THE TEMPERATURE DEPENDENCE OF PLANT ALTERNATIVE OXIDASE AND ITS IMPACT ON RESPIRATION RATES IN NATURE

A thesis submitted in fulfillment of the requirement for the Degree of Doctor of Philosophy in Plant Biology at the University of Canterbury by

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Statement of Originality

The work presented in this thesis is, to the best of my knowledge and belief, original. The material has not been submitted, either in whole or in part, for a degree at this or any other university.

Almost all of the work presented here was completed by the author. A small fraction of the respiration data presented here was measured by students (Sam Thomas, Danielle Bitterman, and Rex Chen), while some Western blots were processed by research assistants (Ian Reeves and Sam Thomas). The grant supporting this research (the Marsden Fund of the Royal Society of New Zealand) was awarded to Matthew Turnbull, Kevin Griffin, Owen Atkin, and Dan Yakir in 2006.

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List of Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
AOX	alternative oxidase
AOX/COX	relative ratio of AOX to COX proteins
AP	alternative pathway
ATP	adenosine triphosphate
COX	cytochrome *c* oxidase
CP	cytochrome pathway
D	oxygen isotope discrimination
D_{KCN}	D in KCN-inhibited tissues
D_{SHAM}	D in SHAM-inhibited tissues
E_0	temperature sensitivity of respiration- related to overall activation energy
ECL	Enhanced ChemiLuminescence
f	fraction of oxygen consumed by respiration
F_V/F_M	chlorophyll fluorescence yield
HRP	horseradish peroxidase
IRMS	isotope ratio mass spectrometer
$^{18}O/^{16}O_{ref}$	the ratio of ^{18}O to ^{16}O of the reference gas
$\delta^{18/16}O$	the ratio of ^{18}O to ^{16}O in a sample relative to that of a standard
$\delta\ ^{18/16}O_m$	measured $d^{18/16}O$
KCN	potassium cyanide
N	leaf nitrogen

NYC	New York City
O_f	fraction of gas that is oxygen
O_{fct}	fraction of gas that is oxygen from contamination
O_{fm}	measured fraction of gas that is oxygen
O_{peak}	area under the oxygen peak on IRMS
PAR	photosynthetically active radiation
Q_{10}	temperature sensitivity of respiration
R	respiration
R/R_o	ratio of 18O to 16O (relative to ambient air)
R_0	rate of respiration at 0 °C
R_{10}	rate of respiration at 10 °C
R_d	dark respiration
R_g	rate of respiration at the growth temperature
R_g/N	R_g on a nitrogen basis
ROS	reactive oxygen species
R_{ref}	rate of respiration at a reference temperature
S	total surface area
SEM	standard error of the mean
SHAM	salicylhydroxamic acid
SLA	specific leaf area
τ_a	electron partitioning through the alternative pathway
τ_c	electron partitioning through the cytochrome pathway
TNC	total nonstructural carbohydrates
T_s	storage time

Abstract

The physiological function of the plant enzyme alternative oxidase has long been a topic of debate. The cyanide-resistant alternative oxidase (AOX), along with the cytochrome *c* oxidase (COX), catalyzes the reduction of oxygen to water in the electron transport chain of mitochondrial respiration. Although respiration via the alternative pathway (AP) results in approximately one third of the ATP production as respiration via the cytochrome pathway (CP), the AP is utilized by all plants and some fungi and animals. This "energy wasteful" pathway has been proposed to reduce oxidative stress in plant cells under a variety of stressful conditions. Virtually all previous work on the AP has been performed on laboratory-grown plants in controlled environment conditions; thus, there is little knowledge of how the AP responds to unstable conditions and multiple environmental stresses in the field. This thesis presents new methodology for studying AP respiration and the AOX protein in field-grown plants, and investigates how the AP responds to natural changes in environmental conditions in the field in several plant species grown in diverse ecosystem types. The experimental work presented here also investigates how AP activity is related to changes in total rates of respiration, and questions whether abundance of the AOX protein determines electron partitioning to the AP.

AP partitioning (or relative changes in AP partitioning) varied over seasonal timescales in each of the experimental studies. Chapter 3 reports on two species of *Chionochloa*, a native New Zealand tussock grass growing along an altitudinal gradient. In Chapter 4, seasonal variation was studied in two tree types: *Populus* x *canadensis,* a deciduous angiosperm, and *Pinus radiata*, an evergreen gymnosperm. *Quercus rubra* trees were studied along an urban-rural gradient originating in New

York City in Chapter 5. In a highly exposed and variable environment, relative changes in AP partitioning in two species of *Chionochloa* were correlated with the previous day's integrated light. In *Quercus rubra,* the AP was instead related to temperature changes: relative AP partitioning increased in response to seasonally low temperature in trees grown at colder, more rural field sites, while at the warmer, urban sites, it increased in response to high summer temperatures. Each of these environmental conditions that were related to increases in the AP (high light, low temperatures, and heat) are potentially stressful to plants. Thus, it is possible that the increases in AP respiration observed in these studies served to oxidize excess reducing equivalents generated through stressful conditions. In Chapter 4, although AP partitioning in *Populus* x *canadensis* and *Pinus radiata* varied seasonally, these changes were not directly related to environmental parameters. However, AP partitioning in *Populus* x *canadensis* was clearly shown to be dependent on measurement temperature.

In each of the studies presented here, changes in the AP were not related to abundance of the AOX protein. AOX protein abundance showed consistent seasonal patterns in the two deciduous angiosperms, *Populus* sp. and *Quercus* sp, and was correlated with seasonal changes in temperature in *Chionochloa* spp. However, the lack of correlation between protein abundance and AP partitioning indicates that the AP is subject to post-translational control and likely varies more rapidly than protein levels. In each of Chapters 3 – 5, there was no clear impact of changes in AP partitioning on rates of total respiration. As the AP produces less ATP than the CP, I hypothesized that increases in AP activity would lead to higher respiration rates in order to meet a plant's energy demands. However, in *Populus* x *canadensis* and *Quercus rubra*,

respiration rates remained stable during sharp increases in AP partitioning, indicating that, at least under certain conditions, increases in AP activity are accompanied by a decrease in the CP.

In some of the first research studying AP partitioning in field-grown plants, this thesis illuminates possible mechanisms, functions, and implications of the AP. Over a range of plant taxa and environmental settings, this work shows that the AP does respond to stressful conditions in the wild, but that this does not result in increased respiration. Lastly, the methods presented here to study AP activity and AOX proteins in the field enable future studies to further probe the specific responses of AOX to natural stresses.

Chapter 1: Introduction, review of literature, and rationale

1.1 REVIEW OF LITERATURE

1.1.1 Global significance: carbon balance

It is now well understood that global temperature rise is a result of human-mediated increases in the concentrations of atmospheric carbon dioxide (CO_2), methane, aerosols, and other greenhouse gases (IPCC, 2007). CO_2 contributes the most to global warming of any greenhouse gas, with global anthropogenic emissions averaging 8.8 Gt C per year from 2000-2005 (including deforestation and other land use change; Denman *et al.*, 2007). However, only roughly a third of anthropogenic CO_2 remains in the atmosphere (Prentice *et al.*, 2001), with the world's oceans absorbing 2.2 Gt C per year (Plattner *et al.*, 2002; Denman *et al.*, 2007). The remaining ~3 Gt C, formerly termed "the missing sink" is now believed to be entering the terrestrial biosphere (Schimel *et al.*, 2001; Houghton, 2003; Denman *et al.*, 2007). The terrestrial CO_2 sink is the least well-understood component of the world's changing carbon cycle, and contributes to large uncertainties in predictions of future atmospheric CO_2 concentrations, and thus, global temperature rise (Friedlingstein *et al.*, 2003; Houghton, 2003; Jones *et al.*, 2003). Overall, the 2007 IPCC report predicts a future decline in the strength of the terrestrial sink, exacerbating climate warming.

The uncertainty in the future role of the terrestrial biosphere as a carbon sink comes not only from a lack of information on current carbon cycling dynamics, but from the difficulty in predicting the response of plant and soil carbon exchange to future environmental change. Regarding plants, carbon uptake is roughly equal to the difference between photosynthesis and respiration. Both processes are known to be sensitive to environmental factors expected to change with global warming: temperature (Wager, 1941; Berry & Bjorkman, 1980), CO_2 (Delucia *et al.*, 1985;

Gonzalez-Meler & Taneva, 2005), drought (Hsiao, 1973; Lawlor & Cornic, 2002), and nitrogen availability (Evans, 1989; Reich *et al.*, 1998).

Although carbon dioxide is sequestered in plants through photosynthesis, plant respiration (*R*) is a major control over the total plant carbon balance. The proportion of daily photosynthetic carbon gain lost through *R* is variable between species and can be quite large (30-80%) (Poorter *et al.*, 1990; Ryan, 1991; Amthor, 2000; Valentini *et al.*, 2000). This flux represents 30-65% of carbon loss at the ecosystem level (Schimel, 1995; Janssens *et al.*, 2001; Xu & Qi, 2001). In fact, one study determined that *R* was the main determinant of total ecosystem carbon balance in northern European forests (Valentini *et al.*, 2000). Small perturbations to *R* caused by climate change will thus have far-reaching consequences to the global carbon cycle.

1.1.2 The response of plant respiration to temperature

R is particularly sensitive to changes in temperature, increasing roughly exponentially as temperature increases (Wager, 1941). Two parameters are generally used to describe the temperature response of *R*: R_{ref}, usually R_0 or R_{10}, refers to the rate of respiration at a basal, low reference temperature, and Q_{10}, the increase in the rate of respiration with a doubling of temperature, describes the temperature sensitivity of respiration. E_0 is a term related to the activation energy of enzymatic reactions, and is also used to describe the temperature sensitivity of *R*. Global climate models generally assume a constant Q_{10} of 2 for all plant species (Ryan, 1991; Aber & Federer, 1992; Schimel *et al.*, 1997; Friedlingstein *et al.*, 2006), thus predicting a substantial increase in global CO_2 release via *R* as climate change progresses. It is now understood that the Q_{10} of plant R is not constant, and changes with temperature range (Wager, 1941; Tjoelker *et al.*, 2001; Atkin & Tjoelker, 2003), water availability

(Bryla *et al.*, 2001; Turnbull *et al.*, 2001), irradiance (Atkin *et al.*, 2000a) and canopy position (Griffin *et al.*, 2002).

