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Respiratory alternative oxidase responds to both low- and high-temperature stress in *Quercus rubra* leaves along an urban–rural gradient in New York

Stephanie Y. Searle[1], Danielle S. Bitterman[2], Samuel Thomas[2], Kevin L. Griffin[3], Owen K. Atkin[4] and Matthew H. Turnbull[1,*]

[1]*School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand;* [2]*Department of Ecology, Evolution, and Environmental Biology, Columbia University, New York, New York, USA;* [3]*Department of Earth and Environmental Sciences, Lamont-Doherty Earth Observatory, Columbia University, Palisades, New York, USA; and* [4]*Functional Ecology Group, Research School of Biological Sciences, The Australian National University, Canberra, Australian Capital Territory, Australia*

Summary

1. Urban–rural transects can be utilized as natural gradients of temperature and also as a tool to predict how plant ecology and physiology might respond to expected global change variables such as elevated temperatures, CO_2 and inorganic nitrogen deposition.

2. We investigated differences in respiration (R) and the balance of electron partitioning through the cytochrome (CP) and alternative (AP) pathways in leaves of mature *Quercus rubra* L. trees along a transect from New York City to the Catskill Mountains over the course of one growing season. In addition, we investigated the effects of elevated temperature on *Q. rubra* seedlings in a controlled environment study.

3. In the field study, we found that urban-grown leaves often respired at greater rates than leaves grown at other sites and that this was likely due to higher leaf nitrogen. At each site, R at the prevailing growth temperature declined steadily throughout the growing season despite higher temperatures at the end of the summer. Differences in R were associated with changes in the relative abundances of cytochrome and alternative oxidase proteins. Oxygen isotope discrimination (D), which reflects relative changes in AP and CP partitioning, was negatively correlated with daily minimum temperature in trees grown at the colder rural sites, but not at the warmer urban sites.

4. In the growth cabinet study, we found that R acclimated to elevated temperatures and that this was accompanied by a steady increase in D.

5. These findings that AP partitioning increases with both high and low temperatures show that the AP may play an important role in plant responses to environmental conditions that elicit stress, and not simply to specific conditions such as low temperature.

Key-words: acclimation, leaf respiration, nonstructural carbohydrates, seasonal variation, oxygen isotope discrimination, cytochrome oxidase, urban ecology

Introduction

Urban ecology is a growing field that addresses ecosystem function in the context of the rapid expansion of urban areas worldwide. City dwellers account for approximately half of the world's population and are growing in numbers (Pickett *et al.* 2001), making it increasingly important to understand how urban forest remnants and city parks perform ecologically, affecting local climate and biogeochemical cycling. Urban environments are also being used in global change research, as they exhibit several environmental factors that are expected in coming decades worldwide (Carreiro & Tripler 2005).

Cities tend to have greater tropospheric CO_2 concentrations, atmospheric nitrogen deposition and higher temperatures (especially at night) compared with rural areas. In New York City (NYC), rates of inorganic nitrogen deposition are twice as high as in surrounding rural areas (Lovett *et al.* 2000). NYC is 3–5 °C warmer than surrounding rural areas at night (depending on season), while maximum daytime temperatures are similar (see Results section). Globally, warming

*Correspondence author. E-mail: matthew.turnbull@canterbury.ac.nz

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is expected to increase more at night than during the day (Easterling *et al*. 1997; Alward, Detling & Milchunas 1999), making urban–rural gradients such as those in NYC especially useful in simulating climate change. Interestingly, unlike other cities, CO_2 concentrations in NYC are only about 15 p.p.m. higher than global ambient concentrations (Hsueh 2009). This makes it possible to simplify an ecological study in New York to exclude CO_2 as a variable and more explicitly investigate the effects of other environmental parameters.

The terrestrial biosphere is currently a major sink for anthropogenically produced CO_2 (Schimel *et al*. 2001; Houghton 2003; Denman *et al*. 2007), but it is uncertain how the strength of this sink will be affected by global changes in environmental conditions (Friedlingstein *et al*. 2003; Houghton 2003; Jones, Cox & Huntingford 2003). In order to accurately predict and prepare for climate change, our understanding of how the carbon balance of plants, including plant respiration (R), responds to environmental factors must be improved. Plant R consumes 30–80% of daily photosynthetically fixed carbon (Poorter, Remkes & Lambers 1990; Ryan 1991; Amthor 2000) and is sensitive to temperature (Wager 1941), nitrogen (Reich *et al*. 1998), drought (Hsiao 1973) and CO_2, although this last point has been debated (Gonzalez-Meler & Taneva 2005). Understanding environmentally dependent variations in plant R will be crucial in estimating the strength of the terrestrial carbon sink, concentrations of atmospheric CO_2, and global surface temperatures in the future.

While R is sensitive to temperature, it can also acclimate to new temperature environments. The instantaneous temperature sensitivity of leaf R is exponential; however, its long-term temperature sensitivity is often much less pronounced, and it may even exhibit no long-term temperature sensitivity at all (Atkin, Bruhn & Tjoelker 2005). For instance, R may be the same when a species is grown and measured at 10 °C as when it is grown and measured at 20 °C; this process is termed 'acclimation'. Moreover, acclimation results in higher rates of R measured at a set temperature in cold-acclimated plants relative to their warm-acclimated counterparts. However, not all plants acclimate, and the degree to which they do varies greatly among species (Larigauderie & Korner 1995; Loveys *et al*. 2002). An improved understanding of the physiological components of R and how they change with environmental variation may aid our understanding of acclimation and ultimately lead to more precise predictions of how R in various plant groups will respond to global environmental change.

A major component of plant respiration is the cyanide-insensitive enzyme alternative oxidase (AOX), which, along with the cytochrome *c* oxidase (COX), catalyses the reduction of oxygen to water in the mitochondrial electron transport chain. Respiration via the alternative pathway (AP) results in, at most, one-third the ATP production of R via the cytochrome pathway (CP) (Vanlerberghe & McIntosh 1997; Millenaar & Lambers 2003). Thus, use of the AP negatively affects the energy efficiency of respiration and would result in greater rates of respiration to meet the same energy demands. Why plants utilize a seemingly energy wasteful pathway is a topic of debate, but it may represent a general stress response (Ribas-Carbo *et al*. 2000; Fiorani, Umbach & Siedow 2005; Sugie *et al*. 2006; Yoshida, Terashima & Noguchi 2007). Rates of AP respiration and AP partitioning have been shown to respond to cold (Gonzalez-Meler *et al*. 1999; Ribas-Carbo *et al*. 2000; Armstrong *et al*. 2008), heat (Rachmilevitch *et al*. 2007), drought (Ribas-Carbo *et al*. 2005b), high ambient CO_2 (Gomez-Casanovas *et al*. 2007; Gonzalez-Meler *et al*. 2009) and excess light (Noguchi *et al*. 2001). Furthermore, the response of AOX to temperature can change over time (Ito *et al*. 1997; Sugie *et al*. 2006; Armstrong *et al*. 2008). It is unclear whether changes in electron partitioning between the AP and CP pathways are associated with concomitant changes in relative abundances of the two oxidases (Guy & Vanlerberghe 2005). Furthermore, the number of studies reporting electron partitioning through the AP is small, and they are typically performed on laboratory-grown herbaceous plants. Thus, our understanding of how AOX affects the response of R to environmental variables in perennial plants in the field is extremely limited.

