Source of nitrogen associated with recovery of relative growth rate in *Arabidopsis thaliana* acclimated to sustained cold treatment

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ABSTRACT

To determine (1) whether acclimation of carbon metabolism to low temperatures results in recovery of the relative growth rate (RGR) of plants in the cold and (2) the source of N underpinning cold-acclimation in *Arabidopsis thaliana*, we supplied plants with a nutrient solution labelled with ^{15}N and subjected them to a temperature shift (23ºC to 5ºC). Whole-plant RGR of cold-treated plants was initially less than 30% of that of warm-maintained control plants. After 14 days, new leaves with a cold-acclimated phenotype emerged, with the RGR of cold-treated plants increasing by 50%; there was an associated recovery of root RGR and doubling of the net assimilation rate (NAR). The development of new tissues in the cold was supported initially by re-allocation of internal sources of N. In the longer-term, the majority (80%) of N in new leaves was derived from the external solution. Hence, both the nutrient status of the plant and the current availability of N from external sources are important in determining recovery of growth at low temperature. Collectively, our results reveal that both increased N use efficiency and increases in nitrogen content *per se* play a role in the recovery of carbon metabolism in the cold.

Keywords: Acclimation, *Arabidopsis*, low temperature, nitrogen uptake, relative growth rate, specific leaf area

INTRODUCTION

As sessile organisms, plants are subjected to changing temperatures on a daily, seasonal or annual basis. For example, temperate-region winter annuals which germinate in the autumn and overwinter in a vegetative state (before flowering in spring) often experience sharp declines in growth temperature during early development, followed by exposure to extended cold through winter: in these conditions the ability to maintain growth in the cold will be advantageous as it may accelerate development in the following spring (Preston & Sandve, 2013). This will be particularly important for short-lived plants such as *Arabidopsis thaliana,* that can establish as seedlings in the late summer/autumn and then overwinter before flowering as day-length and temperatures increase in spring. Similarly, despite air temperatures increasing during spring in temperate regions, plants can still be subjected to sudden, late-season cold spells, often lasting for several days (Lutze *et al*., 1998; Norby, Hartz-Rubin & Verbrugge, 2003; Augspurger, 2013). Despite future climate scenarios predicting warmer average temperatures, such cold spells are still expected to persist throughout this century (Kodra *et al*., 2011). It is therefore important that we understand not only how plants respond to different steady-state growth temperatures, but also how individual plants which are subject to a rapid decrease in temperature respond and re-establish growth during sustained cold exposure.

It is well-documented that growth temperature impacts on the relative growth rate (RGR; increase in mass per unit starting mass and time) of a range of crop and non-crop plants (e.g. Blackman, Black & Kemp, 1955; MacDowall, 1973; Woodward 1979; Loveys *et al.*, 2002, 2003; Tjoelker, Reich & Oleksyn, 1999; Kurimoto *et al*., 2004; Dahal *et al*., 2012; Pyl *et al.* 2012) and on its biomass allocation components

(Porter & Gawith, 1999; Atkin *et al*., 2006; Poorter *et al*., 2012); temperature also impacts on the underlying physiology, for instance on the rates of respiration and photosynthesis, although acclimation in C metabolism can occur with associated changes in tissue N (Atkin *et al*., 2006). The RGR of plants grown at cold-hardening temperatures (≤5^{o}C) can be reduced by up to 80% (MacDowall, 1974; Krol, Griffith & Huner, 1984; Dahal *et al*., 2012). However, one aspect that such studies have not addressed is the initial impact on, and subsequent recovery of, RGR following a shift from warm to low temperature. For example, Kurimoto *et al*. (2004) subjected rice and wheat to a temperature shift (warm to cold) but only reported growth rates after sustained exposure to cold but not growth rates during the transition period itself. Similarly, Pyl *et al*. (2012) calculated RGR for *Arabidopsis* grown at a range of day/night temperatures (12-24^{o}C) but again did not report on the recovery of growth immediately following the change in temperature regime. Thus, how RGR and its components respond to chilling through time has not been elucidated.

Whether whole-plant RGR recovers in cold-acclimated plants will depend on the ability of roots, stems and leaves to re-establish growth following sustained exposure to cold. In turn, recovery of growth rates of each organ will impact on the plant's ability to acquire resources (both above and below-ground). At present, it is unclear to what extent above and below-ground organs recover their growth rates following the onset of the cold acclimation process. If the degree of recovery differs between above- and below-ground organs, above- versus below-ground biomass allocation will be altered as the period of cold-exposure increases. Past studies have reported a higher root mass ratio (RMR; i.e. ratio of root mass to whole plant mass) in plants grown at lower temperatures (Gavito *et al.* 2001; Equiza & Tognetti 2002; Atkin, Scheurwater & Pons 2007), suggesting that roots maintain or recover

89 their growth rates to a greater extent than stems and/or leaves; such increases in the
90 RMR could be important in maintaining nutrient uptake in the cold. Similarly, the rate
91 at which new leaves develop and expand in the cold could contribute to a recovery of
92 growth through increases in the leaf mass ratio (LMR; ratio of leaf mass to whole
93 plant mass) and leaf area ratio (LAR, ratio of leaf area to whole plant mass),
94 resulting in greater assimilate supply (Gorsuch, Pandey & Atkin, 2010). Establishing
95 how each of these factors changes following extended cold treatment (including the
96 timing of any changes in each trait) is essential if we are to develop a more
97 mechanistic understanding of the factors controlling recovery of plant growth in the
98 cold.

99 In plants experiencing sustained cold, lower RGR values are also associated
100 with lower net assimilation rate (NAR; increase in plant mass per unit leaf area and
101 time) (Atkin *et al*. 2006). However, the extent to which NAR declines following initial
102 exposure to cold, and recovers following sustained cold treatment, is unclear. What
103 is known is that the underlying rates of respiration and photosynthesis that contribute
104 to net carbon gain usually decline when a plant is initially challenged with low
105 temperatures (Woodward 1979; Stitt & Hurry 2002; Atkin & Tjoelker 2003). Over a
106 period of several days to weeks, acclimation of leaf-level photosynthesis and
107 respiration then occurs in cold hardy plants such as *Arabidopsis*, resulting in marked
108 increases in the capacity of both processes (Stitt & Hurry 2002; Atkin & Tjoelker
109 2003). Most metabolic acclimation studies have measured carbon exchange at the
110 leaf or root level, with the *assumption* being that such changes in carbon metabolism
111 are integrated at a whole plant level, resulting in a recovery in NAR and hence
112 restoration of growth (Kurimoto *et al*. 2004). Where both whole plant carbon balance
113 and RGR have been measured on the same plants, the experiments have been

conducted at constant temperatures (e.g. Atkin *et al.*, 2007). Therefore, although it may be possible to hypothesise from biochemical analyses and gas exchange measurements that a recovery in NAR and whole-plant growth should occur following transfer to the cold, it remains to be demonstrated that this is the case from growth analysis data.

The whole plant is made up of tissues of different age, each of which may have experienced different thermal conditions during their development. When warm-grown plants are shifted to the cold for sustained periods, more complete metabolic acclimation occurs in newly-developed (ND) than pre-existing (PE) tissues that had previously developed under higher temperature conditions (Strand *et al*. 1997; Atkin & Tjoelker 2003; Campbell *et al*. 2007); hence, the rate at which new tissues develop in the cold is likely to be important for recovery of growth. Changes in the structure and composition of PE leaves occur following a shift to a low growth temperature (Gorsuch *et al*. 2010) but ND leaves which form in the cold differ to a greater extent in leaf morphology and anatomy than their PE counterparts; ND leaves are thicker, contain more cell layers (Boese & Huner 1990; Atkin *et al*. 2006; Gorsuch *et al*. 2010), exhibit increased activity via the phosphorylating cytochrome pathway of mitochondrial electron transport (Armstrong *et al*. 2008) and may differ in protein composition compared to PE leaves (Strand *et al*. 2003; Kaplan *et al*. 2004; Campbell *et al.* 2007). Collectively, such changes in ND leaves can lead to a recovery of photosynthetic and respiratory rates in the cold. To determine how these changes are integrated into NAR and RGR of whole plants, it will be necessary to carry out detailed whole plant growth analyses.

In studies investigating the recovery of growth of plants exposed to cold, it is important to also consider the nitrogen economy of the plant, as changes in plant

139 organic N concentration (PNC) and N productivity (NP, biomass accumulation per
140 unit organic N per unit time) may be associated with recovery of RGR (Poorter,
141 Remkes & Lambers, 1990; Atkin, Botman & Lambers, 1996). N productivity is
142 dependent on the patterns of N allocation in whole plants (i.e. leaves, stem and
143 roots) as well as the proportion of leaf N allocated to the photosynthetic apparatus
144 (Poorter *et al*. 1990; Garnier, Gobin & Poorter, 1995; Poorter & Evans, 1998).
145 Thermal acclimation of respiration in the cold has been associated with an increase
146 in leaf N content (Tjoelker *et al*. 1999; Lee, Reich & Bolstad 2005; Tjoelker *et al*.
147 2008), the abundance of critical proteins (Strand *et al.* 1999) and changes in
148 mitochondrial numbers and volume (Armstrong *et al.* 2006). Consequently, it is
149 commonly assumed that increases in tissue N concentration underpin the cold
150 acclimation process. Assuming this to be so, limitations in N supply could have
151 strong negative effects on the recovery of growth in the cold. N supply could be
152 limited by availability or through temperature affecting uptake and distribution within
153 the plant (Clarkson, Hopper & Jones 1986; Gavito *et al*. 2001). Recovery of N
154 uptake by roots could enable the N requirements of cold acclimation to be met from
155 the soil or alternatively N could be re-allocated from PE to ND leaves.