Not only is the Q_{10} of plant *R* not constant amongst differing environments, but the thermal response of *R* also tends to acclimate to long-term changes in growth temperature in many species; for example, a warm-grown plant shifted to a colder environment will exhibit higher respiration at a set-point measurement temperature (Fig 1.1; Wager, 1941; Rook, 1969; Larigauderie & Korner, 1995; Atkin *et al.*, 2000b; Atkin & Tjoelker, 2003; Ow *et al.*, 2008a; Tjoelker *et al.*, 2008). "Full

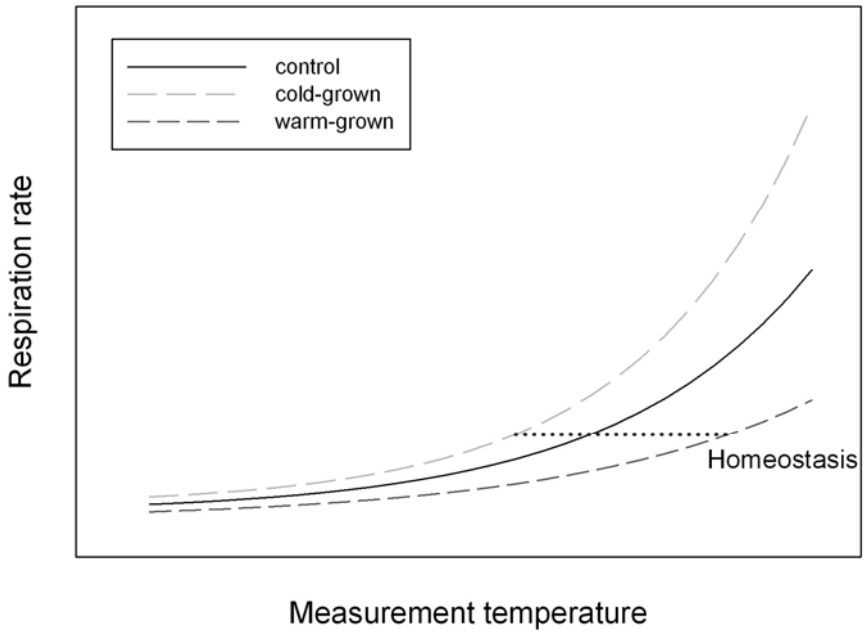


Figure 1.1: Schematic illustrating the process of thermal acclimation of respiration. Cool-grown leaves respire at a higher rate than warm-grown leaves at any set-point temperature. The dotted line indicates the rate of respiration in each plant when measured at its respective growth temperature, illustrating the concept of thermal homeostasis.

acclimation" or 'thermal homeostasis" is achieved when the rate of *R* is the same in plants grown at differing temperatures and measured at their respective growth temperatures. However, full acclimation is not achieved in all species or in all tissues,

and the degree of partial acclimation can vary widely (Larigauderie & Korner, 1995; Collier, 1996; Tjoelker *et al.*, 1999; Loveys *et al.*, 2003).

Thermal acclimation of leaf respiration has been found to occur under a variety of settings and timeframes. Acclimation to growth temperature under controlled laboratory conditions has been reported for several species of coniferous and deciduous trees, as well as herbaceous plants from arctic to temperate biomes (Collier, 1996; Arnone & Korner, 1997; Tjoelker *et al.*, 1999; Loveys *et al.*, 2003; Armstrong *et al.*, 2008). Herbaceous plants and deciduous and coniferous trees have also exhibited acclimation to temperature changes in one week or less under laboratory (Rook, 1969; Atkin *et al.*, 2000b; Bolstad *et al.*, 2003; Armstrong *et al.*, 2008) and field settings (Bolstad *et al.*, 2003). However, Ow *et al.* (2008a; 2008b) found that *R* in deciduous and coniferous tree leaves did not fully acclimate for several weeks until new leaves had developed at the new growth temperature. Interestingly, while evergreen trees have been shown to acclimate to seasonal changes in temperature (Stockfors & Linder, 1998; Atkin *et al.*, 2000b; Vose & Ryan, 2002; Ow *et al.*, 2010), *R* in leaves of deciduous trees appears to steadily decline throughout the growing season (Xu & Griffin, 2006; Marra *et al.*, 2009; Ow *et al.*, 2010; Rodriguez-Calcerrada *et al.*, 2010). Thus, an improved understanding of the differences in respiration between evergreen and deciduous trees as well as how respiratory metabolism functions between these plant groups is needed in order to more accurately factor respiratory acclimation in to global climate models.

1.1.3 Mechanisms of thermal acclimation of respiration

The rate of *R* in any given plant is thought to be a function of the capacity and activity of respiratory enzymes, substrate availability, and the availability of ADP.

Nonstructural carbohydrates (starches and sugars) are often correlated with changes in respiration with temperature in leaves (Tjoelker *et al.*, 1999; Lee *et al.*, 2005; Tjoelker *et al.*, 2008; Ow *et al.*, 2010) and roots (Covey-Crump *et al.*, 2002), and may have greater regulatory control over R at high rather than low temperatures, resulting in changes in Q_{10} rather than R_{ref} (Covey-Crump *et al.*, 2002; Ow *et al.*, 2010). For instance, Covey-Crump *et al.* (2002) found that addition of exogenous glucose to roots of *Plantago lanceolata* stimulated respiration at moderate (15-30 ºC) but not low (5-15 ºC) temperatures. Further evidence for the coupling of respiration with carbohydrate status comes from tracking changes in the rate of respiration with decreasing carbohydrate status throughout the night (Azcòn-Bieto *et al.*, 1983a; Noguchi & Terashima, 1997), and by linking respiration rates with artificially reduced leaf carbohydrate concentration through shading (Whitehead *et al.*, 2004).

Respiration rates have been shown to be stimulated in the presence of uncoupler, which converts ATP to ADP, in *P. lanceolata* roots (Covey-Crump *et al.*, 2002) and soybean mitochondria (Atkin *et al.*, 2002). Moreover, adenylate status was found to directly influence Q_{10} by Atkin *et al.* (2002). Further evidence for adenylate control over respiration comes from comparing ADP availability with the status of the ubiquinone pool, which is presumably closely coupled with the breakdown of glucose. Covey-Crump *et al.* (2007) found that ATP/ADP ratios were highest at low (5 ºC) and high (27 ºC) temperatures, but low at intermediate temperatures; low ADP availability at 5 and 27 ºC was correlated with low reduction state of the ubiquinone pool, suggesting that adenylate status may restrict activity of the mitochondrial electron transport chain at low and high temperatures. Thus, it is clear that at least under certain conditions, ADP may limit respiration.

The rate of respiration must on some level be limited by capacity (i.e. abundance of active respiratory enzymes). There is some evidence that respiration is capacity-limited at low temperatures in particular: addition of exogenous glucose and ADP can stimulate respiration at moderate to high temperatures, but has no effect on *R* at low temperatures in isolated mitochondria (Atkin *et al.*, 2002; Covey-Crump *et al.*, 2002). Thus, in the absence of other regulation, the authors of these studies concluded that *R* must be limited by enzymatic activity in the cold. One question that has not been sufficiently answered to date is whether various respiratory enzymes have different temperature sensitivities. The following sections will discuss key components of the electron transport chain of mitochondrial respiration (Fig. 1.2) and the impact they have on rates of respiration.

1.1.4 Alternative oxidase

Virtually all living organisms possess cytochrome *c* oxidase (COX), which oxidizes products of the mitochondrial electron transport chain and is irreversibly inhibited by cyanide. In addition, plants, some fungi, and a few animals possess a cyanide-resistant alternative mitochondrial respiratory pathway catalyzed by the enzyme alternative oxidase (AOX; Fig 1.2; Vanlerberghe & McIntosh, 1997; Millenaar & Lambers, 2003). This alternative pathway (AP) branches from the cytochrome pathway (CP) at the level of ubiquinone; thus, respiration via the AP is non-phosphylating (at least beyond the ubiquinone pool) and results in approximately one third of the ATP production of CP respiration (Millenaar & Lambers, 2003). Thus, use of the AOX can have a large impact on the carbon use efficiency of respiration.

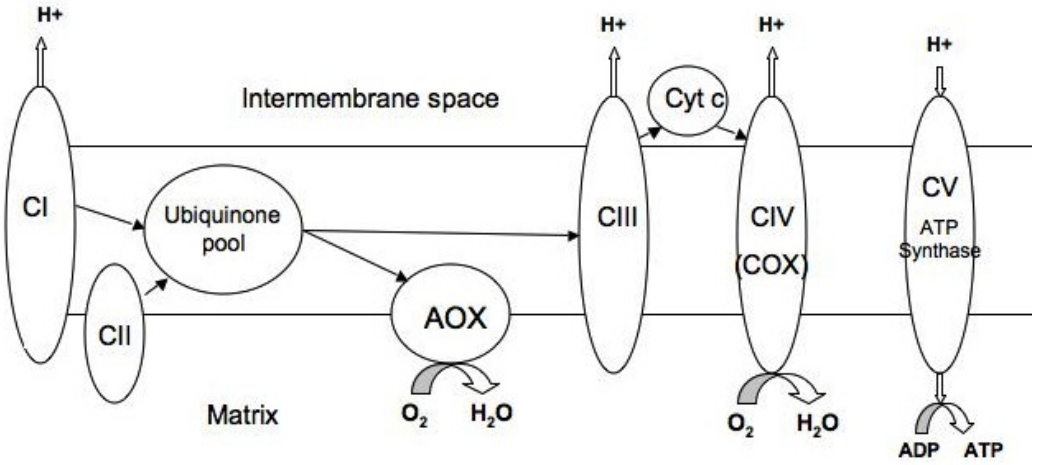


Figure 1.2: Schematic of the electron transport chain of mitochondrial respiration. CI – V = Complexes 1 – V; AOX = alternative oxidase; COX = cytochrome oxidase.

AP respiration was first studied by applying inhibitors to either AOX or COX in order to measure the capacity of the other [potassium cyanide (KCN) to inhibit COX, salicylhydroxamic acid (SHAM) to inhibit AOX], assuming that AOX would only be engaged when the CP was saturated. It is now known that the AP competes with the CP, and can do so at low levels of ubiquinone reduction when pyruvate is added to stimulate AOX (Day *et al.*, 1995; Ribas-Carbo *et al.*, 1995; Day *et al.*, 1996). Thus, capacity does not necessarily reflect *in vivo* electron partitioning through the AP or CP. Currently, the only accepted method with which to measure engagement of, or electron partitioning through, the AP and CP is the oxygen isotope fractionation technique (Guy *et al.*, 1989; Ribas-Carbo *et al.*, 1995). Because AOX and COX use different mechanisms to break the O=O bond, they discriminate against ^{18}O differently. The isotopic fractionation, or discimination (*D*), of oxygen consumed by plant tissue can thus be used to calculate the relative flux through each pathway (Guy *et al.*, 1989; Ribas-Carbo *et al.*, 1995; Ribas-Carbo *et al.*, 1997; Ribas-Carbo *et al.*, 2005a).