In this study, we investigated how respiration and AOX in *Quercus rubra* L. (Northern red oak) differ along an urban–rural gradient originating in New York City. We sampled branches from mature *Q. rubra* trees, which is a native and dominant tree species in north-eastern deciduous forests (Schuster *et al*. 2008), from four sites along a gradient (from Central Park in NYC to the Catskill range in upstate New York) throughout one growing season. We measured the temperature response of total R, relative changes in AP and CP electron partitioning, AOX and COX protein abundances, leaf nitrogen and nonstructural carbohydrates. We sought to understand how changes in environmental conditions along the gradient affect carbon release and its underlying physiology in an ecologically relevant species in the deciduous forest biome.

Based on many studies that have found R to acclimate to changes in temperature (discussed above), we hypothesized that R measured at the prevailing growth temperature would be similar among sites along our urban–rural gradient and throughout the growing season. The AP has been found to increase in response to sustained growth in the cold but also in response to sustained heat (citations above). As a deciduous tree, *Q. rubra* leaves do not experience cold winter temperatures; thus, we hypothesized that AP partitioning in *Q. rubra* leaves would increase with high summer temperatures and that these changes would be related to changes in AOX and COX protein abundances.

Materials and methods

FIELD SITES

We sampled at four sites along a transect from New York City to the Catskill Mountains. The 'urban' site was located on the east side of Central Park in New York City (40·780°N, 73·970°W, 28 m a.s.l.), the 'suburban' site at Lamont-Doherty Earth Observatory in Palisades, NY (41·005°N, 73·950°W, 107 m a.s.l.), the 'rural' site at Black Rock

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Forest near Cornwall, NY (41·430°N, 74·020°W, 115 m a.s.l.) and the 'remote' site near the Ashokan Reservoir in the Catskill Range (41·925°N, 74·248°W, 233 m a.s.l.).

Temperature data at the urban site were obtained from the National Oceanic and Atmospheric Administration (NOAA) weather station on Belvedere Castle in Central Park, logging approximately every hour (http://www.ncdc.noaa.gov). At the suburban site, temperature was measured by the Lamont Atmospheric Carbon Observation Project (LACOP; http://www.ldeo.columbia.edu/outr/LACOP/) logging every 15 min. Temperature at the rural site was measured by the Black Rock Forest Consortium every hour, and at the remote site, it was measured by the authors using a HOBO datalogger (Onset, Bourne, MA, USA) every hour. A HOBO logger was also installed at both the suburban and rural sites. Maximum daily temperature was slightly higher when measured by the HOBO dataloggers than with the established weather stations at these two sites, and the difference between the measured values was used to calibrate a correction for the temperature data at the remote site. Temperature data for all four sites are shown in Fig. 1.

Sun-exposed branches with fully expanded leaves were cut with a pole saw from six mature *Q. rubra* trees at a height of 6–7 m in the early morning at each site six times throughout the growing season: 19–20 May, 9–12 June, 30 June–1 July, 22–23 July, 9–10 August, and 2–3 September. Branches were recut under water and transported to the laboratory at Lamont-Doherty Earth Observatory in darkness. Previous measurements have shown that leaf respiration remains stable under these conditions for several hours (Turnbull *et al.* 2005), and this was confirmed in this study (data not shown).

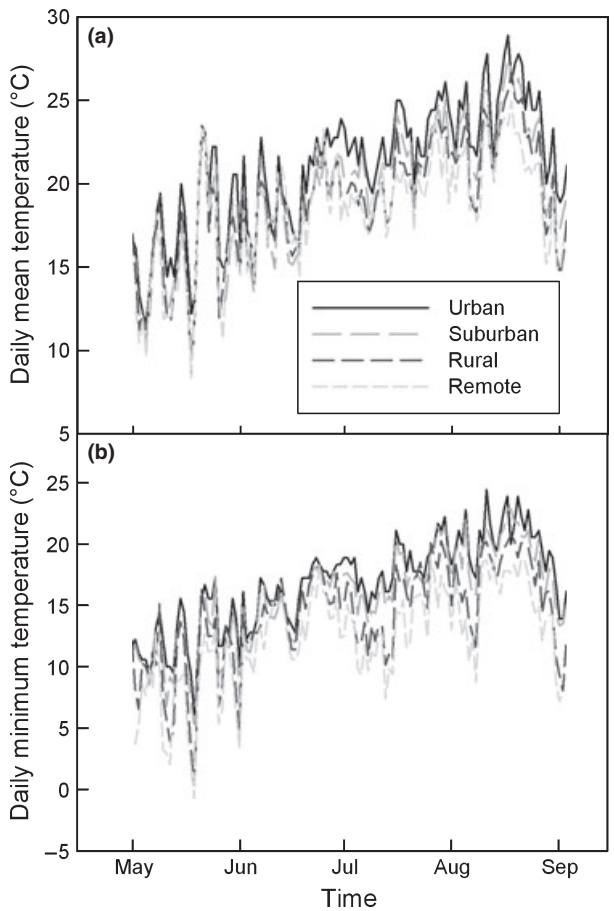


Fig. 1. Daily (a) mean and (b) minimum temperature at all four sites over the course of the field study.

GROWTH CABINET EXPERIMENT

The growth cabinet experiment was performed using six 2-year-old *Q. rubra* seedlings that had been germinated from acorns collected at Black Rock Forest, but grown at a sun-exposed, watered site in Central Park, NY. The seedlings were transported to the Lamont-Doherty Earth Observatory in June 2009 and housed in a greenhouse for 3 weeks prior to being placed in a growth cabinet. Once in the growth cabinet, they were subjected to night/day temperatures of 15/25 °C for 1 week before measurements began, to ensure acclimation to these relatively cool temperatures and moderate light levels. [Acclimation in *Q. rubra* has been shown to occur in as little as 1 day (Bolstad, Reich & Lee 2003)]. Measurements were then taken on days 0, 1, 4 and 7. On day 0, growth chamber temperatures were 15/25 °C. Immediately following measurements, growth chamber temperature was increased to 25/35 °C and remained at this temperature through day 7. Light levels were kept at 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and plants were watered daily throughout the experiment. Different leaves were measured each day.