156 Given the potential role of N for the cold acclimation process, it is important to
157 determine the source of the additional N that accumulates in the tissues of cold
158 acclimating plants: does it come from the redistribution of N within the plant or from
159 new uptake of N from soils? If uptake from external media predominated this would
160 place greater reliance on availability of soil N for the cold acclimation process.
161 Stable isotopes (^{15}N) have been used to follow N assimilation (Dawson et al. 2002;
162 Tcherkez & Hodges 2008) and remobilization in plants (Lestienne, Thornton &
163 Gastal 2006) and it has been shown that leaf N content is derived from both newly

164 acquired N and from reallocation within the plant (Yoneyama, Ito & Engelaar 2003).
165 However, we do not know the relative importance of these two processes in plants
166 subjected to a change in temperature, in particular, when warm-grown plants
167 experience sustained cold treatments.

168 In our study, we exposed *Arabidopsis thaliana* plants to sustained cold to test
169 the hypothesis that RGR recovers in cold-acclimated plants, as suggested by past
170 studies that focused on physiological responses of leaves and roots. *Arabidopsis* is
171 a cold-hardy herbaceous plant which can survive and grow at low and freezing
172 temperatures (Stitt & Hurry, 2002), and hence is a suitable model species for over-
173 wintering short-lived plants. Secondly, we investigated whether, if RGR does indeed
174 recover, the response differed among organs (leaves, stems and roots), and whether
175 recovery of RGR was associated with an increase in NAR, PNC and/or NP. Finally
176 we used ^{15}N to investigate whether the plant relies on redistribution of existing tissue
177 N to support the formation of new tissues in the cold, or if new N is taken up from the
178 soil solution.

179

180 **MATERIALS AND METHODS**

181

182 **Growth conditions**

183 Seeds of *Arabidopsis thaliana* (ecotype Columbia) were sown on Levington F2
184 compost (Scott's Professional, Ipswich, UK). When the plants had roots of at least 3
185 cm length they were carefully removed and the roots washed. The plants were then
186 transferred to fully-aerated modified Hoaglands nutrient solution (2000 μM N; Poorter
187 & Remkes 1990) in 17L hydroponic tanks in Conviron E15 growth cabinets
188 (Conviron, Winnipeg, Canada). Chambers were maintained at 23/18^{o}C day/night

189 temperature regime with 150 μmol m^{-2} s^{-1} PPFD (photosynthetic photon flux density)
190 (400-W metal halide and 400-W high-pressure sodium bulbs) and an 8-h
191 photoperiod. The solution was maintained at a pH of 5.8 and replaced weekly.

192

193 **Experimental procedure**

194 After establishment at 23/18°C plants were transferred to modified Hoaglands
195 nutrient solution (2000 μM N) containing 10% atom excess ^{15}N and plants to be cold
196 treated were shifted to a constant 5°C (150 μmol m^{-2} s^{-1} PPFD; 8-h photoperiod).
197 Control plants remained at 23°C. Repeated harvests were carried out on both sets
198 of plants; the final harvest of 23°C grown plants was taken at 14 d but cold-treated
199 plants were allowed to continue to grow until they reached a similar total dry mass
200 (DM) by day 38. There were four plants per treatment for each harvest date. Leaf
201 area was recorded for warm-grown, pre-existing (PE) leaves and newly-developed
202 (ND) leaves that formed in the cold using a Li-Cor 3100 leaf area meter (Li-Cor
203 BioSciences, Lincoln, NE, USA). Fresh mass (FM) and DM were weighed and
204 recorded (Mettler Delta Range AE166, Mettler-Toledo Ltd, Leicester, UK) for roots,
205 stems (i.e. portion of plant material remaining after roots and leaf blades were
206 removed), PE and ND leaves. Fresh mass of roots was measured following removal
207 of surface water via gentle blotting of roots between layers of absorbent tissues
208 (Kimwipes, Kimberly Clark Professional, West Malling, Kent, UK). Samples were
209 frozen in liquid nitrogen and stored at -20°C and then freeze-dried in an Edwards
210 EF4 Modulyo freeze-drier (Northern Scientific, York, UK).

211

212 **Nitrogen analysis**

213 Replicate samples were pooled for each plant part at each harvest and the dried
214 samples were ground using a hammer mill (31–700 Hammer Mill; Glen Creston,
215 Stanmore, UK). Samples were weighed into tin cups and combusted using a Carlo-
216 Erba elemental analyser NA1500 (Thermo Fisher Scientific, Milan, Italy). ^{15}N
217 analysis was performed using IRMS (Dennis Leigh Technologies) at the Stable
218 Isotope Facility, CEH Lancaster. Tissue nitrate concentration was analysed using the
219 method of Cataldo et al. (1975) and absorbance was measured at 410 nm in a
220 spectrophotometer (ELx 800 universal microplate reader; Bio-Tek Instruments, Inc.,
221 Winooski, VT, USA).

222

223 **Statistical analysis**

224 Statistical analyses, including analysis of variance (ANOVA), analysis of covariance
225 (ANCOVA) and regression analysis, were carried out using SigmaPlot v.12, Excel
226 and SPSS v.19. Levene's test was used to check the homogeneity of variance in the
227 ANOVAs and ANCOVAs. Average RGR values of PE and ND leaves, both for
228 warm-grown and cold-treated plants, were obtained from the slope of the regression
229 line of $\log_e$ DM plots (Cheeseman & Wickens 1986). Differences in RGR between
230 treatments and leaf type were analysed using ANCOVA. LAR (leaf area ratio) values
231 were calculated as in Eqn. 1:

232 $$LAR = LMR \times SLA \quad \text{(eqn 1)}$$

233 where LMR is the leaf mass ratio and SLA is the ratio of leaf area to leaf dry mass
234 ($m^2\ kg^{-1}$). Values of NAR were calculated using the RGR values derived from 1^{st}
235 order regression lines fitted to natural log values of DM versus time (for defined time
236 intervals), and the average LAR over the same time period (Atkin et al. 1998):

RGR = LAR x NAR (eqn 2)

The nitrogen uptake rate (NUR) was calculated as in Garnier (1991), Poorter et al. (1991) and Atkin & Cummins (1994); lines were fitted using a 2nd order polynomial, yielding information on temporal variation in NUR via calculation of the slope of the 2nd order polynomial. For each growth period used to calculate RGR (see above), average values of nitrogen productivity (NP; mg (mmol N)$^{-1}$ d^{-1}) were calculated as:

NP = RGR / PNC (eqn 3)

where PNC is total plant N concentration (mmol N g^{-1}).

RESULTS

Growth and biomass allocation

Warm-grown plants which were transferred to the cold showed an initial reduction in relative growth rate, with a subsequent recovery in RGR once new leaves had developed; new leaves first began to emerge from the central part of the shoot meristem after 14 days in the cold. Comparisons were made: (1) between warm-grown and cold-shifted plants before the emergence of ND leaves at the new growth temperature; and (2) in cold-shifted plants, between plants before and after the emergence of cold-developed ND.

The whole-plant RGR of warm-grown plants over the experimental period was 75 mg g^{-1} d^{-1} (Fig. 1a, Table 1) and warm-developed ND leaves emerged (i.e. became visible) within four days of the start of the experiment. By contrast, when plants were transferred into the cold, RGR of whole plants decreased to 20 mg g^{-1} d^{-1}

during the period prior to the pre-emergence of cold-developed ND leaves, together reflecting 70% and 94% declines in the RGR of shoots and roots, respectively (i.e. root growth was markedly more sensitive to cold than shoot growth). During this period, significant decreases were observed in the RGR of all plant parts except for cold-treated PE leaves (Fig. 1; Table 1; Table S1; Table S2).

ND leaves appeared later on cold- than warm-grown plants; in the cold, they were observed from Day 14 but were not present on all plants until Day 17 at the lower growth temperature (Fig. 1f). Once ND leaves had begun to develop at 5^{o}C there was a 50% recovery in RGR of the whole plant from 20 to 30 mg g^{-1} d^{-1} (Table 1). The RGR of most plant parts recovered to approximately 40%, and of the roots to 45%, of the warm-grown control values (Fig. 1; Table 1). ND leaves exhibited higher growth rates than PE leaves at both temperatures; however, the rate of ND leaf growth in the cold was only 37% of that at the higher growth temperature (Fig. 1f; Table 1). RGR values of all plant parts except PE leaves were numerically, but not significantly, greater after ND leaves had developed in the cold (Table 1; Table S2). Measurements of growth made on cold-grown plants with ND leaves frequently exhibited higher variances than those made prior to the production of these leaves (as evidenced by the Levene test for homogeneity of variance; Table S2). This may have been due to inherent plant variability or to greater between-plant variability in response to the cold and may have masked differences between treatments.