The exact mechanism of AP stimulation in plant tissues *in vivo* is uncertain. The AP is thought to be activated through the reduction state of the AOX protein: the enzyme is more active in the reduced rather than the oxidized dimer form (Umbach & Siedow, 1993; Umbach *et al.*, 1994; Umbach *et al.*, 2006), although the oxidized form is typically not found in plant tissues (Umbach & Siedow, 1997; Millar *et al.*, 1998; Millenaar *et al.*, 1998; Simons *et al.*, 1999). It has been shown that AP activity (Ribas-Carbo *et al.*, 1995; Ribas-Carbo *et al.*, 1997) and capacity (measured with inhibitors; Millar *et al.*, 1996; Vanlerberghe & McIntosh, 1997; Rhoads *et al.*, 1998) are stimulated by pyruvate in isolated mitochondria. It is possible that reactive oxygen species (ROS), such as superoxide, act as a signal to induce AOX (Wagner & Krab, 1995; Vanlerberghe & McIntosh, 1996). Other compounds which are produced in response to oxidative stress may also induce AOX, such as hydrogen peroxide (Ho *et al.*, 2008), salicylic acid (Rhoads & McIntosh, 1992; Lennon *et al.*, 1997; Djajanegara *et al.*, 2002), nitric oxide (Millar & Day, 1996; Huang *et al.*, 2002), ethylene and jasmonic acid (Ederli *et al.*, 2006), nitrate (Escobar *et al.*, 2006) and citrate (Vanlerberghe & McIntosh, 1996; Djajanegara *et al.*, 2002; Ho *et al.*, 2008). Interestingly, Ribas-Carbo *et al.* (2008) found the AP was stimulated by red light in soybean cotyledons, but not by blue or a combination of red and far red light, indicating that the AP may be partially phytochrome-regulated. Lastly, the AP must not necessarily be directly stimulated in order to achieve a response of *D*; changes in electron partitioning to the AP may sometimes be a result of decreases in the CP due to environmental stresses.

Importantly, it is not clear if increases in AOX gene expression and protein abundance lead to increases in AP engagement. Transcript abundance has been linked with protein levels of the AOX in a number of species (Vanlerberghe & McIntosh,

1996; Finnegan *et al.*, 1997; Djajanegara *et al.*, 2002; Karpova *et al.*, 2002; Escobar *et al.*, 2006), as has capacity (Vanlerberghe & McIntosh, 1992; Liang & Liang, 2002; Guy & Vanlerberghe, 2005; Rachmilevitch *et al.*, 2007). However, although changes in AOX protein abundance have been correlated with increased AP engagement in a few studies (Ribas-Carbo *et al.*, 1997; Gonzalez-Meler *et al.*, 1999; Liang & Liang, 2002; Rachmilevitch *et al.*, 2007), more often no correlation is found (Lennon *et al.*, 1997; Millenaar *et al.*, 2000; Guy & Vanlerberghe, 2005; Ribas-Carbo *et al.*, 2005b; Florez-Sarasa *et al.*, 2007; Juszczuk *et al.*, 2007; Vidal *et al.*, 2007; Armstrong *et al.*, 2008; Grant *et al.*, 2008; Florez-Sarasa *et al.*, 2009). As activity of the AP has been shown to be triggered by a variety of chemical stimuli (see above), it is not surprising that abundance of the AOX protein sometimes does not reflect actual engagement. Care should thus be taken in interpreting changes in AOX protein levels or transcript abundance of the genes encoding the AOX protein.

1.1.5 Function of the AOX

Why should plants utilize a respiratory pathway that produces less ATP than the CP? The only example of a direct adaptive role for AOX has been demonstrated in the flowers of aroids, where the generation of considerable heat via AOX volatizes pollinator-attracting compounds (Seymour & Schultze-Motel, 1999; Grant *et al.*, 2010). However, thermogenesis via AOX is unlikely to significantly affect function in other plant organs (Breidenbach *et al.*, 1997).

An early theory explaining AOX activity in non-thermogenic tissues was that the AP served as an "energy overflow" pathway; i.e. that the AP would become engaged only at high levels of reduction of the ubiquinone pool, i.e. when the CP is saturated with excess carbohydrates or limited by ADP availability (Bahr & Bonner,

1973; Lambers, 1982). However, this work was conducted using inhibitors, which may have underestimated the flux of *R* through the AP under low levels of ubiquinone reduction. Later work has generally not supported the energy overflow hypothesis. For instance Millar *et al.* (1998) found large changes in AP capacity but no change in the redox state of the ubiquinone pool, and other studies have found no correlation between AP engagement and carbohydrates (Ribas-Carbo *et al.*, 2000b; Gonzalez-Meler *et al.*, 2001).

In recent years, it has been proposed that the AP plays a key role in plant response to stress. AOX has been shown to be involved in the response of plants to a range of biotic and abiotic stresses. AOX capacity and protein levels are up-regulated during defense against infection (Simons *et al.*, 1999) and exposure to harmful compounds, such as naturally produced cyanide and cyanogenic compounds (Kende, 1993; Millenaar *et al.*, 1998), sulfide (Rennenberg & Filner, 1983), and nitric oxide (Brown & Cooper, 1994; Millar & Day, 1996). AOX capacity and AP activity have been shown to rise in response to low availability of nutrients, such as phosphate (Parsons *et al.*, 1999; Gonzalez-Meler *et al.*, 2001; Juszczuk, IM *et al.*, 2001; Sieger *et al.*, 2005) and nitrogen (Gonzalez-Meler *et al.*, 1997; Sieger *et al.*, 2005). AP contribution is increased during water stress (Gonzalez-Meler *et al.*, 1997; Ribas-Carbo *et al.*, 2005a). There is evidence that high light produces an increase in the AP (Ribas-Carbo *et al.*, 2000b; Noguchi *et al.*, 2001; Ribas-Carbo *et al.*, 2008) as well as AOX protein levels (Dutilleul *et al.*, 2003; Yoshida *et al.*, 2007), although Florez-Sarasa *et al.* (2009) found no response of *D* to differences in growth light levels. The AP has been found to increase in response to heat stress in roots (Rachmilevitch *et al.*, 2007), while Murakami & Toriyama (2008) found that transgenic rice seedlings with elevated levels of AOX were more tolerant to heat stress than the wild type. There is

substantial evidence that AOX plays a role in plant response to low temperature stress: *D* has been shown to increase in response to low temperature (Gonzalez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000a; Armstrong *et al.*, 2008), while the concentration of AOX protein often increases following transfer to low temperatures (Vanlerberghe & McIntosh, 1992; Gonzalez-Meler *et al.*, 1999; Mizuno *et al.*, 2008), as do AOX transcript abundances (Ito *et al.*, 1997; Sugie *et al.*, 2006). Interestingly, Ribas-Carbo *et al.* (2000a) found that AP engagement increased markedly more in a chilling-sensitive cultivar of maize than in a chilling-tolerant cultivar, supporting the notion that AOX plays a role in plant response to stress, and not just cold tolerance.

The response of the AP to stressful environmental conditions may function to prevent excess ROS accumulation, which may act as both a consequence and signal (see above) of stress. ROS accumulate during mitochondrial response to environmental stresses such as cold exposure (Purvis & Shewfelt, 1993; Fiorani *et al.*, 2005; Sugie *et al.*, 2006) drought (Sgherri & Navariizzo, 1995; Bartoli *et al.*, 2004), nutrient deficiency (Juszczuk, I *et al.*, 2001) and infection (Wang *et al.*, 2007) and can cause severe harm to the mitochondria (Rich & Bonner, 1978). Evidence that AOX can play a preventative role in ROS production is as follows. Mitochondrial ROS production is higher in transgenic cells with anti-sense AOX genes relative to the wild type in tobacco (Maxwell *et al.*, 1999) and in *A. thaliana* when exposed to low temperatures (Fiorani *et al.*, 2005; Sugie *et al.*, 2006), moderate light and drought (Giraud *et al.*, 2008), and KCN (Umbach *et al.*, 2005). Antioxidants and antioxidant proteins increase in *A. thaliana* plants with anti-sense AOX (Giraud *et al.*, 2008; Watanabe *et al.*, 2008), in *Nicotiana tabacum* with anti-sense AOX and P limitation (Sieger *et al.*, 2005) and in *Nicotiana sylvestris*, concomitantly with a rise in AOX transcripts during light exposure (Dutilleul *et al.*, 2003).

It is not well understood how quickly the AP responds to environmental change. There is evidence that the AP is more temperature sensitive than the CP, resulting in an instantaneous temperature dependence of *D*. The balance of electron partitioning through the AP and CP has been found to be highly sensitive to measurement temperature in *Arabidopsis thaliana* (Armstrong *et al.*, 2008) and a small temperature dependence was found in leaves of *Vigna radiata* (Gonzalez-Meler *et al.*, 1999) and *Nicotania tabacum* (Guy & Vanlerberghe, 2005). Each of these three studies found higher *D* when leaves were measured at high (30 ºC) than at low (5 or 10 ºC) temperatures. Guy & Vanlerberghe (2005) found a decrease in AP contribution to *R* when extending this temperature range to 30 - 40 ºC. These results contrast with Macfarlane *et al.* (2009), who found that *D* was not sensitive to measurement temperature in *Curcubita pepo, Nicotania sativa,* and *Vicia faba,* and with Atkin *et al.* (2002), who found that the AP was not more temperature sensitive than the CP in isolated mitochondria of soybean cotyledons. Thus, it is still uncertain if *D* changes with measurement temperature, and how this varies between species.

The AP has been shown to be sensitive to changes in temperature on timescales of one day to several months. Ribas-Carbo *et al.* (2000a) found that electron partitioning through the AP was greater when measured one day after cold stress in a chilling-sensitive maize cultivar than in warm-treated plants. Several studies have shown AP respiration to be different in plants grown under different environmental conditions: low vs. high temperature in *V. radiata* (Gonzalez-Meler *et al.*, 1999), CO_2 in *A. thaliana* (Gonzalez-Meler *et al.*, 2009), and light in *Spinacia oleracea*. Armstrong *et al.* (2008) and Rachmilevitch *et al.* (2007) tested the response of *D* in *A. thaliana* and *Agrostis stolonifera* to low and high temperatures, respectively, over a period of days. Armstrong *et al.* (2008) found AP respiration

increased after 4 days of cold exposure, but by day 10 there was no difference in *D* between cold and warm-treated plants. Similarly, Rachmilevitch *et al.* (2007) showed that AP engagement rose after 7 days of heat treatment, but fell to pre-treatment levels after 28 days. Thus, it is clear that the timescale of AP responses varies depending on species and type of stress.

1.2 RATIONALE FOR PRESENT STUDY

From the literature review above, it is clear that further research is needed to understand the function of the alternative oxidase. In particular, relatively few studies have been performed using the oxygen isotope fractionation technique to study the AP. This is largely due to the limited availability of the high precision isotope ratio mass spectrometers (IRMS) needed to make these measurements. A complete lack of respiratory oxygen isotope discrimination measurements in the field is also likely due to the limited availability of equipment. Additionally, measurements of *D* using conventional systems (Robinson *et al.*, 1992; Ribas-Carbo *et al.*, 1995; Armstrong *et al.*, 2008) are time-consuming; only a few plant samples can be measured in one day (Armstrong, personal communication). This conventional system can also only be used on plant material grown at, or near, the mass spectrometer facility. Nagel *et al.*, (2001) developed an off-line system using sealed vials (Exetainers, Labco, High Wycombe, UK) to store respired gas until they could be analyzed on an IRMS. One of my major objectives for this work was to modify this off-line system to allow rapid and accurate measurement of respiratory oxygen isotope discrimination that can be used with virtually all plant types and under field conditions.