RESPIRATION MEASUREMENTS

For the field study, branches of *Q. rubra* were transported to the laboratory and placed in a dark growth cabinet to manipulate temperature. For the growth cabinet study, plants were measured *in situ*. Dark respiration measurements were taken following the method described in Ow *et al.* (2010), using a portable infrared gas analysis system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA) at 10, 14, 18, 22 and 26 °C. The temperature response of respiration was modelled using a modified Arrhenius equation (Ryan 1991; Turnbull *et al.* 2003; Kruse & Adams 2008):

$$R = R_{10} \cdot e^{\frac{E_0}{g}\left(\frac{1}{T_{10}} - \frac{1}{T_a}\right)} \quad \text{(eqn 1)}$$

where R is the respiration rate, R_{10} is the respiration rate at a reference temperature, T_{10} is the reference temperature (here 10 °C), T_a is the measurement temperature of R, g is the ideal gas constant (8·314 J mol^{-1} K^{-1}) and E_0 is the temperature sensitivity of respiration, which is related to the overall activation energy. Bolstad, Reich & Lee (2003) found leaf respiration in *Q. rubra* to acclimate to changes in temperature in 1 day; thus, we calculated respiration at the prevailing growth temperature (R_g) by substituting R_{10} and E_0 values into eqn 1 where T_a was the minimum temperature the day prior to measurement. Nonlinear curve fitting was performed using the Marquardt–Levenberg algorithm (Sigma Plot, v8.0; SPSS Inc. Chicago, IL, USA).

OXYGEN ISOTOPE MEASUREMENTS

Changes in partitioning through the two terminal oxidase pathways in respiratory electron transport were assessed *in vivo* based on the premise that the CP and the AP discriminate against the heavier isotope of oxygen (^{18}O) to different extents (Guy *et al.* 1989; Ribas-Carbo, Robinson & Giles 2005a). We adopted an 'off-line' method modified from that of Nagel, Waldron & Jones (2001) that has been previously described (Searle *et al.* 2011a). *Quercus rubra* leaves were

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cut into large sections and placed in 12-mL exetainers (Labco, High Wycombe, UK). One half pellet of potassium hydroxide was present to absorb excess CO_2. Four exetainers were filled with varying amounts of leaf material in order to achieve a range of oxygen consumption. Samples were incubated in a water bath at 25 °C. After incubation, a syringe was inserted into each exetainer in order to sample the gas; a second water-filled syringe was inserted to allow water to replace the volume of gas removed. The sample gas was then injected into a 3·8-mL, pre-evacuated exetainer.

Gas samples were stored before being analysed on a Delta IV isotope ratio mass spectrometer (IRMS) equipped with GasBench II (Thermo Fisher Scientific, Waltham, MA, USA). Precision gas mixtures were used to calibrate a slight nonlinearity in the response of the IRMS. Exetainers were found to be contaminated with a small amount of gas (5–15% of total volume) before sampling. The amount and composition of this contamination were accounted and corrected for with nitrogen blanks. (Four exetainers were injected with oxygen-free nitrogen gas with each set of samples.) Stored samples were determined to leak at a rate of 0·7% per month, and so storage time was kept to a minimum (< 8 months). The specific effect of storage on the O_2/N_2 ratio of samples was determined and corrected for by storing standard gases of varying O_2/N_2 for various periods of time before measurement. Leakage depended on both storage time and the original concentration of oxygen present in the exetainer. These effects were found to be strongly linear and were corrected for in stored samples. The specific effect of storage on $^{18/16}O$ was determined empirically: a large set of *Pinus radiata* samples were collected in May 2009, and half were analysed immediately, while half were stored for 10 months. The calculated discrimination value of each set was used to calculate $^{18/16}O$ at specific values of oxygen drawdown; the $^{18/16}O$ ratio could then be directly compared between stored and freshly analysed samples. A strong linear relationship between $^{18/16}O_{stored}$ and $^{18/16}O_{fresh}$ was determined and utilized to correct for changes in $^{18/16}O$ owing to storage in samples. A linear rate of leakage over time was assumed. After applying these corrections, no systematic effect of storage time on discrimination values was found.

Calculations of oxygen isotope fractionation were made as described by Guy *et al.* (1989) and Ribas-Carbo *et al.* (2005a,b) with modifications to yield a value of discrimination against ^{18}O (D). We used changes in the O_2/N_2 ratio to reflect oxygen drawdown and did not include samples with > 50% oxygen consumption. We analysed all replicates of each species together; thus, the standard error presented represents the combined error of measurement and of biological variation. We did not use the R^2 value of discrimination regressions to determine the strength of the fit; following Guy *et al.* (1989), we report the standard error of the regression, which is more appropriate given the large and variable number (18–24) of points used in each calculation of D. Typically, a cut-off of $R^2 > 0{\cdot}995$ is applied to discrimination regressions using a small number of points; for example, such a regression using five points would have a standard error of c. 1·15. We validated this method by producing isotopic endpoints and uninhibited D values for *Phaseolus vulgaris* leaves similar to those reported in Noguchi *et al.* (2001). Additionally, our colleague measured KCN-inhibited D in cotyledons of *Glycine max* to be similar to that reported in Gonzalez-Meler *et al.* (1999) and Ribas-Carbo *et al.* (1997) (A. Kornfeld, unpublished data).

Calculation of electron partitioning through the AP and CP depends on the knowledge of endpoints; discrimination is measured on potassium cyanide- and salicylhydroxamic acid-treated tissues, which inhibit COX and AOX, respectively. Discrimination in uninhibited tissues is then compared to the isotopic endpoints of AOX and COX to calculate electron partitioning through each pathway. However, despite exhaustive efforts, we were not able to obtain isotopic endpoints for the AP and CP in *Q. rubra*. There appears to have been diffusional issues preventing proper gas sampling after the leaves had been incubated in potassium cyanide. However, residual respiration rates in leaves sampled along the transect in 2005 that were inhibited by both KCN and SHAM were measured by the authors to be relatively consistent among sites (12–20%; S. Y. Searle, unpublished data); thus, it is unlikely that changes in discrimination reflect activities of oxidases other than AOX and COX. In the absence of isotopic endpoints, we interpret changes in discrimination as relative changes in electron partitioning through the AP and CP.