The differences in RGR among the different plant organs resulted in changes in biomass allocation. Leaf mass contributed to over half of the initial plant mass in warm-grown control plants (Fig. 2a). In both temperature treatments, the ratio of PE leaves to total plant mass (PE LMR) declined over time (Fig. 2a; Table S1) but this was more rapid in warm-grown control plants (P=0.015; Table S2). The leaf mass

285 ratio of ND leaves (ND LMR) increased over time (Fig. 2a) but these changes
286 occurred more slowly at 5°C (P=0.013; Table S2). There was a slight but significant
287 increase in total LMR over time in both treatments (Table S1). No significant
288 changes in RMR were observed prior to the development of new leaves (Fig. 2d;
289 Table S1). However, following the emergence of ND leaves there was a small but
290 significant increase in RMR over time at 5°C (Fig. 2d; Table S1), although the slope
291 was not significantly greater than that prior to the production of these leaves (Table
292 S2). Averaged over all measurement dates, the root:shoot ratio of cold-grown plants
293 was consistently slightly lower than that of warm-grown plants (0.14 compared to
294 0.17 respectively; P=0.016).

295 Any change in biomass allocation in the cold may be related to the slower
296 overall rate of growth and may therefore be due to differences in developmental
297 stage. Hence, the mass ratio data are shown plotted against the total DM, as an
298 indicator of plant development (Poorter & Pothmann 1992), in Figure 2 (e-h). Total
299 LMR was similar for both treatments at a similar plant DM (Fig. 2e; Table S2);
300 however, the proportion of plant mass allocated to PE and ND leaves differed: for
301 any given total plant mass PE LMR was numerically higher in cold-grown plants as
302 they retained these leaves for longer than warm-grown plants but the ND LMR was
303 lower (Fig. 2f: P=0.013; Table S2). RMR was consistently, but not significantly,
304 lower in the cold once new leaves have started to appear (Fig. 2h).

305 Mature cold-developed leaves had lower final specific leaf areas (SLA) than
306 warm-grown leaves (Fig. 3a & b; Table 2). PE and ND leaves showed a similar
307 decrease in SLA over time but the SLA of cold-developed ND leaves was lower from
308 emergence than in cold-shifted PE leaves. The reduction in SLA in the cold was due
309 to a combination of changes in leaf thickness (as indicated by the ratio of leaf fresh

mass per unit area, LFMA; Dijkstra 1989; Gorsuch *et al*. 2010) and leaf density (as indicated by the leaf dry mass content (DMC); Table 2; Table S3). Leaf DMC was higher in ND leaves at both temperatures whereas LFMA was similar in both PE and ND leaves in the cold but lower in ND leaves at 23ºC (Table 2). LFMA increased more slowly in PE leaves in the cold (Table S3). Leaf DMC also demonstrated higher variance in cold-shifted plants.

LAR was calculated (eqn. 1) as the product of LMR (Fig. 2a) and SLA (Fig. 3a & b). On average total LAR was lower in plants transferred to the cold compared to warm-grown control plants (Fig. 3c-f; mean 26.4 compared to 29.9 m^2 kg^{-1}), even following the appearance of ND leaves (averaging 17.6 m^2 kg^{-1} over this period). At 23ºC there was a decline in PE LAR, which was largely compensated for by an increase in ND LAR at this temperature. However, at 5ºC there was a gradual decline in total LAR ($P \leq 0.000$; Table S1) as PE LAR decreased but was not fully compensated for by the increase in ND LAR.

NAR was calculated from the measured data using LAR and RGR (Eqn. 2). In the cold NAR was reduced to 30% of that of the control prior to the production of ND leaves but recovered to 68% of the control value once new leaves had been produced (Table 1).

Taken together, the growth and biomass allocation data demonstrate that while initial exposure to cold greatly inhibited growth, particularly of the roots, extended cold treatment was associated with recovery of growth rates, with the recovery of growth being most marked in the roots. This recovery of growth rate in the cold was associated with changes in leaf density and thickness and a rise in the net assimilation rate.

334

335 **Nitrogen uptake and distribution of N**

336 The fact that RGR and NAR both recovered in plants exposed to cold for >14 days
337 suggests that there was a concomitant increase in rates of carbon metabolism –
338 indeed, past studies have shown that both photosynthesis and respiration increase
339 in capacity following formation of ND in the cold (Strand *et al*. 2003; Gorsuch *et al*.
340 2010; Armstrong *et al*. 2006, 2008). Given the reliance of metabolic capacity on
341 protein investment, and thus N supply, one question of interest was whether tissue N
342 concentrations increased during the cold acclimation process.

343 Total plant nitrogen content (PNC) varied slightly between treatments (Table
344 1) and mass-based total N concentration (N_{mass}) varied between tissues (Fig. 4). The
345 total N_{mass} of PE leaves was 65 mg g^{-1} DM at the start of the experiment. At 23ºC
346 this decreased slightly during the first few days of growth to *c.* 58 mg g^{-1} DM (Fig.
347 4a). In plants shifted to the cold there was a drop in the total N_{mass} of PE leaves over
348 the first 10 days to approximately 46 mg g^{-1} DM, followed a further decline to c. 44
349 mg g^{-1} DM by day 38. The total N_{mass} of ND leaves of warm-grown plants was
350 greater than that for cold-developed ND leaves (Fig. 4a; Table S4) averaged over all
351 dates. The stems and roots of warm-grown plants had lower mean N_{mass} than leaves
352 (Fig. 4b & c; Table S4). At 5ºC the total N concentration of stems and roots, apart
353 from an initial drop for stems, increased slightly over time (Fig. 4 b & c).

354 Total N_{mass} consists of both inorganic nitrate and organic N pools. Organic
355 N_{mass} as a proportion of total N_{mass} varied with plant organ and growth temperature
356 (Fig. 4; Table S4). Levels were similar throughout the experiment in tissues grown at
357 23ºC with the highest proportion in roots and ND leaves. The proportion of organic
358 N_{mass} to total N_{mass} increased over time in the roots, stems and PE leaves of plants

359 exposed to the cold, resulting in higher relative levels of organic N_{mass} in all tissues in
360 cold-grown compared to warm-grown plants (Table S4). In cold-developed ND
361 leaves the proportion of total N_{mass} present as organic N_{mass} was high throughout
362 their development (0.97).

363 Total N content expressed on an area basis (N_{area}) varied little with time at
364 23^{o}C, whereas it increased over time in both PE and ND leaves in the cold (Fig. 5)
365 reaching a final concentration 37% and 98% greater for PE and ND leaves than their
366 warm-grown counterparts. Similarly, the organic N_{area} values for cold-grown PE and
367 ND leaves were 83% and 124% greater than the controls respectively. Organic N_{area}
368 as a proportion of total N_{area} increased from 0.71 to 0.95 in PE leaves in the cold but
369 was constantly at a high level (0.97) in the ND leaves in this treatment. Values of
370 total and organic N_{area} in cold-shifted PE leaves were similar to those of the warm-
371 grown leaves for the first ten days.

372 N turnover in pre-existing (PE) leaves resulted in almost 60% being replaced
373 by N from the hydroponic solution (Fig. 6a) whereas 78% of the nitrogen
374 accumulating in newly-developed (ND) leaves came from the nutrient solution (Fig. 6
375 b). Similar final values (of the proportion of N derived from the nutrient solution)
376 were found in both warm-grown and cold-acclimated leaves, but accumulation and
377 turnover were slower at 5^{o}C. 60-70% of the N accumulating in stems and roots
378 came from the nutrient solution at both temperatures (Fig. 6c & d) but over a longer
379 time period at 5^{o}C. Nitrogen was taken up from the solution quite rapidly at 23^{o}C but
380 uptake was slower at 5^{o}C, although similar levels of uptake were achieved overall
381 (Fig. 7a). The nitrogen uptake rate (NUR) of the roots declined over time (Fig. 7b).
382 NUR in warm-grown plants was initially approximately double the rate in cold-grown
383 plants but declined to a similar level by d14. The rate of decline in NUR was slower

384 at 5ºC (Fig. 7b). N uptake at 23ºC was for the most part greater than that at 5ºC for
385 any given plant size indicating that there was a temperature effect on N uptake over
386 and above that caused by slower rate of development at lower temperatures (Fig.
387 7c). NUR was also greater in warm-grown plants than for a cold-developed plant of
388 similar developmental stage (Fig. 7d).

389 NP was reduced to 28% of the control rate when plants were first place in the
390 cold (Table 1). However, post emergence of ND leaves, NP of the cold-treated
391 plants recovered to 37% of the control rate (Table 1).

392

393 **DISCUSSION**

394 Our study investigated the impact of cold on *Arabidopsis* in order to assess whether
395 the cold-acclimation of leaf and root level energy metabolism seen in many studies
396 (e.g. Stitt & Hurry 2002; Atkin & Tjoelker 2003) integrates at whole plant level,
397 manifesting as a recovery of growth rate in the cold. Our results indicate that relative
398 growth rate does indeed recover in plants exposed to low temperatures for a
399 sustained period, being underpinned by a recovery of the net assimilation rate and
400 nitrogen productivity. Given the reliance of metabolism on nitrogen for synthesis of
401 additional protein needed to enhance metabolic capacity, we asked if the recovery of
402 growth was reliant on uptake of additional N, in addition to the recovery of nitrogen
403 productivity.