As there have been no published reports on AP respiration under field settings, little is known about the response of the AP to natural variation in environmental

conditions, or about the combined effect of different stresses (e.g. low temperatures and high light). Thus, it is difficult to ascertain the function of AOX and its impact on plant respiration on a larger scale. Additionally, almost all of the published research on the topic to date has concerned investigations of herbaceous plants. Therefore, I investigated changes in AP engagement and respiration in various species and functional plant groups in the field. In an effort to identify the possible biochemical stimuli affecting changes in AP engagement in the field, I measured AOX protein abundance, carbohydrate status, leaf nitrogen and chlorophyll fluorescence yield, all of which have been theoretically linked to the AP. Utilizing natural environmental gradients and observing changes in physiology over seasons, I sought to understand how the AP responds to natural variation in temperature and other environmental factors.

In this thesis, I attempt to address the following questions:

1. How rapidly does respiration acclimate to natural changes in temperature in the field?
2. Does relative electron partitioning through the enzyme alternative oxidase respond to natural changes in temperature in the field?
3. Do changes in the electron partitioning through the alternative pathway (AP partitioning) contribute to the thermal acclimation of respiration?

1.3 OVERVIEW OF THESIS

This thesis is divided into 6 chapters addressing the following topics:

Chapter 1

General introduction to the background information of this thesis. The global significance of plant respiration is discussed, along with a literature review on the thermal acclimation of respiration and the alternative oxidase (AOX) and its response to environmental change.

Chapter 2

This chapter describes the methodology used in this work. Standard measurements of respiration, carbohydrates, leaf nitrogen, and chlorophyll fluorescence are described. Also detailed are the processes through which I developed methodologies for measuring oxygen isotope discrimination of respiration in the field as well as detecting abundance of the AOX protein in field-grown plants using Western analysis.

Chapter 3

This chapter reports on an investigation of how respiration and AOX in the alpine tussock grasses *Chionochloa pallens* and *C. rubra* respond to temperature. Measurements were conducted over the course of one year, and plants were sampled along an altitudinal gradient on Mt. Hutt, New Zealand, in order to extend the range of temperatures experienced naturally. The main objectives of this study were to determine if respiration in *Chionochloa* spp. acclimates to long-term changes in temperature, if this acclimation is underpinned by responses of AOX to temperature changes, and how quickly these changes occur. For the last objective, an intensive

short-term project was conducted that tracked changes in respiration and AOX in these plants over the timescale of days.

A version of this chapter has been published as an article in *New Phytologist* and is included in Appendix A.

Chapter 4

Seasonal changes in respiration and AOX are studied in stands of mature *Pinus radiata* and *Populus* x *canadensis* trees in Lincoln, New Zealand. Previous work found that respiration in both species acclimated to seasonal changes in temperature (Ow, 2009). A key objective of this chapter was to determine whether this acclimation was a result of changes in AOX. Additionally, I investigated whether the instantaneous temperature response of alternative pathway (AP) respiration changed with season, and whether it impacted the temperature response of total respiration. An over-arching question in this chapter was whether the responses of the AP to short and long term changes in temperature differed between an evergreen gymnosperm (*P. radiata*) and a deciduous angiosperm (*P.* x *canadensis*)

A version of this chapter has been published as an article in *Physiologia Plantarum* and is included in Appendix B.

Chapter 5

This study investigated how respiration and AOX change along an urban-rural gradient in New York City, USA. Using native *Quercus rubra* trees, seasonal variation in respiration along the gradient was also studied. This project was

supplemented with a growth cabinet study subjecting *Q. rubra* seedlings to heat stress. In particular, this chapter addresses the question of whether the AP in *Q. rubra* responds to naturally low or high temperatures.

A version of this chapter has been published as an article in *Functional Ecology* and is included in Appendix C.

Chapter 6

This chapter discusses the results of the experimental chapters in the context of previously published literature. The response of the AP to various environmental changes and possible biochemical mechanisms are discussed. This chapter also discusses how the work presented here has furthered the understanding of the scientific community of AOX, and suggests future direction for the field.

Chapter 2: Methods

2.1 PLANT SAMPLING

In all studies presented in this thesis, samples were excised from study plants and were transported to the laboratory for measurement. With *Chinochloa* spp. (Chapter 3), leaves from one tiller were cut at the base of the shoot, wrapped in damp paper towels, placed in an open plastic bag, and transported inside a cool, dark, temperature-controlled bin. Small branches of *Pinus radiata, Populus* x *canadensis* (Chapter 4), and *Quercus rubra* (Chapter 5) were cut from mature trees, recut under water in a bucket, and transported to the laboratory under a dark cloth. Turnbull *et al.* (2003) showed that respiration remains stable for several hours in well-hydrated, excised plant tissues. This was also shown here for *C. pallens* (Fig. 2.1) and *Q. rubra* (Fig. 2.2).

2.2 SPECIFIC LEAF AREA

For *Chionochloa* spp., a length of 15 cm was cut from one leaf from each sample plant, well above the base of the leaf. The width of the leaf at each end of the segment was measured using calipers, and the total leaf area was calculated by treating the leaf segment as a trapezoid. For *P. radiata*, the diameter of each fascicle was measured at the base, and total surface area of the three needles in the fascicle was calculated as:

$$S = \left(\frac{\pi dl}{3} + dl \right) \times n \qquad (2.1)$$

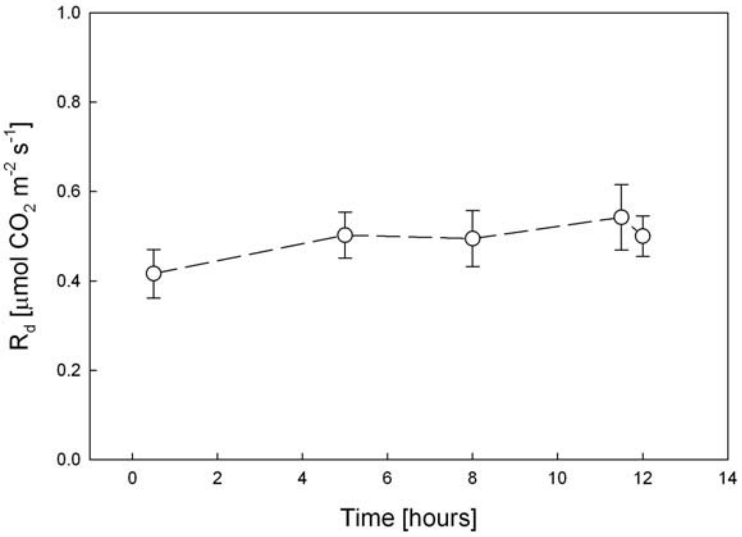


Figure 2.1: Dark respiration (R_d) in excised *C. pallens* tillers sampled from plants grown in an enclosure at the University of Canterbury. Leaves were kept darkened, hydrated, and were measured at a constant room temperature of 20 °C. N = 6; F = 0.638; p = 0.648. Values shown are mean ± SEM.

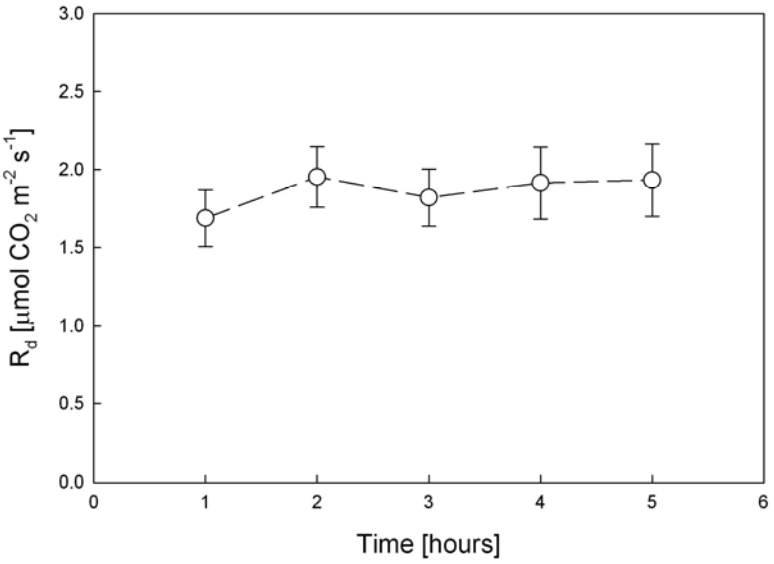


Figure 2.2: Dark respiration (R_d) in *Q. rubra* leaves on excised branches sampled from Lamont-Doherty Earth Observatory. Branches were kept darkened, hydrated, and were measured at a constant room temperature of 25 °C. N = 6; F = 0.281; p = 0.887. Values shown are mean ± SEM.

Where S = total surface area, d = diameter, l = length of needle present in chamber, and n = number of needles per fascicle, after Turnbull *et al.* (1998). Leaf disks of a known area were taken from leaves of *P.* x *canadensis*, and the area of two complete leaves of *Q. rubra* was measured using a leaf area meter (Li-Cor, Lincoln, NE, USA). Leaf sections of known area for all species were placed in paper bags and oven-dried at 60 °C. Dried leaves were then weighed, and specific leaf area (SLA) was calculated as the area of fresh leaves divided by the dry weight.

2.3 RESPIRATION MEASUREMENTS

Plants were placed in dark growth cabinets for measurement of respiration. Measurements were made at four or five temperatures for each study. One leaf (or set of leaves for *Chionochloa* spp. and *P. radiata*) was marked and used for measurement of *R* at all temperatures on each day of measurement. All respiration measurements were made using an LI-6400 gas exchange system (Li-Cor, Lincoln, NE, USA). Leaves of *P.* x *canadensis* and *Q. rubra* were large enough to cover the entire area of the leaf chamber of the gas exchange system. Three or four leaves of *C. pallens* and *C. rubra* were arranged lengthwise in the leaf chamber. The width of each leaf at the middle of the chamber was measured; the area of the leaf section enclosed in the chamber was then calculated treating each leaf as a trapezoid. Two fascicles of *P. radiata*, consisting of six needles total, were arranged lengthwise in the chamber. Total surface area of these needles was calculated according to Eq. 2.1. All respiration measurements for *Chionochloa* spp. and *P. radiata* were corrected for differences in area between the leaf surfaces and the area of the leaf chamber. The temperature of the growth cabinet was changed to match the temperature of the leaf chamber of the gas exchange system with each set of measurements. Relative humidity inside the leaf

chamber was maintained at 50-75%. Measurements were made after each leaf was present in the chamber for three minutes in order to allow the system to equilibrate. With each new measurement temperature, plants were allowed to rest at the new temperature for 30 minutes before measurement in order to allow the plant to adjust, and also to allow the gas exchange system to equilibrate to the change in temperature. Approximately 20 measurements were made per leaf at each temperature. These values were screened for outliers, and then averaged.