IMMUNOBLOTTING

Leaf samples were snap-frozen in liquid nitrogen, freeze-dried, ground with a ball mill and shipped to the University of Canterbury for analysis. Samples were prepared in a buffer solution containing 2-mercaptoethanol and loaded into 4–12% gradient polyacrylamide gels for SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane using iBLOT (Invitrogen, San Diego, CA, USA). Immunoblotting was performed using Snap i.d. (Millipore, Billerica, MA, USA). Blots were first analysed for AOX with an anti-AOX monoclonal antibody (Elthon, Nickels & McIntosh 1989) at a dilution of 1 : 300 and detected using an anti-mouse horseradish peroxidase (HRP) conjugate and a noncommercial enzymatic chemiluminescence (ECL) solution, modified from the study of Haan & Behrmann (2007). Blots were photographed with a cryo-cooled digital camera (Chemigenius, Syngene, Cambridge, UK). After detection of AOX, the HRP conjugate was deactivated by incubating the blot in 15% H_2O_2. The blot was then reanalysed with anti-COX (Agrisera, Vannas, Sweden) at a dilution of 1 : 600 and detected using an anti-rabbit HRP conjugate and ECL Advance (GE Healthcare, Waukesha, WI, USA). Bands were quantified using densitometry (ImageJ; NIH, Bethesda, MD, USA). Representative images of protein bands are shown in Fig. 2.

A standard sample of *Q. rubra* sampled in July from the suburban site was run on each blot and used to standardize all samples; i.e. the brightness of each sample's protein band was divided by the brightness of the standard band on that blot. This protein abundance for each sample was then divided by the mean abundance of all analysed samples for each of AOX and COX in order to equalize the distribution of these two proteins. In order to simultaneously view changes in AOX and COX abundances, relative AOX protein abundance was then divided by relative COX abundance; thus, we report the AOX/COX ratio. An AOX/COX ratio of 1 represents the mean AOX/COX ratio of all samples in this study.

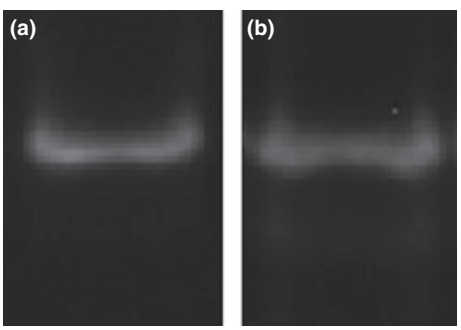


Fig. 2. Representative images showing protein bands of (a) alternative oxidase and (b) cytochrome *c* oxidase from *Quercus rubra*.

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LEAF CHARACTERISTICS

Approximately three leaves from each tree were sampled for the analysis of specific leaf area (SLA), nonstructural carbohydrates and nitrogen (N). These samples were taken from cut branches *c*. 2 h after field sampling (late morning). These leaves were analysed on a leaf area meter (Licor), dried in an oven at 65 °C, weighed and ground with a ball mill. SLA was calculated as leaf area divided by weight. Soluble sugar and starch were analysed following the method of Tissue & Wright (1995). Leaf nitrogen was analysed on a Delta plus IRMS in the Stable Isotope Laboratory at Washington State University (Seattle, WA, USA). Mass fractions were determined from total area of IRMS peaks calibrated against standards measured on a stand-alone thermal conductivity detector.

STATISTICAL ANALYSIS

Statistical analyses were performed using R 2.4.0 (R Development Core Team). As different leaves were used on each sampling date, a two-way analysis of variance (ANOVA) was used to detect changes in parameters over time and between sites. Simple linear regressions were used to test correlations between protein abundances, carbohydrates, nitrogen and respiratory parameters. A Chow test was used to detect differences in oxygen isotope discrimination between measurement days in the growth cabinet experiment. Comparisons and regressions were considered significant if $P < 0{\cdot}05$.

Results

FIELD STUDY

Daily average and minimum field temperatures are shown for all four sites in Fig. 1. Temperatures generally rose from the beginning of the study to late August and declined rapidly thereafter. Averaged over the course of this study, temperatures at the rural, suburban and urban sites were 0·5, 1·2 and 6·7 °C warmer than at the remote site, respectively. Nighttime temperatures were as much as 12 °C warmer at the urban site relative to the remote site. Temperatures along the gradient were generally cooler in 2009 than in recent years. For instance, the maximum temperature at the rural site in 2009 was *c*. 2 °C lower than in 2005, 2006 or 2008 (data not shown).

Results of two-way ANOVAs for respiratory parameters and the AOX/COX protein ratio are shown in Table 1. The rate of respiration at 10 °C (R_{10}) declined rapidly from May to June at all sites and continued to decline slowly through the end of the growing season (Fig. 3a); the change in R_{10} over time was significant. There was no significant difference in R_{10} between sites over the course of the experiment. The temperature sensitivity of respiration (E_0) was variable throughout the growing season without any clear trend at each of the sites (Fig. 3b). E_0 was not significantly different between sites, nor did E_0 change significantly over time. Respiration at the growth temperature (R_g; the minimum temperature the day prior to measurement) generally declined throughout the growing season at all sites (Fig. 3c); however, the decline in R_g was considerably less than that exhibited by rates of R_{10}. R_g changed significantly over time and was significantly different between sites. Respiration on a nitrogen basis (R_g/N) also changed significantly over time and was significantly different between sites (Fig. 3d) although the difference between sites was smaller than with R_g (Table 1). There was a significant interaction between site and time with R_g, but not with R_{10}, E_0 or R_g/N.

R_g showed a significant positive correlation with leaf nitrogen (N) when all sites and sampling dates were pooled (Fig. 4; $R^2 = 0{\cdot}218$, $P = 0{\cdot}021$). Leaves at the urban site contained significantly greater nitrogen concentrations than leaves at the other sites (Table 2). Soluble starch, sugar and SLA values are also shown in Table 2. With all sites pooled, there was a statistically significant but weak negative correlation between R_g and leaf starch concentration ($R^2 = 0{\cdot}191$, $P = 0{\cdot}033$), with greater respiration at the growth temperature occurring when leaf starch was low. There was no trend between R_g and leaf soluble sugar. There was a weak positive correlation between R_g and the AOX/COX protein ratio when all sites were pooled ($R^2 = 0{\cdot}148$, $P = 0{\cdot}063$), with a greater ratio of AOX to COX protein abundance being associated with higher rates of R_g (Fig. 5). The AOX/COX protein ratio changed significantly both over time and between sites, but there was no time–site interaction (Table 1).