404 There was a recovery of whole-plant RGR in the cold after 17 days (Fig. 1).
405 Initially exposure to the cold resulted in a drop in the RGR of the whole plant to less
406 than 30% of the warm-grown plants. This initial value for growth in the cold was
407 similar to that calculated assuming a temperature coefficient (Q_{10}) of 2.0 for growth

(an actual rate of 20 mg g^{-1} d^{-1} compared to a calculated rate for the assumed Q_{10} of 21.5 mg g^{-1} d^{-1}; Atkin & Tjoelker 2003). Following the development of new leaves at 5°C there was a recovery in growth to 30 mg g^{-1} d^{-1}; i.e. an increase of 50% compared to the earlier cold-grown RGR. Not surprisingly, the RGR of PE leaves in warm-grown plants was low relative to warm-grown ND leaves and whole roots, reflecting the fact that growth of PE leaves is restricted to expansion and carbon deposition of already formed cells. In comparison, the RGR of warm-grown ND leaves was markedly higher, reflecting the deposition of dry mass in newly divided and expanding cells. The recovery of RGR of cold-treated plants could, in part, be explained by acclimation of leaf-level photosynthesis following exposure to the cold. However, an increase in leaf-level photosynthesis will not necessarily result in an increase in the rate of whole plant C gain, as this will also depend on the effect of cold treatment on the efficiency of light interception and stomatal conductance of whole shoots, as well as on the rates of respiratory CO_2 release by whole shoots and roots. Our data strongly suggest that previous reports of leaf-level photosynthesis increasing in cold acclimated plants (e.g. Stitt & Hurry 2002) are indeed associated with a recovery of whole plant growth, suggesting that whole-shoot photosynthesis also acclimates.

The impact of cold on growth differed between organs, with the greatest reduction being in the RGR of roots, indicating that root growth in this *Arabidopsis* ecotype is particularly sensitive to low temperature. This differential effect of temperature on the growth rate of roots and shoots can vary between species and cultivars (Kurimoto *et al*. 2004) and a number of authors have reported increases in the R:S ratio in the cold (Gavito *et al*. 2001; Equiza & Tognetti 2002; Atkin *et al*. 2007; Poorter *et al*. 2012). Changes in biomass allocation would have implications

433 for resource capture, particularly light versus nutrient acquisition. However, in our
434 experiment, recovery of growth appeared to occur at approximately the same time in
435 both roots and shoots. Some of the differences between warm- and cold-grown
436 plants may have been due to slower growth at 5^{o}C rather than due to fundamental
437 changes in biomass allocation as there were no significant changes in biomass
438 allocation with growth temperature at an equivalent plant dry mass, other than the
439 retention of PE leaves for longer and the lower production of ND leaves in the cold
440 (Fig. 2).

441 Plants transferred to the cold had started to produce new leaves by 14-17
442 days and this new leaf material had characteristics of a cold-acclimated phenotype
443 with lower SLA (Fig. 3) and higher DMC than warm-developed material (e.g. Boese
444 & Huner 1990; Equiza & Tognetti 2002, Atkin *et al*. 2006). Increases in DMC have
445 been attributed to an increase in the ratio of cytoplasmic to vacuolar volume (Strand
446 *et al*. 1999; Hurry *et al*. 2000). Gorsuch *et al*. (2010) subjected *Arabidopsis* to a
447 temperature shift from 25 to 5^{o}C and noted similar increases in DMC but more
448 pronounced increases in leaf thickness (measured both as the ratio of fresh mass
449 per unit leaf area, LFMA, and as actual leaf thickness). This was associated with
450 increased numbers of cell layers in the cold and changes in carbohydrate content
451 (Gorsuch *et al*. 2010). This increase in cell layers (with associated chloroplasts and
452 mitochondria) likely contributes to the restoration of respiratory (Gorsuch *et al*. 2010)
453 and photosynthetic (Strand *et al*. 1997; Gorsuch *et al*. 2010; Pons 2012) rates on an
454 area basis.

455 Alongside leaf morphological and anatomical changes, a major re-engineering
456 of leaf energetic metabolism also occurs as part of the cold acclimation syndrome

457 (Strand *et al*. 1999; Stitt & Hurry 2002; Atkin & Tjoelker 2003; Armstrong *et al*. 2006,
458 2008). Cold-acclimation of metabolic pathways occurs over a period of several days
459 (Atkin & Tjoelker 2003) during which there may be recovery in the rates of both
460 respiration and photosynthesis in PE leaves (Stitt & Hurry 2002; Atkin & Tjoelker
461 2003). Subsequently, further increases in respiration and photosynthesis occur once
462 ND leaves form in the cold (Strand *et al*. 1999; Stitt & Hurry 2002; Atkin & Tjoelker
463 2003; Armstrong *et al*. 2006, 2008; Gorsuch *et al*. 2010). It is presumed that it is
464 these changes in carbon metabolism, with the net result of recovery in NAR, that
465 lead to restoration of growth (Kurimoto *et al*. 2004). Here, our results indicate that
466 the recovery of RGR seen in the cold-grown plants once new leaves had been
467 produced may have been through such an increase in NAR (i.e. productivity per unit
468 leaf area; Pons 2012) and NP, rather than through an overall increase in the amount
469 of photosynthetic tissue; here the leaf area supporting the total plant mass (i.e. total
470 LAR; Fig. 3) actually decreased in cold-shifted plants despite the production of new
471 leaves, and a slight overall increase in total LMR. Up-regulation of carbon
472 metabolism may have occurred to some extent in both PE and ND tissues, but would
473 be expected to occur to a greater extent in those leaves that developed at the new
474 growth temperature (Atkin & Tjoelker 2003; Campbell *et al*. 2007; Gorsuch *et al*.
475 2010).

476 To support acclimation and the recovery of growth in the cold it is often
477 assumed that additional N is necessary (Martindale & Leegood 1997; Stitt & Hurry
478 2002), presumably to underpin the increase in NAR and the development of new
479 tissues. Indeed, N levels are often reported to be greater in cold-grown material (e.g.
480 Tjoelker *et al.* 1999; Lee *et al*. 2005; Tjoelker *et al*. 2008). Because photosynthesis
481 and respiration rates scale with N concentration, on a mass or area basis (across

482 species and environments) (Evans 1989; Reich, Walters & Ellsworth 1997; Reich *et*
483 *al*. 1998; Wright *et al*. 2004), the increases in N content would likely have resulted in
484 up-regulation of carbon metabolism. Recovery of growth did occur in the cold,
485 suggesting that recovery in the rate of respiration and photosynthesis had indeed
486 taken place, as has previously been reported in *Arabidopsis* under similar growth
487 conditions to that used in our study (Gorsuch *et al*. 2010). This occurred despite
488 similar mass-based organic N concentration in ND leaves at both temperatures (Fig.
489 4). However, there was an increase in the area-based N values from about ten days
490 in leaves shifted to the cold (Fig. 5) which can only partially be explained by an
491 increase in leaf thickness (LFMA; Gorsuch *et al*. 2010). Hence, if photosynthesis
492 and respiration rates had acclimated, then this may have been due in part to an
493 increase in the N concentration of the tissues per unit leaf area and partly to an
494 increase in the efficiency of N use, with the reallocation of N from inorganic to
495 organic pools.

496 From an N economy perspective, the recovery in RGR appeared to be
497 associated more with increased NP, rather than increased PNC, following the
498 emergence of ND leaves in the cold, i.e. the efficiency of N use did indeed increase
499 during the cold acclimation process and could be due to an increased proportion of
500 total leaf N allocated to the photosynthetic system (Evans 1989; Poorter & Evans
501 1998). Given that NP is dependent on the rate of net carbon gain per unit plant N
502 (Lambers, Chapin & Pons 2008), this suggests that cold acclimation was associated
503 with increased photosynthetic N use efficiency (PNUE) and/or decreases in
504 respiration per unit N. Acclimation of respiration and photosynthesis have been
505 shown to occur on an area basis in *Arabidopsis* (Gorsuch *et al.* 2010) and this could
506 have been associated with increased total and organic N per unit area in the cold

(Fig. 5). The allocation of organic N to the respiratory and photosynthetic pathways will be important (Poorter *et al*. 1990; Garnier *et al*., 1995; Poorter & Evans, 1998; Lambers *et al*. 2008) and further work is needed to elucidate how the partitioning of N changes when a plant is challenged with the cold.

The development of new, cold-acclimated leaves in plants shifted to low temperature was supported by N from three possible sources: reallocation from existing tissues, movement between inorganic and organic pools of N and by uptake of new N by the roots (Yoneyama *et al*. 2003). These mechanisms contributed different proportions of the final N content of the new tissues and may have been important at different stages of leaf growth. Firstly, the N_{mass} lost from PE leaves in the first ten days (Fig. 5) may have contributed to the initial development of new leaves at low temperatures. Reallocation of N from leaves is a recognised part of the process of senescence (Hörtensteiner & Feller 2002) and underpins the development of new tissues (Yoneyama *et al*. 2003). During the period of the experiment, these leaves were retained by the plants and did not appear to senesce earlier than their warm-grown counterparts despite the loss of N. Reallocation of N from PE leaves with a consequent decrease in PE leaf N content may be a common response in changing conditions where new tissues are formed (cf. shade conditions: Pons & Pearcy, 1994). The protein turnover required to facilitate remobilization of N within the plant will have an energetic cost (Noguchi *et al*. 2001) and will be dependent on the supply of energy from photosynthesis and respiration.