The temperature response of respiration was modeled using a modified Arrhenius equation (Ryan, 1991; Turnbull *et al.*, 2003; Kruse & Adams, 2008):

$$R = R_{10} \cdot e^{\frac{E_o}{g}\left(\frac{1}{T_o} - \frac{1}{T_a}\right)} \qquad (2.2)$$

where R is the respiration rate, R_{10} is the respiration rate at a reference temperature (T_0) of 10ºC, 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. Non-linear curve fitting was performed using the Marquardt-Levenberg algorithm (Sigma Plot, v8.0 SPSS Inc. Chicago, Illinois).

Respiration at the growth temperature (R_g) was calculated by substituting R_{10} and E_0 values that had been determined for each plant into Eq. 2.2 with T_a being equal to the assessed growth temperature; R then equaled R_g.

2.4 LEAF NITROGEN AND CARBOHYDRATES

Sub-samples were taken from each plant sample, placed in a paper bag, oven-dried at 60 °C, and ground to powder with a ball mill. A portion of this powder was analyzed on a Europa Scientific 20/20 isotope analyzer at the Waikato University stable isotope unit (Hamilton, New Zealand) for %N.

Soluble sugar and starch were analyzed following the method of Tissue & Wright, (1995). Between 15 and 20 mg of powdered leaf sample was mixed with an extraction buffer of 12:5:3 methanol:chloroform:water. Samples were centrifuged in order to separate the supernatent and pellet. The supernatent was allowed to separate over a period of hours. A portion of the methanol fraction, assumed to contain soluble sugars, was removed. The pellet, assumed to contain starch, was mixed in perchloric acid in order to hydrolyze the starch. Portions of methanol extract and of perchloric acid extract were analyzed similarly: phenol, water, and sulfuric acid were added to the sample, and the resulting mixture was analyzed on a spectrophotometer at 490 nm. A set of glucose standards were also analyzed in order to enable conversion of spectrophotometer readings to absolute glucose values, and to characterize the linearity of the response of the spectrophotometer to glucose concentration. Using this conversion, and accounting for the dilution of the initial sample powder in the reagents used, soluble sugar and starch were calculated as a percentage of dry leaf mass. Soluble sugar and starch were summed to calculate total nonstructural carbohydrates (TNC).

2.5 OXYGEN ISOTOPE DISCRIMINATION

2.5.1 Theory

Currently, the only accepted method to measure the activity of the alternative pathway (AP) is the oxygen isotope fractionation technique. Both alternative oxidase (AOX) and cytochrome oxidase (COX) discriminate against ^{18}O, but do so to different extents, with the AP typically discriminating against ^{18}O more heavily than does the cytochrome pathway (CP) (Ribas-Carbo *et al.*, 2005a). Therefore, assessment of the oxygen isotope signature of the residual oxygen from a plant's respiration can be used

to infer the oxygen isotope discrimination (*D*) of respiration in that plant, and thus the electron partitioning between the AP and CP.

For calculation of *D*, I follow the method of Guy *et al.* (1989). They write:

$$\ln\frac{R}{R_o} = -\ln\frac{^{16}\mathrm{O}}{^{16}\mathrm{O}_o}[1-\alpha] \qquad (2.3)$$

where R is the ratio of $^{18}O/^{16}O$ of the residual oxygen from respiration, R_o is the same ratio of ambient air before respiration occurs, ^{16}O is the amount of ^{16}O in the residual gas from respiration, $^{16}O_o$ is the amount of ^{16}O in ambient air before respiration, and $[1-\alpha]$ is the slope of a linear regression of $\ln R/R_o$ against $-\ln(^{16}O/^{16}O_o)$, or discrimination (*D*). Guy *et al.* (1989) write that $^{16}O/^{16}O_o$ is essentially equal to the fraction of oxygen consumed by respiration (*f*). Therefore, we can write:

$$D = \frac{\ln R/R_o}{-\ln f} \qquad (2.4)$$

which is mathematically equivalent to

$$D = \frac{\ln R}{-\ln O_f} \qquad (2.5)$$

where O_f is the fraction of total gas that is oxygen after respiration has occurred (this is not relative to the fraction of total gas that is oxygen before respiration has occurred, which would be *f*). Faraday cup multiplication factors for the different isotopes of oxygen were corrected for before the isotopes were added together for the

total concentration of oxygen. Thus, I analyzed discrimination of oxygen isotopes without reference to the initial composition of ambient air. The initial concentrations of O_2 and $^{18}O/^{16}O$ were measured and were found to be similar on days for which *D* measurements were compared. Following Henry *et al.* (1999), I did not force regressions through the origin. Lastly, the term (ln*R*) must be multiplied by 1000 in order to calculate *D* on a per mil basis:

$$D = \frac{\ln R \times 1000}{-\ln O_f} \qquad (2.6)$$

Calculation of the absolute flux of electrons via the AP and CP requires that the isotopic endpoints of both AOX and COX are known for a particular species; i.e. to what extent the AP or CP discriminate against ^{18}O in the absence of the other pathway. This is achieved by applying chemicals that inhibit one or the other pathway [salicylhydroxamic acid (SHAM) to inhibit AOX, and potassium cyanide (KCN) to inhibit COX]. Measurement of these isotopic endpoints requires full inhibition of each pathway; this is tested by applying both inhibitors to a plant simultaneously, and measuring the total rate of respiration, which should be close to zero. I calculated electron partitioning to the AP following Guy *et al.* (1989):

$$\tau_a = \frac{D_s - D_c}{D_a - D_c} \qquad (2.7)$$

where τ_a is electron partitioning through the AP, D_s is the value of discrimination of the uninhibited sample, D_c is discrimination through the CP, and D_a is discrimination

through the AP. An illustration of the calculation of *D* in inhibited and uninhibited tissues is shown in Figure 2.3.

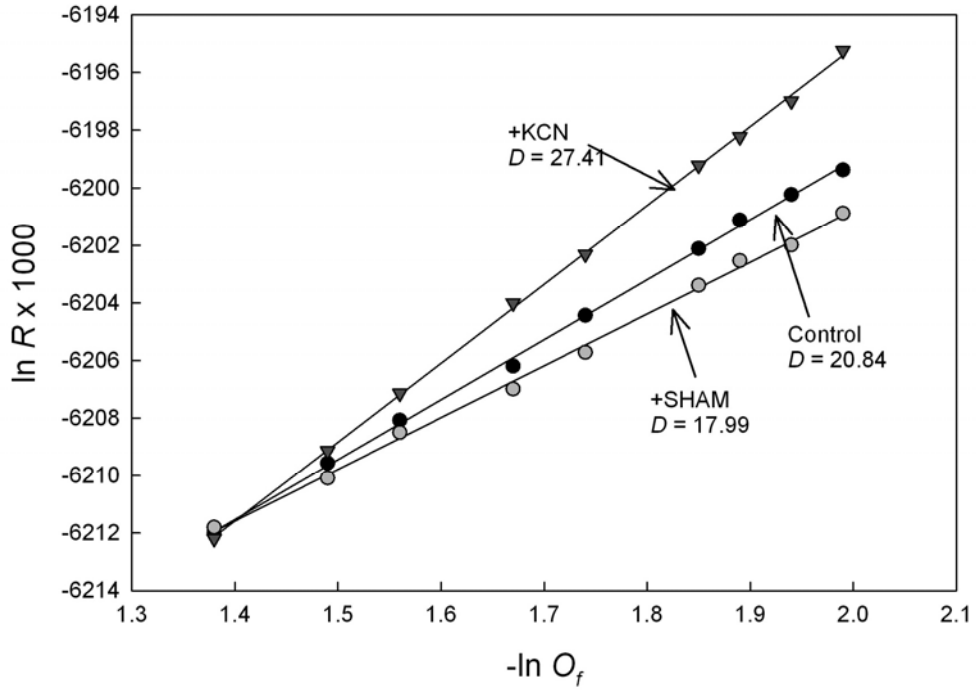


Figure 2.3: Illustration showing the calculation of discrimination (*D*) as the slope of ln *R* x 1000 against –ln O_f. Typical values across species are shown for uninhibited (control), SHAM-inhibited, and KCN-inhibited plants.

2.5.2 Exetainer method for measurement of oxygen isotope discrimination

Typically, oxygen isotope discrimination is measured using an on-line method, in which the cuvette containing the plant sample is directly connected to the mass spectrometer (Guy *et al.*, 1989; Robinson *et al.*, 1992; Ribas-Carbo *et al.*, 1995; Armstrong *et al.*, 2008). This method requires constant access to an isotope ratio mass spectrometer (IRMS) while experiments take place. As an alternative, Nagel *et al.* (2001) developed an off-line method in which plants were incubated for a period of

time in a cuvette with an oxygen electrode measuring oxygen depletion, after which the residual gas from respiration was injected into pre-evacuated Exetainers for storage before measurement on an IRMS. One limitation to this method is that only one sample can be measured at a time in the specialized cuvette.

In order to measure oxygen isotope discrimination on ecologically-relevant numbers of field-grown plants, it was necessary for me to develop a method that would allow simultaneous incubation of many samples, as field conditions vary daily and so as many as 24 samples must be measured in 1-2 days. In collaboration with Ari Kornfeld, a fellow PhD candidate, I modified the method of Nagel *et al.* (2001) to allow rapid measurement. This method involved incubation of plant material inside large Exetainers (Labco, High Wycombe, UK), after which the resultant gas was transferred to small Exetainers for storage until an IRMS could be accessed for measurement of oxygen isotope fractionation.

Leaves were cut into large pieces (in order to minimize wounding effects) and placed in large (12 mL) Exetainers. In order to prevent the build-up of excess CO_2, one half pellet of potassium hydroxide (KOH) was present in each Exetainer, placed inside the severed end of a microcentrifuge tube in order to prevent direct contact between leaves and the KOH. Three to four Exetainers were filled with varying amounts of material from each plant sample in order to produce a range of oxygen consumption and isotope discrimination. Exetainers were sealed and placed in a rack in a water bath set to a certain temperature. Samples were incubated in darkness. Depending on species, samples were incubated for a certain period of time in order to achieve a maximum of 50% oxygen depletion. When measurements at multiple temperatures were needed a second water bath was employed, and samples being incubated at lower temperatures were allowed to incubate for longer periods of time

than warm-incubated samples in order to achieve a similar degree of oxygen depletion.