Discrimination is plotted against the minimum temperature the night prior to sampling for each site in Fig. 6a. Simple linear regression analysis showed a significant relationship between discrimination and temperature in the rural ($R^2 = 0{\cdot}836$, $P = 0{\cdot}011$) and remote ($R^2 = 0{\cdot}748$, $P = 0{\cdot}026$) sites, but not in the urban ($R^2 = 0{\cdot}006$, $P = 0{\cdot}881$) or suburban ($R^2 = 0{\cdot}002$, $P = 0{\cdot}941$) sites. D at each site is shown over time in Fig. 6b.

GROWTH CABINET EXPERIMENT

R_{10} dropped sharply from day 0 at 15/25 °C to day 1 at 25/35 °C and remained at a low rate through days 4 and 7 (Fig. 7a). R_{10} was significantly lower on days 4 and 7 than it had been on day 0 ($P = 0{\cdot}034$). E_0 rose from day 0 to 1 and declined from day 1 to 7; however, these changes were not

Table 1. Results of two-way ANOVA for R_{10}, E_0, R_g, R_g/N and the alternative oxidase (AOX)/cytochrome *c* oxidase (COX) protein ratio

	R_{10}	E_0	R_g	R_g/N	AOX/COX
Time	**< 0·001; 106·4**	0·617; 0·252	**< 0·001; 23·056**	**< 0·001; 21·832**	**0·003; 9·016**
Site	0·804; 0·062	0·764; 0·091	**< 0·001; 30·898**	**< 0·001; 14·240**	**0·041; 4·272**
Interaction	0·999; 0	0·723; 0·126	**0·0324; 4·682**	0·050; 3·905	0·554; 0·3511

Values shown are P; F. Bold indicates statistically significant effects.

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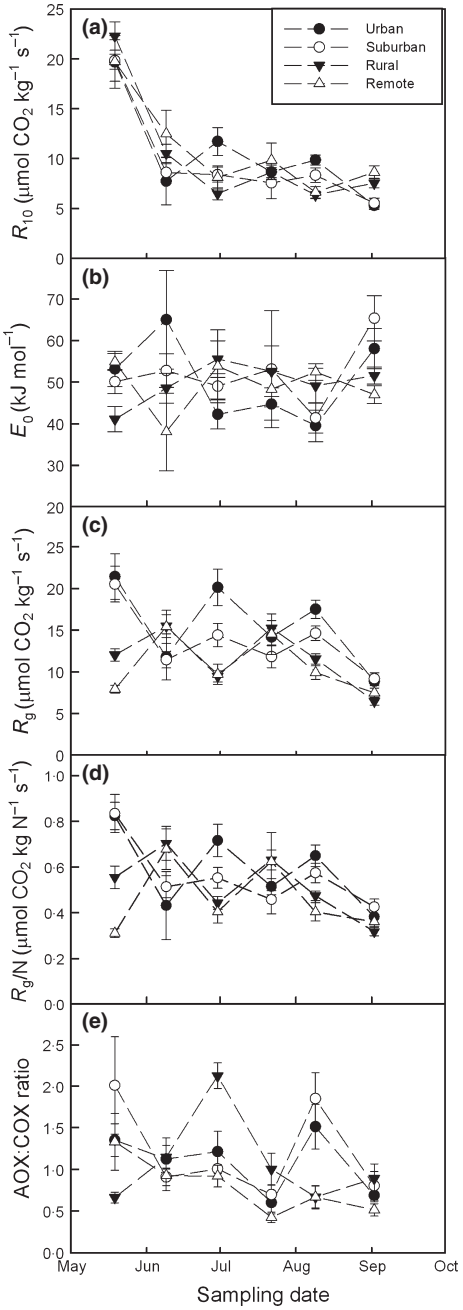


Fig. 3. Respiratory parameters for each site over the growing season. (a): R_{10}; (b): E_0; (c): Respiration at the growth temperature (R_g; the minimum temperature the day prior to measurement) over the growing season; (d): R_g on a nitrogen basis (R_g/N) over the growing season; (e): the alternative oxidase/cytochrome *c* oxidase ratio over the growing season. Values shown are mean ± SEM, where $n = 6$.

significant ($P = 0{\cdot}201$; Fig. 7b). R_g rose from day 0 to 1 with the increase in temperature but declined again on days 4 and 7 (Fig. 7c). Figure 7c also shows the rate of respiration that would be predicted at the elevated growth temperature on days 1–7 using R_{10} and E_0 values measured on day 0. Measured R_g was significantly lower than predicted values on days 4 and 7 ($P = 0{\cdot}042$; $0{\cdot}040$), indicating acclimation to the higher temperatures.

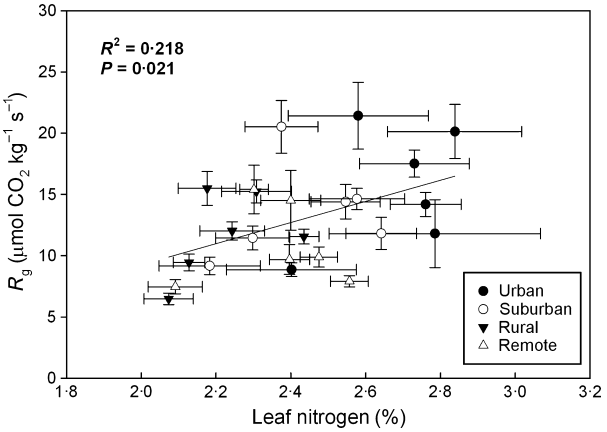


Fig. 4. Respiration at the growth temperature (R_g) plotted against leaf nitrogen (N) in the seasonal study. Values shown are mean ± SEM, where $n = 6$. The line represents the linear regression through data pooled from all sites.

Leaf soluble sugars were relatively constant through the growth cabinet experiment ($P = 0{\cdot}456$; Fig. 7d). Leaf starch declined with the warming treatment and was significantly lower on day 4 than on day 0 ($P < 0{\cdot}001$; Fig. 7d). Discrimination was similar between days 0 and 1 but rose significantly on days 4 and 7 ($P = 0{\cdot}020$; $< 0{\cdot}001$), indicating a greater contribution of the AP to respiration at higher growth temperatures (Fig. 8a). The AOX/COX protein balance declined with the warming treatment and was significantly different between days 0 and 4 ($P = 0{\cdot}003$; Fig. 8b).