Secondly, recovery of growth may have been supported in these plants from the reallocation of stored nitrate into organic compounds on a gradual and continuing basis over the course of the experiment (Fig. 5). In warm-grown plants, a greater proportion of N was present as NO_3^-, but this pool was depleted in cold-grown

tissues and was at its lowest (3%) in ND leaves at 5ºC. Similarly, Clarkson, Jones & Purves (1992) demonstrated a change in the NO_3^- pool of both roots and shoots with temperature, with a lower proportion of N present as NO_3^- at lower temperatures. However, although the proportion of organic N was higher in both PE and ND leaves at 5ºC, the organic N_{mass} was similar in these organs at both temperatures. In cold-shifted PE leaves the organic N content was maintained despite a reduction in total N content in these leaves. There was a different response in the roots and stems of cold-shifted plants where both the proportion of N present in organic form and the organic N concentration increased over time compared to the controls. It is possible that roots and stems were acting as storage buffer organs for N during a transition period (Noquet *et al.* 2004).

Thirdly, N was taken up from the nutrient solution and contributed to turnover in existing leaf tissue (60%) and the development of ND leaves, where ultimately 80% of the N was derived externally (Fig. 7). Although tissues in the cold-shifted plants had similar final uptake of ^{15}N to warm-grown plants, uptake and turnover of N were slower in the cold. Low temperatures could impact on the uptake of N through a direct effect on the processes of uptake and assimilation (Clarkson *et al*. 1986; Atkin & Cummins, 1994; Volder, Bliss & Lambers 2000) or indirectly through modifying root growth (Clarkson *et al*. 1986). Indeed, the control of the balance between C and N supply and demand is complex and possibly mediated through amino acids (Foyer, Parry & Noctor (2003). In our experiment, low temperatures initially reduced root growth but as the new leaves developed there was also a concurrent increase in root DM: this resumption of root growth did not, however, seem to have contributed to an increase in N uptake rate. Whilst the ^{15}N study strongly suggested that the development of ND leaves was supported primarily by N derived from the nutrient

557 solution, this accumulated over a period of nearly 40 days and N reallocated from
558 pre-existing tissue or from stored nitrate would be important in the early development
559 of those leaves i.e. both stored N and new uptake may be required to underpin
560 acclimation.

561 Recovery of growth occurred while the uptake of new N from the solution was
562 still low; this suggests that recovery in nutrient uptake may not be important in the
563 early stages of acclimation. Initially, low root temperature would have impacted
564 directly on N uptake or assimilation. As shoot growth recovered, both plant demand
565 for N (Clement, Hopper & Jones 1978; Clarkson *et al*. 1992; Fitter 1997; Jeuffroy,
566 Ney & Ourry 2002) and the supply of carbohydrates to the root (Clement *et al*. 1978;
567 Mengel and Viro 1978; Lestienne *et al*. 2006) would have increased and could have
568 contributed to increased N uptake. As uptake is dependent on respiratory energy
569 (van der Werf *et al.* 1988; Lambers *et al*. 2008; Zhang, Burns & Turner 2008) cold-
570 acclimation could help to maintain N uptake through up-regulation of respiration
571 within the plant (Atkin & Tjoelker 2003). The response may, however, vary between
572 species due to differing respiratory costs of ion uptake (e.g. as observed in
573 comparisons of fast and slow growers, Scheurwater *et al.* 1998). Reduced
574 carbohydrate supply in the cold may also reduce root growth (Hermans *et al.* 2007)
575 which then results in a smaller surface area over which N uptake may occur.

576 In conclusion, our results have shown that the rate of growth recovered in
577 *Arabidopsis* plants experiencing a shift to cold temperatures and that this recovery in
578 growth was likely due to an increase in NAR. Both increased N use efficiency and
579 increases in nitrogen content *per se* may have played a role in the recovery of
580 carbon metabolism in the cold. Reallocation of N within the plant, between pools
581 and between organs, and N supply to the plant are also important in underpinning

cold-acclimation. Future work is needed to assess whether these findings are maintained under conditions where N availability is limiting.

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Tables

Table 1. Relative growth rate (RGR) of total plant and of root, stem and leaf portions and leaf area ratio (LAR), net assimilation rate (NAR), plant nitrogen content (PNC) and nitrogen productivity (NP) for whole plant. Values for cold-shifted plants were calculated separately for the time period before and after the emergence of newly developed (ND) leaves, with values for pre-existing leaves also shown (PE). RGR values were obtained by regression of $\log_e$ plots of dry mass (DM) against time (see Fig. 1). LAR and PNC values were calculated as means over each time period. NAR was derived by calculation from RGR and LAR values and NP by calculation from RGR and PNC values.

			5ºC			
	Plant part	**23ºC**	**Pre-emergence of ND leaves**	**% of control**	**Post-emergence of ND leaves**	**% of control**
RGR	Whole plant	75.0	20.0	27	29.8	40
(mg g^{-1} d^{-1})	Total shoot	74.3	22.4	30	29.0	39
	Stem	64.1	19.9	31	24.3	38
	Total leaves	79.0	23.5	30	30.9	39
	PE leaves	58.2	21.8	38	21.8	38
	ND leaves	312.2	n/a	n/a	114.7	37
	Root	80.2	4.5	6	36.3	45
LAR (m^2 kg^{-1})		29.9	26.4	88	17.6	59
NAR (g m^{-2} d^{-1})		2.5	0.8	32	1.7	68
PNC (mmol N g^{-1})		2.9	2.7	93	3.1	107
NP (mg (mmol N)$^{-1}$ d^{-1})		26.1	7.3	28	9.7	37

826 **Table 2**. Mean values for specific leaf area (SLA), leaf thickness (as indicated by the ratio of fresh mass per unit leaf area, LFMA) and leaf dry

827 matter content (DMC) averaged over all harvests ± SE, n values: 23ºC PE =7, 23ºC ND = 5, 5ºC PE = 12, 5ºC ND = 7.

Growth temperature	**SLA (m^2 kg^{-1})**		**LFMA (g FM m^{-2})**		**Leaf DMC (g DM g FM^{-1})**	
	PE	**ND**	**PE**	**ND**	**PE**	**ND**
23ºC	50.5 ± 3.2	51.2 ± 2.1	229.4 ± 7.0	177.8 ± 3.8	0.09 ± 0.00	0.11 ± 0.00
5ºC	34.7 ± 3.6	27.2 ± 1.1	213.5 ± 2.90	215.4 ± 9.2	0.15 ± 0.01	0.17 ± 0.01
Effect of temperature	P=0.000		P=0.085		P=0.000	
Effect of leaf type	P=0.336		P=0.000		P=0.006	
Interaction	P=0.256		P=0.000		P=0.693	

828 **Figure Legends**

829

830 **Figure 1.** Increase in (a) total plant dry mass (DM) and the dry mass of (b) shoot, (c)
831 root, (d) stem, (e) total leaves and (f) pre-existing (PE) and newly-developed (ND)
832 leaves for plants grown at 23^{o}C and plants shifted to 5^{o}C. DM values were
833 transformed to $\log_e$. Values are means ± SE (n=4).
834 Plots (a-e): 23^{o}C grown plants closed triangles, 5^{o}C grown plants with PE leaves
835 only, closed circles; 5^{o}C grown plants with ND leaves, open circles. Plot (f); 23^{o}C
836 grown plants PE leaves closed triangles 23^{o}C grown plants ND leaves, open
837 triangles, 5^{o}C grown plants PE leaves, closed circles; 5^{o}C grown plants ND leaves,
838 open circles.

839 **Figure 2.** Leaf mass ratio (LMR), stem mass ratio (StMR) and root mass ratio (RMR)
840 for plants grown at 23^{o}C and plants shifted to 5^{o}C. Mass ratio values are plotted
841 against time in days (a-c) and against total plant dry mass (DM) (d-f). Values are
842 means ± SE (n=4).
843 Plots (a & e): 23^{o}C grown plants, closed triangles; 5^{o}C grown plants, closed circles.
844 Plots (b & f) 23^{o}C grown plants pre-existing (PE) leaves, closed triangles; 23^{o}C
845 plants newly-developed (ND) leaves, open triangles; 5^{o}C grown plants PE leaves
846 only, closed circles; 5^{o}C grown plants ND leaves, open circles. Plots (c, d, g & h):
847 23^{o}C grown plants, closed triangles; 5^{o}C grown plants with PE leaves only, closed
848 circles; 5^{o}C grown plants with ND leaves, open circles.

849 **Figure 3.** Specific leaf area (SLA; a & b) and leaf area ratio (LAR; c-f) of pre-existing
850 (PE) and newly-developed (ND) leaves for 23 and 5^{o}C grown plants. Values are
851 means ±SE (n=4).

SLA values are plotted against time in days (a) and total plant dry mass (DM) (b): 23^{o}C grown plants PE leaves, closed triangles; 23^{o}C plants ND leaves, open triangles; 5^{o}C grown plants PE leaves, closed circles; 5^{o}C grown plants ND leaves, open circles. (Note: The high value of SLA for PE leaves at Day 0 can be attributed to one plant with leaves that may not have been fully expanded at the time of measurement.) Leaf area ratio (LAR) for plants grown at 23^{o}C (c & d) and 5^{o}C (e & f) are plotted against time in days (c & e) or total plant dry mass (DM) (d & f): 23^{o}C grown plants - total LAR, dotted triangle; pre-existing (PE) leaves, closed triangles; newly-developed (ND) leaves, open triangles; 5^{o}C grown plants - total LAR, dotted circle; PE leaves, closed circles; ND leaves, open circles.

