of developmental stage in fully expanded leaves of both WD and CD treatments (data not shown). However, LDMA values increased markedly, with increasing DM of PE leaves that shifted to the cold, suggesting that the changes in LDMA and associated traits reflected the impacts of the temperature treatment far more than leaf size and/or age. Moreover, the response of photosynthesis at 5 °C (Fig. 5) opposed the typical age-related decline in net assimilation (Flexas *et al.* 2007), and there was no evidence for previously described age-related increases in glucose and fructose, or a decrease in sucrose (Wingler *et al.* 1998; Ludewig & Sonnewald 2000). Thus, while we cannot rule out potential effects of senescence in PE leaves exposed to cold for several weeks, it seems likely that the data principally reflect the overriding influence of growth temperature.

CONCLUDING STATEMENTS

We have demonstrated that for some leaf traits (e.g. anatomy and leaf gas exchange), development in the cold was essential for maximizing differences between cold and warm-acclimated phenotypes, whereas for others (e.g. carbohydrate profiles and $\delta^{13}C$), development temperature was less crucial, so long as near fully expanded PE leaves experience extended periods of cold. This finding has important implications for predictions of how seasonal changes in growth temperature might affect the carbohydrate profiles and the isotopic signature of leaves.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Typical appearance of *Arabidopsis* plants from this investigation. (a) Before cold treatment start, (b) after 6 d of cold exposure, (c) after 28 d of cold exposure, (d) after 56 d of cold exposure, at which point cold-developed leaves of these plants were harvested, and (e) entirely warm-grown plants, from which warm-developed leaves were harvested. All but (e) are approximately to scale.

Figure S2. Expansion of specific, marked leaves, as estimated from length and width measurements using the equation described in the Methods. Filled symbols represent cold-exposed leaves, open symbols are leaves that remained in the warm. (a) Warm-grown, pre-existing leaves that were part-expanded at treatment start (squares) and a younger, smaller pre-existing insertion at treatment start (triangles). (b) Newly developed leaves: the third-youngest visible leaf at treatment start (squares) and the first leaf to emerge from the meristem following treatment start (triangles). Bars refer to standard errors.

Figure S3. Other gas exchange parameters measured during the investigation ($\pm$SE). (a–d) cold-exposed, pre-existing leaves; (e–h) leaves that developed fully in the warm (WD) or cold (CD). Readings were carried out at 25 °C (open symbols) and 5 °C (filled symbols). C_i, internal CO_2 concentration; VPD_L, leaf vapour pressure deficit; iWUE, instantaneous water use efficiency. Lowercase letters denote significantly different groups as determined using an least significant difference (LSD) post hoc test performed as part of a one-way analysis of variance.

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