Discussion

Our study sought to understand how changes in environment provided by an urban–rural gradient impact on respiratory processes of a common deciduous tree of broadleaved forests of North America. Importantly, this study is the first to combine measurements of *in vivo* respiratory CO_2 release with estimates of respiratory ^{18}O discrimination along an urban–rural environmental gradient. We show that while *Q. rubra* has the potential to thermally acclimate respiratory processes (Fig. 7), there was little evidence of this across the urban–rural environmental gradient (Fig. 3). However, the gradient did have marked effects on leaf respiration rates, as evidenced by site-to-site differences in respiration (measured at the prevailing growth temperature – R_g), protein abundance and ^{18}O discrimination (D, indicative of relative changes in the AP and CP, responded to both high and low temperatures in *Q. rubra*).

We found that the most important factor associated with differences in R_g along the gradient was leaf nitrogen (N). R_g was significantly different between sites, often being higher at the urban site than at the other sites (Fig. 3c; Table 1). Urban-grown leaves had significantly higher N than leaves grown at other sites (Table 2), and respiration was positively correlated with N (Fig. 4). Nitrogen has been reported to be an important factor in determining respiration rates in a

Table 2. Leaf nitrogen (*N*), nonstructural carbohydrates and specific leaf area (SLA) in the seasonal study. Statistical significance is shown, with bold indicating significant effects

Site	Sampling period	*N* (%)	Soluble sugar (%)	Starch (%)	SLA (m^2 kg^{-1})
Urban	1	2·58 ± 0·19	12 ± 1·3	7·2 ± 0·6	15·7 ± 1·3
	2	2·79 ± 0·28	7·9 ± 0·6	5·5 ± 0·2	12·8 ± 0·7
	3	2·84 ± 0·18	9·2 ± 0·4	6·6 ± 0·3	11·4 ± 0·9
	4	2·76 ± 0·09	8·0 ± 0·6	6·6 ± 0·8	12·4 ± 1·1
	5	2·73 ± 0·15	6·3 ± 0·6	10·2 ± 0·6	9·9 ± 0·5
	6	2·40 ± 0·17	5·8 ± 0·4	6·5 ± 0·6	10·6 ± 0·5
Suburban	1	2·38 ± 0·10	13·2 ± 1·7	7·1 ± 0·8	15·8 ± 0·8
	2	2·30 ± 0·10	8·6 ± 0·5	5·6 ± 0·6	11·7 ± 0·5
	3	2·55 ± 0·09	8·3 ± 0·4	8·8 ± 0·8	10·4 ± 0·4
	4	2·64 ± 0·09	8·0 ± 0·3	8·9 ± 0·3	11·3 ± 0·4
	5	2·58 ± 0·13	7·9 ± 0·9	8·7 ± 0·9	10·5 ± 0·5
	6	2·18 ± 0·14	6·0 ± 0·8	7·2 ± 0·9	12·1 ± 0·4
Rural	1	2·24 ± 0·09	18·6 ± 1·9	7·8 ± 0·5	19·2 ± 2·7
	2	2·18 ± 0·08	11·2 ± 0·6	6·3 ± 0·4	10·8 ± 1·0
	3	2·13 ± 0·04	12·0 ± 0·6	9·3 ± 1·1	10·9 ± 0·6
	4	2·31 ± 0·09	11·4 ± 1·3	7·0 ± 0·3	12·0 ± 0·8
	5	2·44 ± 0·04	8·1 ± 0·7	12·5 ± 1·4	10·4 ± 0·6
	6	2·07 ± 0·07	6·7 ± 1·1	7·2 ± 1·3	9·5 ± 0·3
Remote	1	2·56 ± 0·05	19·4 ± 1·0	12·0 ± 0·9	18·5 ± 0·8
	2	2·30 ± 0·04	11·3 ± 0·6	5·8 ± 0·5	12·0 ± 0·4
	3	2·40 ± 0·05	14·3 ± 0·7	7·9 ± 1·1	9·8 ± 0·5
	4	2·40 ± 0·08	11·7 ± 1·3	6·8 ± 0·5	11·6 ± 1·2
	5	2·48 ± 0·05	5·4 ± 0·5	13·8 ± 0·9	10·4 ± 0·4
	6	2·09 ± 0·07	5·8 ± 0·8	9·4 ± 0·5	10·3 ± 0·3
Statistical significance (*P*)	Over time	0·208	**< 0·001**	**< 0·001**	**0·007**
	Between sites	**< 0·001**	0·981	**< 0·001**	0·055
	Interaction	0·656	0·158	**0·040**	**0·004**

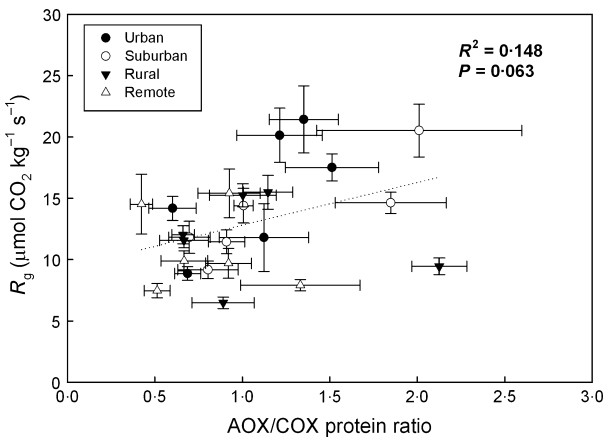


Fig. 5. Respiration at the growth temperature (R_g) plotted against the alternative oxidase/cytochrome *c* oxidase protein ratio in the seasonal study. Values shown are mean ± SEM, where $n = 6$. The dotted line represents the linear regression through data pooled from all sites.

variety of species (Reich *et al.* 1998; Tjoelker, Reich & Oleksyn 1999; Wright *et al.* 2006; Tjoelker *et al.* 2008), including *Q. rubra* (Xu & Griffin 2006), and is an especially important driver in urban ecosystems, where atmospheric nitrogen deposition is a major factor in urban pollution (Lovett *et al.* 2000). The results shown here provide further field evidence for a link between *N* and respiration rates.

In the absence of acclimation, one might expect that R_g would *increase* in line with the increasing growth temperature across the urban–rural gradient. However, our controlled environment experiment demonstrated that rapid acclimation can occur in *Q. rubra*. In this experiment, R_g acclimated substantially to the 10 °C warming regime, with respiration at the growth temperature being only slightly higher after 4 and 7 days of warming than it had been before the warm treatment was imposed. This finding provides further field support for those of Bolstad, Reich & Lee (2003), who found *R* in *Q. rubra* seedlings acclimated to changes in growth temperature. It also suggests that differences in R_g observed across the urban–rural transect were unlikely to be a result of the 6·7 °C average temperature difference between the urban and rural areas. Rather, differences in leaf nitrogen were likely responsible for the site-to-site differences in R_g.