After the period of incubation, the residual gas from respiration was transferred to a smaller (3.8 mL) Exetainer that had been pre-evacuated by the manufacturer. Two syringes, one empty and one filled with distilled water, were inserted into the larger incubation Exetainer. Pressure was gently applied to the water-filled syringe in order to slowly fill the Exetainer with water. The displaced gas entered the empty syringe, which was then removed and inserted into a pre-evacuated 3.8 mL Exetainer. Pressure was allowed to equilibrate between the gas syringe and the small Exetainer, after which the gas syringe was pressed lightly in order to overcome the force of friction in the syringe to achieve full equilibration. The gas syringe was flushed with ambient air three times between samples in order to eliminate sample-to-sample contamination. The small Exetainers containing gas samples were then stored in darkness at room temperature until the IRMS became available.

2.5.3 Measurement of oxygen isotopes using an IRMS

Samples were analyzed using a Delta IV isotope ratio mass spectrometer (IRMS) equipped with GasBench II (Thermo Fisher Scientific, Waltham, MA, USA). The GasBench was equipped with an auto-sampler. A needle with two airstreams, one flowing helium gas outwards and the other pulling sample gas in, was automatically inserted into each sample-filled Exetainer. Helium gas replaced a quantity of sample gas that flowed into the GasBench. Sample gas then flowed through an 8-port Valco valve (Valco, Houston, TX, USA), where either a 50 μL or a 100 μL sample loop was filled with sample gas. A small sample loop was chosen in order to reduce the total

amount of oxygen inserted into the IRMS to bring the amount of oxygen measured into the accurate measurement range of the equipment. Sample gas then flowed from the Valco valve to an auto-diluter, which diluted the sample gas with helium, also to limit the amount of oxygen inserted into the IRMS. Sample gas then passed through two tubes filled with Nafion to remove water, after which it passed through a 5Å gas chromatography (GC) column (Varian Australia, Mulgrave, VI, Australia) to separate molecular constituents of the sample gas. This gas was then injected into the IRMS.

At the start of each day's measurements, a diagnostic was run on the IRMS to check the accuracy and precision of oxygen readings: a reference oxygen gas from a tank was injected six times into the IRMS. The peak areas from these injections were found to be very similar, and the standard deviation of the six measurements of $\delta^{18/16}O$ was always less than 0.2. At the beginning of each day's run, and approximately every 6 samples thereafter, a peak center was performed, in which the reference oxygen gas was injected into the IRMS, and the three Faraday cups were automatically adjusted until each achieved a peak reading on masses 32, 33, and 34 (corresponding to $^{16}O^{16}O$, $^{17}O^{16}O$, and $^{18}O^{16}O$, respectively). Before each sample was analyzed, between two and five injections of reference oxygen gas were analyzed on the IRMS in order to check that these readings were stable.

I measured both oxygen and nitrogen in my samples on the IRMS. The purpose of this was to use the ratio of oxygen to nitrogen gas (O_f) as a measure of the concentration of oxygen in samples (ambient air is 21% oxygen, 78% nitrogen, and 1% argon). The GC column separated oxygen and nitrogen gas such that nitrogen reached the IRMS 20-40 seconds after oxygen. This allowed time to shift the Faraday cups to measure masses 28, 29, and 30 ($^{14}N^{14}N$, $^{15}N^{14}N$, and $^{16}N^{14}N$ respectively) after the oxygen isotopes were measured. As I only required the total sum of peak areas of

all isotopes of nitrogen (i.e. the total amount of nitrogen in the sample), a peak center was only performed every several months, and nitrogen reference gas was not analyzed with each sample.

The GC column and the IRMS were "baked" (the temperature inside the system was raised to 200 °C) once a week during measurement periods in order to volatilize and flush out any particles that may have adhered to the inside of the equipment.

2.5.4 Corrections for non-linearity of the IRMS

Three corrections were applied to the data: for non-linearity of the IRMS, leakage due to storage time, and initial contamination in the Exetainers, in that order.

A slight non-linearity in the response of measured O_2/N_2 ratio (O_f) and $\delta^{18/16}O$ to actual concentration of oxygen was characterized using precision gas mixtures (BOC, Munich, Germany). Small Exetainers were flushed for >1 minute with mixtures of 0, 5, 10, 15, and 20% O_2 in N_2 and were analyzed immediately on the IRMS. This test was performed four times and the average response of measured O_f and $\delta^{18/16}O$ to actual oxygen concentration was used to correct for the non-linearity of the IRMS. The measured $\delta^{18/16}O$ was found to increase slightly with increasing oxygen concentration, with an average slope of 0.01135 across the datasets. I assumed measurement to be most accurate at very low concentrations of oxygen (although correcting measured values to the highest concentrations of oxygen may be more accurate), and so corrected for the apparent increase in $\delta^{18/16}O$ at increasing concentrations of oxygen, and corrected thus:

$$\delta^{18/16}O = \delta^{18/16}O_m - (O_{peak} \times 0.01135) \qquad (2.8)$$

where $\delta^{18/16}O_m$ is the measured isotopic ratio and O_{peak} is the area under the oxygen peak, summed for masses 32, 33, and 34 (i.e. the amount of oxygen measured in the sample, not the O_2/N_2 ratio). The IRMS was found to overestimate O_f slightly with a slope of 0.9462 for the regression of real O_f against measured O_f, and an intercept of 0.0008. Thus, the non-linearity of the response of O_f to oxygen concentration was corrected as:

$$O_f = O_{fm} \times 0.9462 + 0.0008 \qquad (2.9)$$

where O_f is the actual ratio of O_2N_2 and O_{fm} is the measured ratio of O_2/N_2.

2.5.5 Corrections for storage time

All of the following analyses were performed after correction of measurements for non-linearity as described above. The small Exetainers were found to leak at a rate of approximately 0.7% per month, as estimated by storing N_2 blanks for varying periods of time before measurement and comparing the amount of oxygen detected within. This leakage, presumably including leakage of sample out of the Exetainers as well as leakage of ambient air in, tended to result in measurements of samples that were closer in composition to ambient air than the original samples were. To develop a correction for the effect of storage time on measured O_f, 5 sets of Exetainers were flushed with 0, 5, 10, 15, and 20% O_2 in N_2 (precision gas mixes) and stored for varying periods of time, up to 8 months. As expected, leakage increased with storage time, but this relationship depended on the concentration of oxygen in the original sample – leakage increased as true O_f decreased (i.e. more leakage occurred in

samples that were more different than ambient air). I found a strong correlation between true O_f and the slope of measured O_f to storage time; the slope of this regression was -1.709 x 10^{-4} with an intercept of 2.7 x 10^{-5}. Thus, I corrected for the effect of storage time on O_f as:

$$O_f = O_{f_m} - T_s \times (-1.709 \times 10^{-4} \times O_{f_m} + 2.7 \times 10^{-5}) \qquad (2.10)$$

where T_s is the storage time before analysis on the IRMS (days).

As the precision gas mixtures were presumed to all have the same isotopic signature, it was not possible to use these to determine the effect of storage time on the $\delta^{18/16}O$ of samples. As with O_f, leakage with storage time would presumably dilute samples with high $\delta^{18/16}O$ more so than samples with low $\delta^{18/16}O$. I determined this effect empirically: I collected a large set of gas samples (48) from needles of six branches of *Pinus radiata* (collected at Landcare Research, Lincoln, NZ), analyzed half immediately, and analyzed the other half after storing for 10 months. By varying the amount of leaf material in the incubations, I was able to produce a wide range of $\delta^{18/16}O$ in samples. O_f for stored samples was corrected according to Eqn 2.10. Calculated D for samples measured immediately was 21.39‰, and for stored samples was 17.89‰. The raw $\delta^{18/16}O$ could not be directly compared between samples measured immediately and those stored, as this depended on the exact amount of leaf material used in each sample. I used the D value calculated for each of stored and immediately measured samples to calculate what $\delta^{18/16}O$ would be at set values of O_f. Thus, I could directly compare these calculated $\delta^{18/16}O$ values between stored and immediately analyzed samples. I regressed $\delta^{18/16}O$ (measured immediately) against $\delta^{18/16}O_m$ (stored), and fitted the equation:

$$\delta^{18/16}O = (1.2543 \text{ x } \delta^{18/16}O_m) + 1.4248 \quad (2.11)$$

I applied this equation to the measured $\delta^{18/16}O$ values in the stored set, and the resultant *D* value was 21.36 ‰, very similar to the *D* value of immediately analyzed samples. The above equation assumes a constant storage time of 297 days, which is the period for which these samples were stored. To apply this equation to samples that have been stored for more or less than 297 days, I modified the degree of leakage as:

$$\delta^{18/16}O = \delta^{18/16}O_m \times (1 + 0.2543 \times (T_s/297)) + 1.4248 \quad (2.12)$$

where T_s is the storage time, in days, of any particular set of samples. This correction assumes a linear rate of leakage and compares the number of storage days of any particular sample to the number of storage days used in this pine experiment. Ideally, this assumption would be tested by collecting a large set of gas samples from a similar set of plants and storing them for varying periods of time (e.g. 50 d, 100 d, 150 d, 200 d, etc.) before analysis. However, due to my limited access to the IRMS, this was not possible.

After applying storage corrections to samples, no influence of storage time was found on discrimination values in all datasets (i.e. storage time was not correlated with *D*). Thus, I concluded that while storing samples may contribute to overall measurement error of *D* it does not do so systematically.

2.5.6 Corrections for contaminating gas in Exetainers

The pre-evacuated Exetainers were found to be contaminated with gas occupying 5-15% of the volume of the 3.8 mL Exetainers. If not corrected for, this contamination would skew measurements by diluting the isotopic signature of the residual gas from respiration. Following non-linearity and storage time corrections (above), I corrected for contamination with the following protocol: on each day that I collected gas samples, I injected three or four pre-evacuated small Exetainers with oxygen-free nitrogen. I analyzed these "N_2 blanks" with the other samples on the IRMS. The average amount of oxygen detected in this set of N_2 blanks (i.e. the sum of peak areas for masses 32, 33, and 34) was divided by the amount of oxygen in each sample; this fraction was the fraction of contaminating oxygen. The original O_f was calculated as:

$$O_f = \frac{1 - O_{fct}}{1/O_{fm} - \left(O_{fct} \times 1/0.334\right)} \qquad (2.13)$$

where O_{fct} is the fraction of measured oxygen that was contamination and O_{fm} is measured fraction of O_2 to N_2. I empirically determined 0.334 to be the average ratio of O_2 to N_2 of the contaminating gas by measuring both O_2 and N_2 in pre-evacuated Exetainers that I injected with helium. The original $\delta^{18/16}O$ was calculated as:

$$\delta^{18/16}\mathrm{O} = \frac{\delta^{18/16}\mathrm{O}_m - \left(\delta^{18/16}\mathrm{O}_{ct} \times \mathrm{O}_{ct}\right)}{1 - \mathrm{O}_{ct}} \qquad (2.14)$$

where $\delta^{18/16}O_m$ is the measured $\delta^{18/16}O$ and $\delta^{18/16}O_{ct}$ is the $\delta^{18/16}O$ of contamination. $\delta^{18/16}O_{ct}$ was empirically determined as the $\delta^{18/16}O$ in N_2 blanks, after correcting for linearity and storage time.