Figure 4. Mass-based total and organic nitrogen concentration (N_{mass}; mg g^{-1} DM) of (a) leaves, (b) stems and (c) roots of plants grown at 23^{o}C or shifted to 5^{o}C over the course of the experiment. 23^{o}C grown plants pre-existing (PE) leaves, closed triangles; 23^{o}C plants newly-developed (ND) leaves, open triangles; 5^{o}C grown plants PE leaves, closed circles; 5^{o}C grown plants ND leaves, open circles; total N, solid line; organic N dashed line.

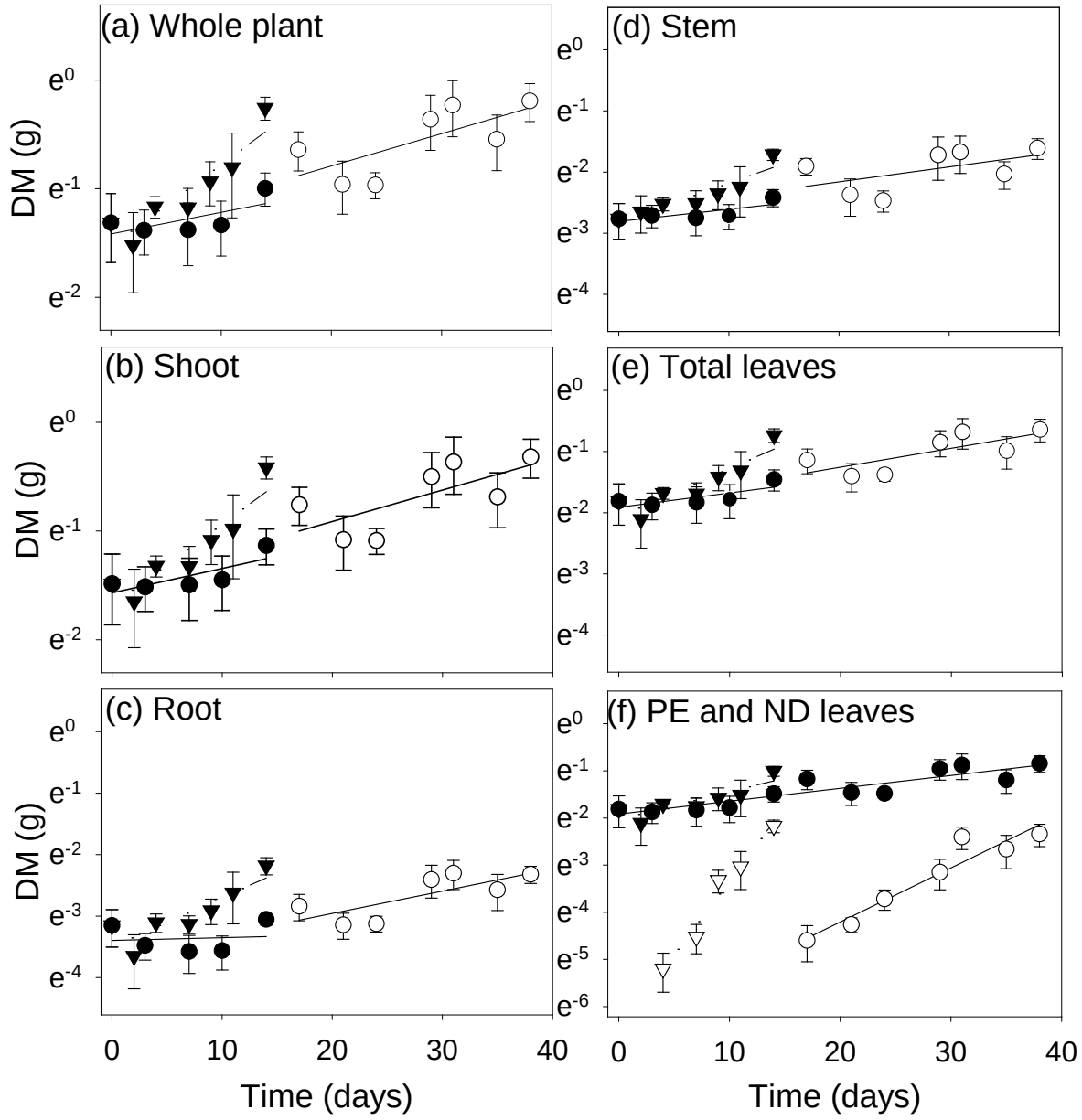
Figure 5. Leaf area-based total and organic nitrogen content (N_{area}; g m^{-2}) of pre-existing (PE) and newly-developed (ND) leaves of plants grown at 23^{o}C or shifted to 5^{o}C over the course of the experiment. 23^{o}C grown plants PE leaves, closed triangles; 23^{o}C plants ND leaves, open triangles; 5^{o}C grown plants PE leaves, closed circles; 5^{o}C grown plants ND leaves, open circles; total N, solid line; organic N dashed line.

874 **Figure 6.** Uptake of ^{15}N from the hydroponic solution by (a) pre-existing (PE) leaves
875 (b) newly-developed (ND) leaves, (c) stems and (d) roots of plants grown at 23 or
876 5^{o}C: 23^{o}C grown plants, closed triangles; 5^{o}C grown plants, closed circles.

877 **Figure 7.** Nitrogen uptake (a & c) on a whole plant basis (mg ^{15}N g^{-1} plant DM) and
878 (b & d) expressed as N uptake rates (mg ^{15}N g^{-1} root DM d^{-1}) plotted against time in
879 days (a & b) and total plant dry mass (DM) (c & d): 23^{o}C grown plants, closed
880 triangles; 5^{o}C grown plants, closed circles.

881

882 Figure 1.



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885 Figure 2.

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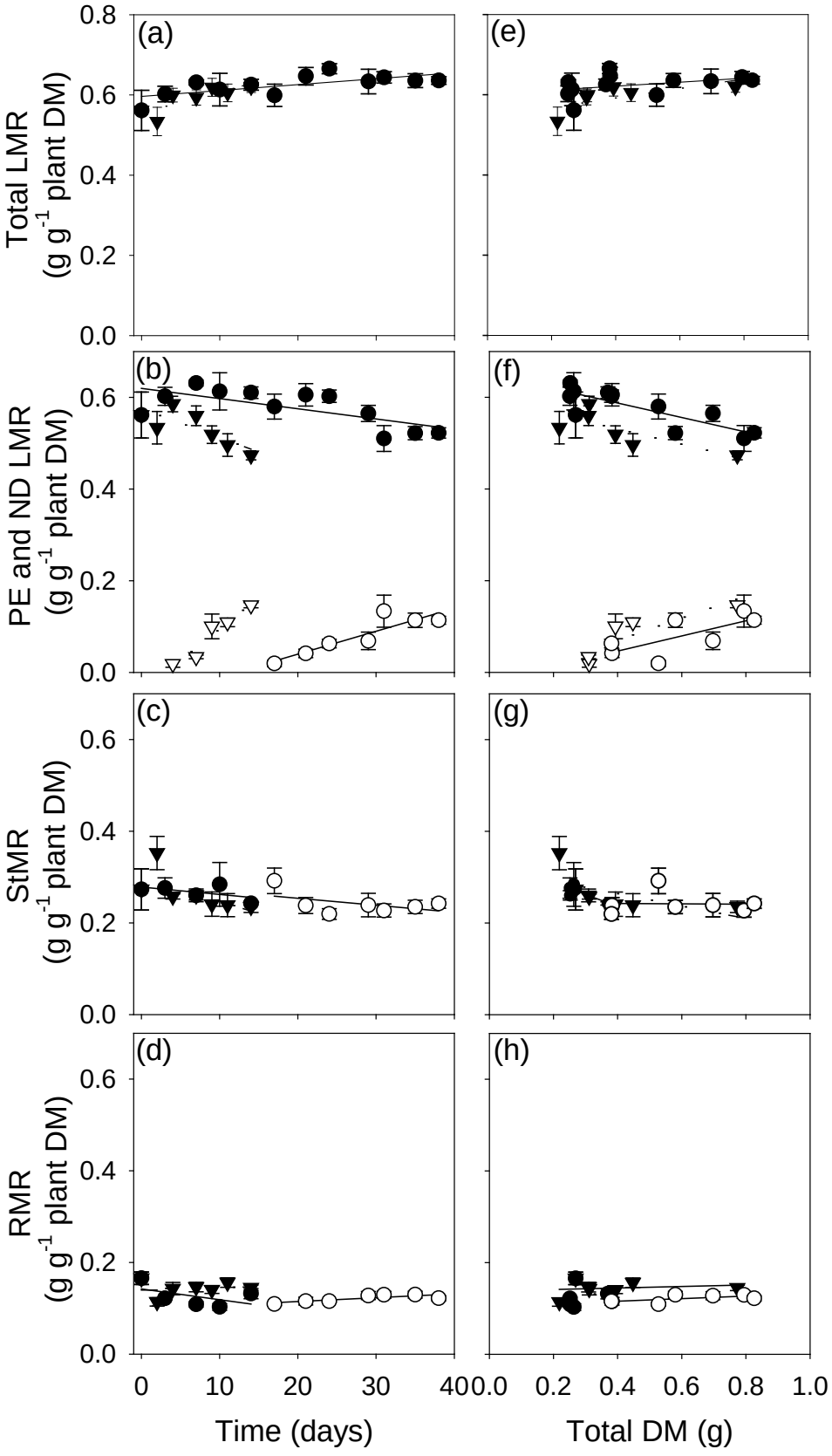