Despite strong evidence for thermal acclimation of *R* from the cabinet experiment, R_g in the field actually declined throughout the growing season despite climbing temperatures from May through August (Fig. 3c, Table 1). Although several studies have found evergreen leaf respiration to acclimate to seasonal changes in temperature (Stockfors & Linder 1998; Atkin, Holly & Ball 2000; Vose & Ryan 2002; Ow *et al.* 2010), the extent to which thermal acclimation is responsible for seasonal changes in *R* in deciduous trees in the field is not easily disentangled from other processes. Respiration in deciduous trees has often been found to decline steadily throughout the growing season (Searle & Turnbull 2011b;

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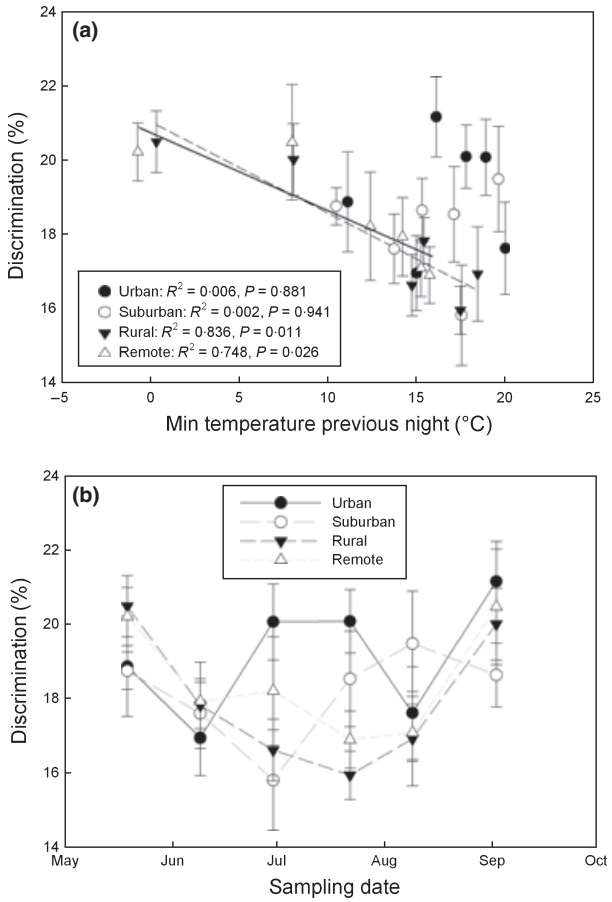


Fig. 6. (a) Discrimination plotted against the minimum temperature the night prior to measurement in the seasonal study for each site. Higher discrimination values indicate greater relative contribution of the alternative pathway (AP) to respiration, and lower values indicate greater cytochrome pathway contribution. Linear regressions are shown for the rural (dotted line) and remote (solid line) sites. Statistics for linear regressions for each site are shown in the figure legend. (b) Discrimination at each site over time. Values shown are mean ± SEM, where $n = 6$.

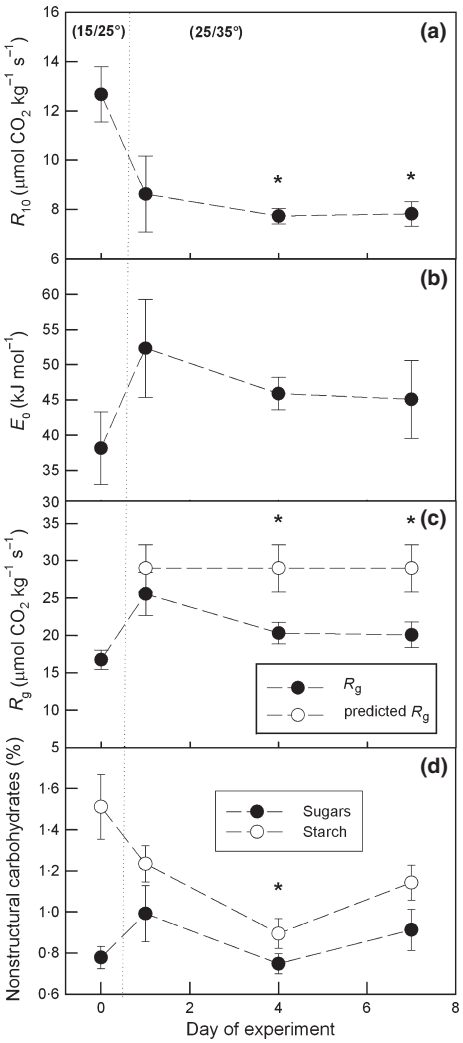


Fig. 7. Respiratory parameters and leaf carbohydrates over day of the growth cabinet experiment. (a): R_{10}; (b): E_0; (c): measured R_g and R_g predicted at the new growth temperature by the R_{10} and E_0 values measured on day 0; (d): Leaf starch and soluble sugar. On day 0, plants were acclimated to night/day temperatures of 15/25 °C; on days 1–7, plants were exposed to temperatures of 25/35 °C. Stars indicate significant differences from day 0 in (a, b, d) and between measured and predicted R_g in (c). Values shown are mean ± SEM, where $n = 6$.

Ow *et al.* 2010; Xu & Griffin 2006; Rodriguez-Calcerrada *et al.* 2010; Marra *et al.* 2009), although why R declines in deciduous leaves is not fully understood. The complexities of environmental, developmental and physiological drivers mean that establishing a mechanism for the seasonal decrease in R in deciduous tree leaves is difficult. However, this decline has crucial implications for estimating carbon uptake in deciduous forests worldwide, especially in north-eastern US forests, which are currently considered a major carbon sink (Myneni *et al.* 2001; Pacala *et al.* 2001; Schuster *et al.* 2008).

Another factor that was associated with the seasonal decline in R_g was changes in key respiratory proteins. There was a relationship between R_g and the ratio of AOX to COX protein abundance when data were pooled between sites and sampling dates, although this relationship was weak (Fig. 5). Our field results are similar to those reported in Searle & Turnbull (2011b) where a seasonal decline in R_g in leaves of *Populus* × *canadensis* was accompanied by a decline in the AOX/COX balance. We have also found that the AOX/COX balance was associated with the thermal acclimation of R_d in the alpine grass *Chionochloa pallens* (Searle *et al.* 2011a).