All samples for oxygen isotope analysis for the tussock project (Chapter 3) were collected before I had determined N_2 blanks would be necessary for contamination corrections. Thus, to correct for contamination in the samples of this project I used average values of all other N_2 blanks for the amount of oxygen and the isotopic signature of contamination.

The total error (using the propagation method) associated with corrections for linearity, storage time, and contamination was 0.6% for the measurement of the O_2/N_2 ratio and 7.6% for the measurement of $^{18}O/^{16}O$.

2.5.7 Final calculations of oxygen isotope discrimination

After correcting for non-linearity, storage time, and contamination, in that order, I was able to calculate discrimination for plant samples. I converted values of $\delta^{18/16}O$ for my samples to an absolute isotopic ratio following:

$$R = \frac{^{18}O}{^{16}O_{ref}} \times (\delta^{18/16}O + 1) \qquad (2.15)$$

where R is the ratio of ^{18}O to ^{16}O and $^{18}O/^{16}O_{ref}$ is the ratio of ^{18}O to ^{16}O of the reference gas (from which $\delta^{18/16}O$ was calculated). I was then able to insert R and O_f into Eqn 2.6 to calculate discrimination.

It is typical to calculate a D value for each plant sample using 3-7 measurements of O_f and R, then to average these values for a number of plant samples for a treatment mean of D. I analyzed all values of O_f and R for each treatment together in one regression (e.g. 4 incubations per plant sample, with 6 plant replicates in a treatment = 24 measurements per treatment) to maximize statistical power. I

report the standard error of the regression of ln R against – ln O_f as the standard error of D for the treatment.

I excluded occasional outliers, as well as all samples with more than 50% oxygen drawdown. These samples tended to have atypical isotopic values, usually much lower than would be predicted by the rest of the data. It is likely that this phenomenon is due to the fact that the AOX has a lower affinity for O_2 at low concentrations of O_2 than does COX.

Relative electron flow through the AP is typically calculated by comparing discrimination results to the isotopic endpoints of each oxidase as shown in Eq. 2.7. This calculation was made for *Populus* x *canadensis* in Chapter 4. However, endpoints could not be determined for *Chionochloa* spp., *Pinus radiata*, or *Quercus rubra*. Inhibition of the AP and CP is typically confirmed by applying both inhibitors (KCN and SHAM) to a plant sample simultaneously, and observing less than 10% residual respiration when measured using oxygen electrodes. Full inhibition of respiration could not be achieved in both species of *Chionochloa* despite exhaustive efforts. The methods attempted include various concentrations of inhibitors (2 – 100 mM) and incubation times (10 – 180 minutes), vacuum infiltration, gaseous infiltration of KCN, slicing leaf tissues and stripping of the cuticle; none of these methods resulted in lower than a 30% residual respiration rate when both inhibitors were applied. In *P. radiata* and *Q. rubra*, full inhibition was observed when measuring respiration on the oxygen electrodes; however, the measured isotopic endpoints were very similar for both the AP and CP and were unusually low. Low isotopic endpoints were measured in leaves of *Nothofagus* sp. that had not been allowed to air-dry after vacuum infiltration and incubation in inhibitors (Turnbull, personal communication). It was concluded that diffusion of oxygen through inter-

cellular spaces in the leaves was greatly altered due to the presence of buffer/inhibitor. Thus, although I allowed foliage of *P. radiata* and *Q. rubra* to air-dry for several hours prior to gas collection for isotopic analysis, it is possible that inter-cellular diffusion was still an issue. Needles of *P. radiata* are thicker than broad leaves, and liquid may take far longer to evaporate from the interstices between mesophyll cells of the leaves. To the best of my knowledge, there have been no previous reports of oxygen isotope discrimination being measured in gymnosperms; thus, it would be unsurprising if evergreen needles exhibit unprecedented diffusional issues for oxygen isotopes. For unknown reasons, leaves of *Q. rubra* did not return to pre-inhibition weights after seven hours of drying, suggesting that liquid was still present in the inter-cellular spaces in the leaf. In the absence of measured endpoints for the AP and CP in these species, I interpret changes in discrimination as relative changes in the balance of electron partitioning through the AP and CP.

2.5.8 Validation of method

In order to validate this method of measuring oxygen isotope fractionation, I sought to replicate results of a previously published study. I grew *Phaseolis vulgaris* under similar conditions to those reported in Noguchi *et al.* (2001). Seeds were planted in 10 cm square pots with 15 cm depth using potting soil with slow release fertilizer. Pots were placed in a growth cabinet set to 12h:12h light:dark, 21:28 °C night:day, 70% humidity at all times, and 720 μmol photon m^{-2}. Plants were watered daily. After one month, I excised fully developed, mature leaves from 6 plants and measured their oxygen isotope discrimination following the method detailed above. Samples were incubated at 23 °C. In order to measure isotopic endpoints of these plants, I cut a leaf from each of the 6 plants into 2 cm^2 pieces and incubated these in either 5 mM KCN

or 30 mM SHAM in a buffer solution of 10 mM HEPES and 10 mM MES for 30 minutes. Leaves were then rinsed and allowed to air dry until they had reached their original fresh weight before incubation and analysis as above. Values of *D* for control and inhibited leaves were similar to those obtained by Noguchi *et al.* (2001): I measured 25.8‰ ± 0.5, 21.6‰ ± 0.4, and 20.5‰ ± 0.4 for KCN-inhibited, SHAM-inhibited, and control treatments respectively, compared with 26.7‰ ± 1.0, 19.0‰ ± 2.2, and 22.1‰ ± 2.7 (Noguchi *et al.,* 2001). Thus, I concluded that this methodology for measuring *D* was sufficient to produce accurate results.

2.6 RESPIRATORY PROTEIN ANALYSIS

2.6.1 Immunoblotting protocol

Leaf samples were snap frozen in liquid N_2 and stored for a period of time in a -80 °C freezer. Samples were freeze-dried in batches and then ground to a powder using a ball mill. 15-20 mg of each sample was weighed into a microcentrifuge tube. At this step, a membrane extraction was performed on tussock samples (see section 2.6.3 below). A heated denaturing buffer containing 42% 2-mercaptoethanol was added to samples (see Table 2.1 for concentrations for different species), which were immediately vortexed until smooth and placed in a heating block at 95 °C for 10 minutes. Samples were then centrifuged at 10,000 g at room temperature for 5 minutes. Between 25 and 30 μL of sample extract was loaded into wells of 12-well, 4-12 % SDS gels (NuPAGE, Invitrogen, Carlsbad, CA, USA) with MES running buffer (Invitrogen). A mix of protein markers (MagicMark, Invitrogen; Precision Plus, Biorad, Hercules, CA, USA) was loaded into one of the outer wells. The other outer well was left empty. Electrophoresis was performed at 200 V for 50 minutes. Proteins were transferred to a nitrocellulose membrane using iBlot (Invitrogen).

Immunoblotting was performed using Snap i.d. (Millipore, Billerica, MA, USA). Antibody concentrations used for different species are listed in Table 2.1. Antibodies were diluted in blocking solution, which was 0.5% Enhanced ChemiLuminescence (ECL) Advance Blocking Agent (GE Healthcare, Waukesha, WI, USA) in Tris-Buffered Saline with 0.1% Tween as a surfactant (TBS-t). A monoclonal AOX antibody (Elthon *et al.*, 1989) was first applied, followed by an anti-mouse horseradish peroxidase (HRP) conjugate. Blots were then incubated in either a homemade or commercial ECL solution (see section 2.6.5 below) before being photographed using a cryo-cooled digital camera (Chemigenius, Syngene, Cambridge, UK). Blots were then incubated in 15% hydrogen peroxide in order to de-activate the existing HRP-conjugates. A polyclonal antibody against the subunit II of COX (Agrisera Co., Vannas, Sweden) was then applied to the blots, followed by an anti-rabbit HRP-conjugate. Blots were similarly incubated in an ECL solution and photographed. Digital images of the blots were analyzed using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA). Bands were quantified with "integrated density" (density x area of band), adjusted for background noise on the blot.

Table 2.1: Concentrations of denaturing buffer and antibodies used for different species. The concentration of antibody in blocking solution is shown.

	Denaturing buffer (uL / mg sample)	anti-AOX	anti-mouse	anti-COX	anti-rabbit
Chionochloa pallens	12.5	3.33×10^{-3}	1.00×10^{-4}	8.33×10^{-4}	5.00×10^{-5}
C. rubra	12.5	3.33×10^{-3}	1.00×10^{-4}	16.7×10^{-4}	5.00×10^{-5}
Pinus radiata	25	2.50×10^{-3}	1.67×10^{-5}	4.17×10^{-4}	1.25×10^{-5}
Populus x *canadensis*	25	1.67×10^{-3}	1.67×10^{-5}	4.17×10^{-4}	1.25×10^{-5}
Quercus rubra	25	1.67×10^{-3}	2.50×10^{-5}	6.25×10^{-4}	1.67×10^{-5}

2.6.2 Performing immunoblotting on freeze-dried samples

Immunoblotting with AOX antibodies is typically performed on either isolated mitochondria (Elthon *et al.*, 1989) or fresh-frozen leaf tissues that are ground in liquid N_2 (Campbell *et al.*, 2007). I worked on freeze-dried samples rather than fresh-frozen for the following reasons: [1] leaves of *Chionochloa* spp. were too stiff and fibrous to grind sufficiently using liquid N_2 and a mortar and pestle, [2] grinding and weighing samples while attempting to keep the samples frozen greatly increases the chances of momentary thawing and oxidation of samples, and [3] using a ball mill to grind dry samples results in much greater particle uniformity between samples than grinding frozen. While it is possible that some proteins are degraded during the freeze-drying process, this is likely to have occurred similarly for all samples; thus, as long as all samples were prepared following the same procedure, relative comparisons between samples can still be safely made.

Once freeze-dried, I stored samples in a -20 °C freezer to ensure dryness. Samples were stored for a maximum of one year before analysis. Using a sample of *Griselinia littoralis*, I showed that proteins were still intact and detectable after storage at room temperature and humidity for 6 months.

2.6.3 Membrane extraction

I performed a "membrane extraction" on samples of *Chionochloa* spp. before applying the protein denaturing buffer. A cold (4 °C) buffer solution containing sodium ascorbate and a plant protease inhibitor was mixed with samples, which were then centrifuged at 10,000 g at 4 °C for 10 minutes. The supernatent, assumed to contain cytosolic and vacuolic contents, was discarded. The pellet was assumed to contain cell membranes and walls (AOX is thought to be attached to cellular

membranes). Samples were kept on ice throughout this procedure. The heated protein denaturing buffer was then applied to the pellet.