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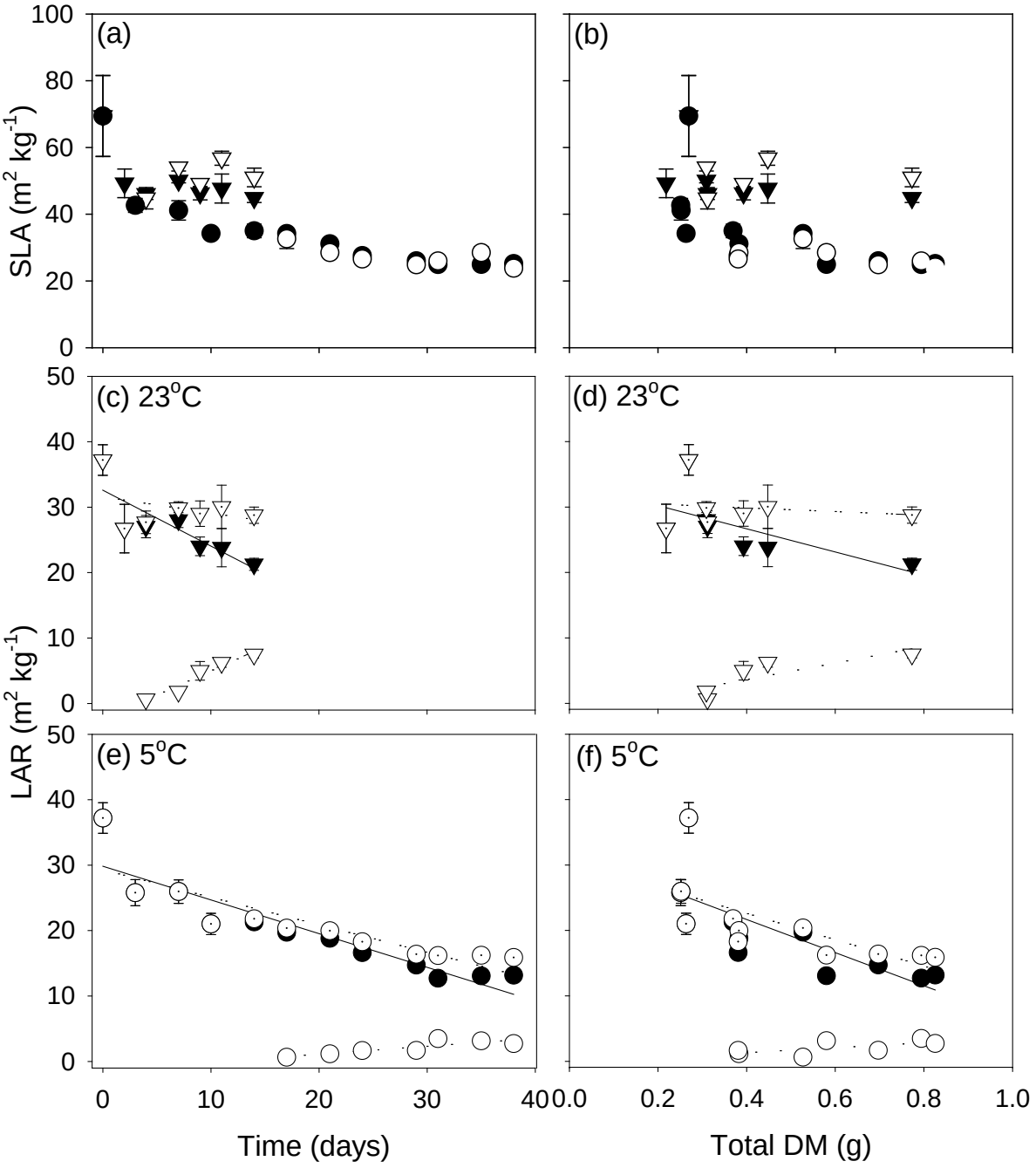

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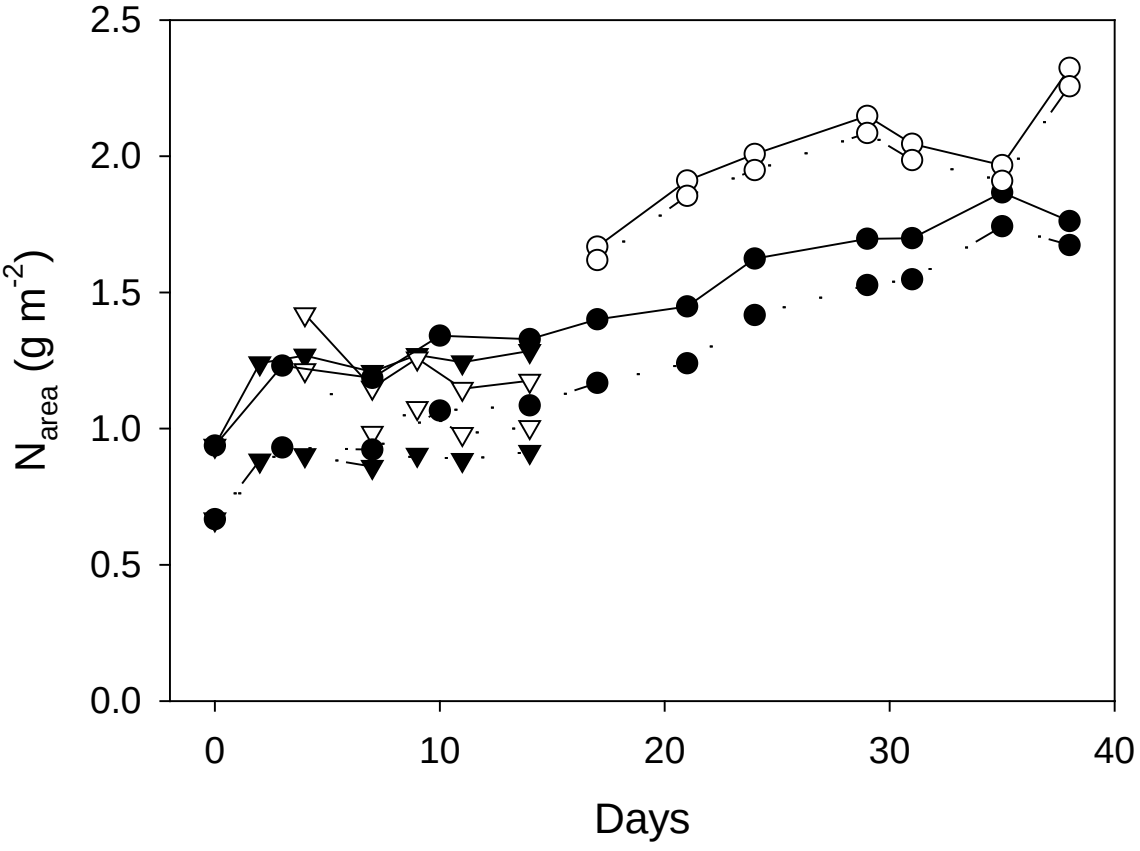
893 Figure 4.