Contrary to our hypothesis, abundance of AOX and COX proteins in both the field and growth cabinet study was not correlated with discrimination (D), which here we interpret as indicating relative changes in electron partitioning between the AP and CP. This is consistent with other studies, which have also found no relationship between AOX protein abundance and discrimination or AP engagement (Vidal *et al.* 2007; Armstrong *et al.* 2008; Guy & Vanlerberghe 2005; Lennon *et al.* 1997; Ribas-Carbo *et al.* 2005b). The lack of correlation may be partially attributable to the fact that the AOX protein exists in either an oxidized form or a reduced dimer

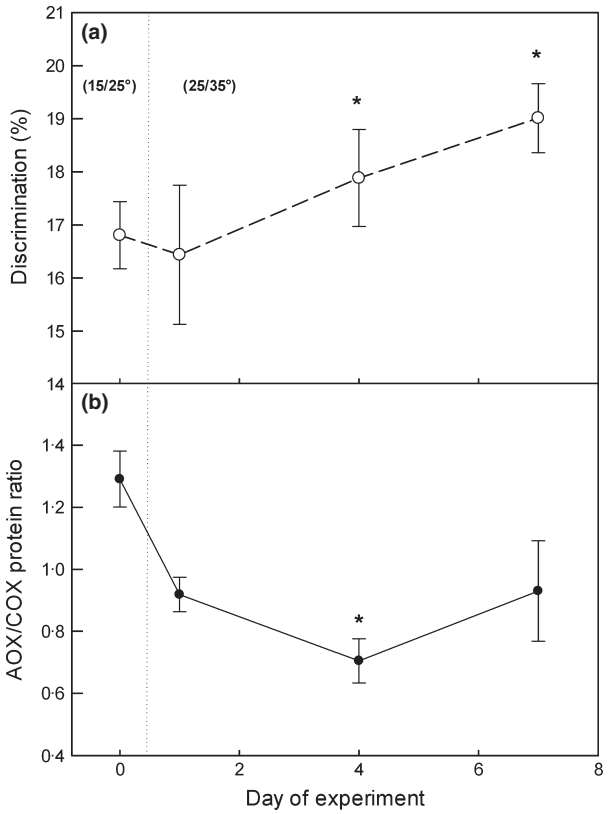


Fig. 8. (a) Discrimination over day of the growth cabinet experiment. Higher discrimination values indicate greater relative contribution of the alternative pathway (AP) to respiration, and lower values indicate greater cytochrome pathway contribution. (b): alternative oxidase/cytochrome *c* oxidase protein ratio over day of experiment. On day 0, plants were acclimated to night/day temperatures of 15/25 °C; on days 1–7, plants were exposed to temperatures of 25/35 °C. Stars indicate significant differences from day 0. Values shown are mean ± SEM, where $n = 6$.

and is thought to be active in only the oxidized state (Umbach & Siedow 1993; Umbach, Wiskich & Siedow 1994; Umbach, Fiorani & Siedow 2005). Clearly, changes in activity and AP/CP partitioning may be quicker than, and not necessarily reflect, turnover of the AOX and COX proteins.

Respiratory oxygen isotope discrimination (D) in leaves from the rural and remote sites showed strong negative correlations with the minimum temperature the previous night (Fig. 6), indicating that at these colder sites, the AP was up-regulated relative to the CP following lower temperatures. D has been negatively correlated with temperature in *Chionochloa rubra* and *C. pallens* in the field (Searle *et al.* 2011a), and discrimination has been found to increase in leaves exposed to cold in controlled environment studies (Gonzalez-Meler *et al.* 1999; Ribas-Carbo *et al.* 2000; Armstrong *et al.* 2008). Our results provide additional field support for the notion that the AP responds to low (night-time) temperatures.

In contrast, there was no relationship between variations in D and overnight minimum temperature in leaves from the urban and suburban sites (Fig. 6a). Figure 6b shows that D was highest in the rural and remote leaves at the beginning and end of the season, when temperatures were relatively cool, but some of the highest values of D at the urban and suburban sites occurred during mid-summer, when air temperatures reached 33 °C in the city. This suggests that the higher temperatures at the more urban sites may have elicited a heat response. These field results were supported by those of the growth cabinet experiment in which we subjected *Q. rubra* seedlings to high temperatures (night/day 25/35 °C) that are similar to maximum temperatures reached at the urban and suburban sites. D rose significantly from day 0 to 4 and 7 (Fig. 8a), indicating a clear response to heat. Although this finding is consistent with our hypothesis that D would respond to heat, it provides a contrast with the results from the rural and remote sites in the field study and with previous studies (listed above) linking AP engagement with cold stress. Rachmilevitch *et al.* (2007) found higher AP partitioning with heat stress in roots of a heat-adapted *Agrostis* species, while Murakami & Toriyama (2008) reported increased heat tolerance in transgenic rice seedlings over-expressing AOX. Our seemingly contradictory results point towards a notion that is currently forming in the literature: that AOX functions to reduce various types of stress in plants and does not respond to changes in specific environmental variables such as low temperature (Ribas-Carbo *et al.* 2000; Fiorani, Umbach & Siedow 2005; Sugie *et al.* 2006; Yoshida, Terashima & Noguchi 2007).

We did not find a link between D and R_g or other respiratory parameters (i.e. R_{10}, E_0). In particular, D in the growth cabinet study rose during the 7 days of warm treatment, while R_g acclimated and remained fairly stable. The increase in D likely resulted from both an increase in the AP as well as a decrease in the CP in order to maintain a stable rate of respiration. Although D cannot be directly compared with respiration rates measured by CO_2 release, the discrepancy between these measurements (i.e. the respiratory quotient) is not likely to be variable enough to invalidate comparison of long-term trends. Initially, we thought that an increase in the AP would result in greater respiration, as R via the AP would likely be associated with greater rates of tricarboxylic acid cycle activity (and thus higher rates of CO_2 release) in order to meet the same energy demands as R via the CP (depending on whether the external NAD(P)H dehydrogenase is used as an input of electrons to the ubiquinone pool). On the contrary, the balancing between the two oxidative pathways prevents an increase in R_d while up-regulating the AP in response to stress.

In summary, our findings provide novel insights into plant respiratory function and its metabolic underpinnings along an 'urban–rural' environmental gradient. The implications of these findings for the carbon balance of trees in the deciduous forest biome are significant. Several environmental factors that are stressful to plants are expected to rise with global environmental change, such as high temperatures, drought and excessive inorganic nitrogen deposition. While the stresses these changes elicit will likely lead to complex patterns of response in respiratory metabolism (and potentially widespread increases in AP respiration), our results indicate that the response of the AP to climatic change is not likely to be

associated with an increase in stand-level or regional plant respiration.

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