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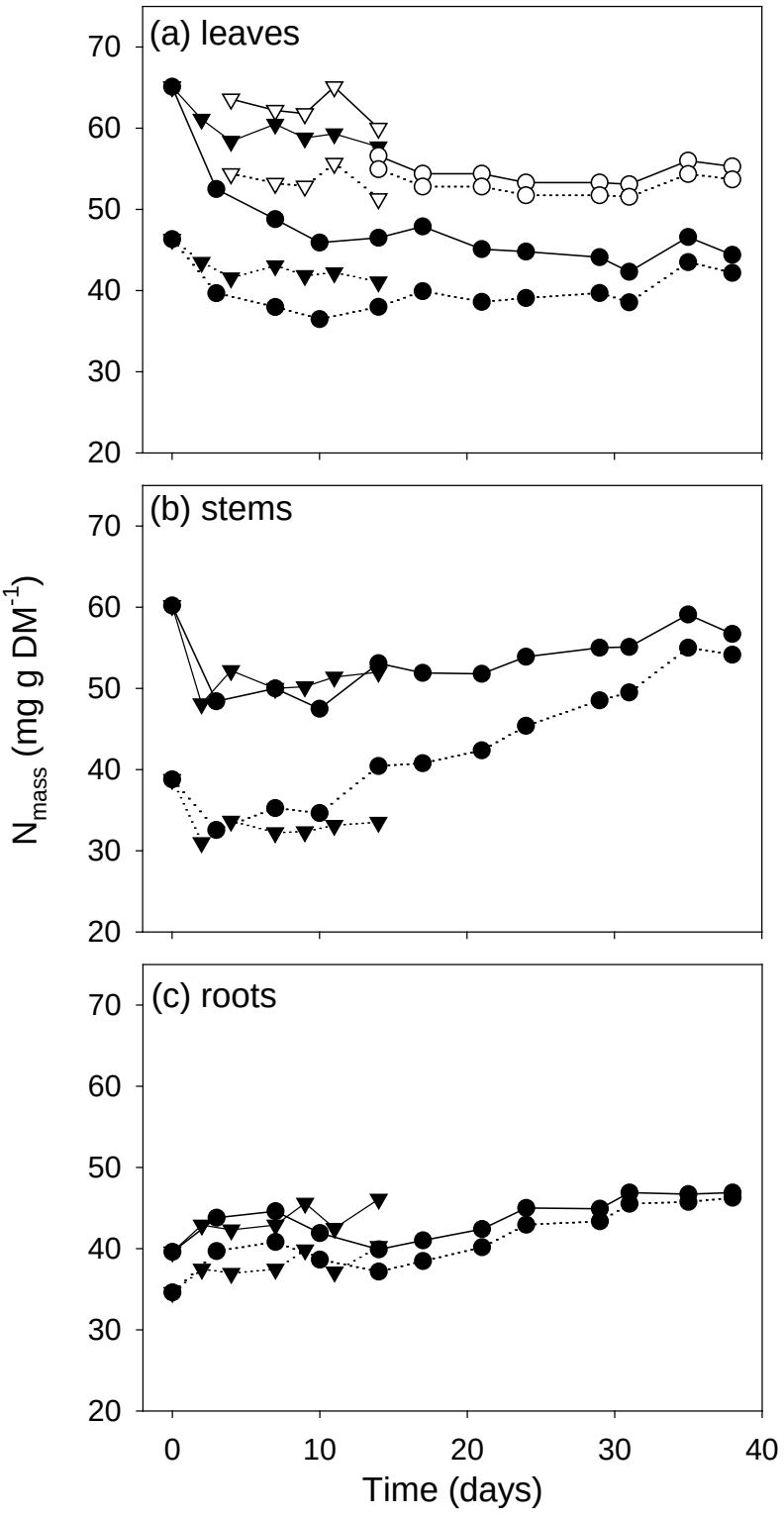

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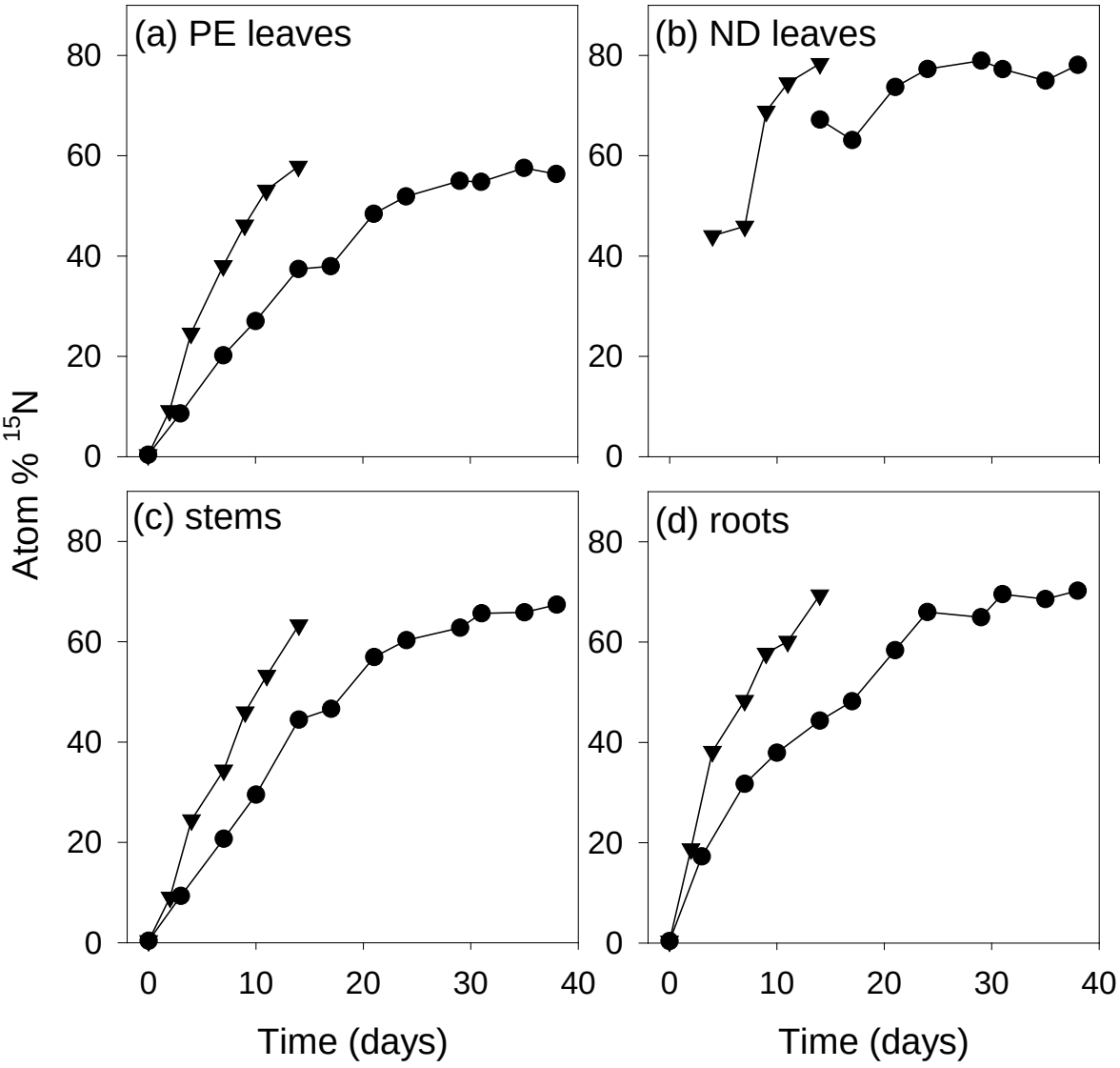

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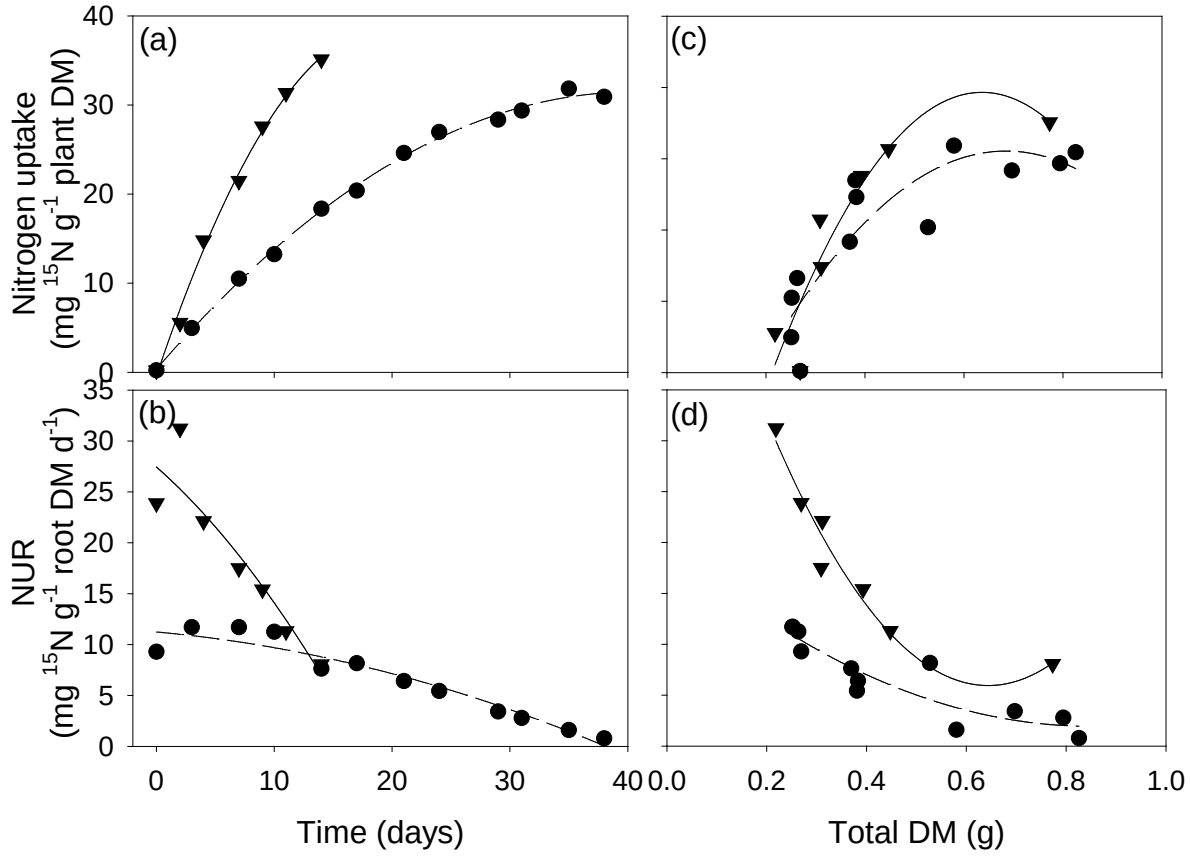
901 Figure 7.



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