The membrane extraction procedure was found to improve the quality of blots for *Chionochloa* spp. It was not performed on *Pinus radiata, Populus* x *canadensis,* or *Quercus rubra*, because with these species, especially *Q. rubra*, a gel-like substance formed in the samples once the membrane extraction buffer was applied. I presume that this is due to pectin or another gel-forming substance in tree leaves. This substance prevented effective centrifugation. Clear protein bands were observed on blots of *P. radiata*, *P.* x *canadensis,* and *Q. rubra* samples when not performing the membrane extraction, so this procedure was deemed unnecessary for these species.

2.6.4 Enhanced chemiluminescence (ECL) substrate for HRP conjugates

Commercially available ECL substrates are costly; thus, I used an ECL solution that was developed by Haan & Behrmann (2007) for imaging of *Chionochloa* spp. blots. This substrate was not as effective as the commercial ECL solutions, requiring application of greater concentrations of antibodies in order to produce detectable protein bands. This substrate did not produce clear results for *P. radiata*, *P.* x *canadensis,* and *Q. rubra* samples. In an effort to produce results for these species in a cost-effective manner, I developed a stronger ECL solution by increasing the concentration of all reagents in the Haan & Behrmann (2007) recipe six-fold. This "6x ECL" recipe was used for imaging of all AOX blots for these species. However, excess noise was present on blots using COX antibody and the 6x ECL solution for these three species; thus, commercial ECL (GE Healthcare) was applied to blots for detection of COX for *P. radiata*, *P.* x *canadensis,* and *Q. rubra* samples.

Representative images of Western blots are shown for all species for both AOX and COX in Figure 2.4.

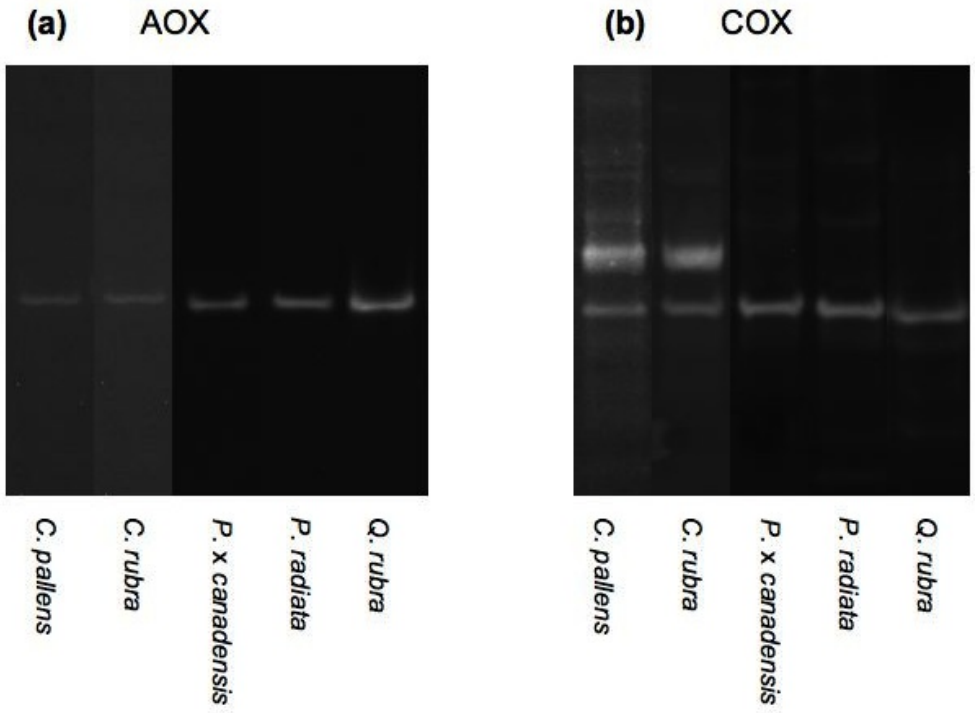


Figure 2.4: Representative lanes from Western blots showing **(a)** AOX and **(b)** COX protein bands for each species: *Chionochloa pallens, C. rubra, Populus* x *canadensis, Pinus radiata,* and *Q. rubra*. In **(b)**, the thick protein bands above the COX bands are porin bands that were not used in analysis in Chapter 3.

2.6.5 Standardizing abundances of AOX and COX proteins to total mitochondrial protein

Some studies quantify the abundance of the mitochondrial voltage-dependent anion channel porin as a measure of changes in total mitochondrial protein and report abundances of AOX and COX protein relative to this protein (Noguchi *et al.*, 2005; Armstrong *et al.*, 2008; Florez-Sarasa *et al.*, 2009). Armstrong *et al.* (2008) state that their reason for using this approach is that porin abundances remain stable under a wide range of environmental conditions. I considered this approach, and measured

abundance of porin in all *Chionochloa* spp. samples. However, due to the large range in measured porin (a range of 0.19 – 3.09 for *C. pallens* and 0.08 – 3.10 for *C. rubra* when relative to a standard), I concluded that either [1] porin is not stable under changing environmental conditions and is thus not an appropriate standard to use, or [2] the porin antibody we were using was not properly developed or stored. Additionally, the porin bands detected for *Chionochloa* spp. appeared to be double-banded, leading to the suspicion that the porin antibody I used was not specifically binding to only one protein. Figure 2.5 compares a porin band with a AOX and an COX band for one sample of *C. rubra*.

(a) **(b)** **(c)**

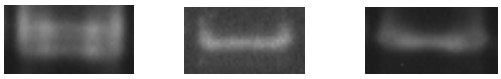


Figure 2.5: Images of **(a)** porin, **(b)** AOX, and **(c)** COX protein bands in a sample of *C. rubra*.

I also considered using total protein from whole leaf extracts as a standard against which to report AOX and COX protein abundances. I measured total protein for all *C. pallens* samples, but finding a significant correlation between total protein and chlorophyll fluorescence yield ($R^2 = 0.49$; data not shown), I concluded that changes in total protein were likely largely influenced by changes in photosynthetic protein and were thus not an accurate measure of total mitochondrial protein.

Thus, all abundances of AOX and COX reported in this thesis reflect relative abundances of these proteins only.

2.6.6 Relative abundances of AOX and COX proteins

I included a plant standard on every blot. This standard was a large batch of one typical sample for each species with average abundances of AOX and COX protein. Each species had a separate standard. The integrated density of every sample protein band was divided by the integrated density of this standard; thus, all results for each species were standardized to each other and could be fairly compared within each species. This procedure removed the complication of differing brightness between blots that could have been caused by adjusting the brightness of each blot on the digital camera or by methodological error.

In order to fairly compare the range and distribution of AOX and COX proteins, I then standardized the distribution of all samples for each protein and species by dividing all protein abundances by the average protein abundance for each set of samples. Thus, a value of 1 represents the average abundance of AOX or COX protein for a particular species over the course of the experiment. I then divided these relative values of AOX by the relative value of COX for each sample to produce an AOX/COX protein ratio. An AOX/COX protein ratio of 1 represents the average AOX/COX ratio for each species within an experiment. Because the distribution of values for AOX and COX abundances was equalized, changes in the AOX/COX ratio accurately reflect relative changes in these two proteins.

2.7 CHLOROPHYLL FLUORESCENCE

Measurements of chlorophyll fluorescence yield were made using a mini pulse-amplitude modulator (Mini-PAM, Walz, Effeltrich, Germany). Leaves were dark-adapted for 20-30 minutes using clips lined with black felt. A saturating pulse was then applied, and the maximum quantum yield of PSII (F_V/F_M, or chlorophyll

fluorescence yield) was measured. For *Chionochloa* spp., 2-4 leaves were grouped together in order to produce a large enough area for measurement. For *P. radiata*, the needles of 2-3 fascicles were pressed flat together to produce a larger area. The uneven surface of the pine needles is likely to have generated error in the F_V/F_M measurements.

For all measurements of *Chionochloa* spp. in the seasonal experiment, measurements of chlorophyll fluorescence were conducted on plants *in situ* in the field. For all other species and for *Chionochloa* spp. in the short-term experiment, measurements were made in the laboratory 1-3 hours after excising tissue in the field. The extra period of dark-adaptation during transport is likely to have caused slightly inflated values of F_V/F_M. Twenty minutes is a standard period of time for dark-adaptation before measurement of F_V/F_M, yet a longer period of time is required for complete relaxation of all PSII reaction centers. Thus, while measurements made after 20 minutes of dark-adaptation in the field represent non-maximal but standard values of F_V/F_M, the measurements made after dark transport to the laboratory would have been higher than these standard values. However, it is likely that F_V/F_M values after a long period of dark adaptation can be compared between plants of the same species in the same project.

2.8 STATISTICAL ANALYSIS

Apart from modeling the temperature response of respiration, for which Sigmaplot was used (see section 2.3), all statistical analyses were performed using R 2.4.0 (R Core Development Team). Simple linear regressions were used to test correlations between variables. A one-way ANOVA was used to test differences between treatments or days of an experiment. A two-way ANOVA was used to test differences

in respiratory parameters over time and among treatments in Chapter 5. A time series analysis was not used to test changes in variables over time as different leaves were sampled each day. A Chow test was used to detect differences between regressions for oxygen isotope discrimination analysis. All results were considered significant if $p < 0.05$.

Chapter 3: Leaf respiration and alternative oxidase in field-grown alpine grasses respond to natural changes in temperature and light

A version of this chapter has been published as an article in **New Phytologist** *and is included in Appendix A.*

3.1 INTRODUCTION

Plant respiration is highly temperature sensitive, and furthermore can acclimate to long-term changes in growth temperature (Larigauderie & Korner, 1995; Tjoelker *et al.*, 1999; Atkin *et al.*, 2000b; Atkin & Tjoelker, 2003; Ow *et al.*, 2008a; ; Tjoelker *et al.*, 2008). The physiological mechanisms leading to acclimation are still not fully understood. Nonstructural carbohydrates (starches and sugars) are often correlated with changes in respiration and its temperature response (Azcòn-Bieto *et al.*, 1983a; Tjoelker *et al.*, 1999; Lee *et al.*, 2005; Tjoelker *et al.*, 2008), as is adenylate status (Hoefnagel *et al.*, 1998; Atkin *et al.*, 2002; Covey-Crump *et al.*, 2007). It is also possible that the cyanide-insensitive alternative oxidase (AOX), which catalyzes the reduction of O_2, may contribute towards the process of thermal acclimation. AOX produces at most a third of the ATP generated by respiration via the cytochrome *c* oxidase (COX) catalyzed pathway (Vanlerberghe & McIntosh, 1997; Millenaar & Lambers, 2003). Thus, in theory, an increased proportion of respiration via the alternative pathway (AP) would result in greater total rates of respiration in order to meet the same energy demands.

Why would plants respire via an "energy-wasteful" pathway? AOX has been proposed to be involved in the stress response of plants to cold (Gonzalez-Meler *et al.*, 1999; Fiorani *et al.*, 2005; Armstrong *et al.*, 2008), heat (Rachmilevitch *et al.*, 2007; Murakami & Toriyama, 2008), drought (Ribas-Carbo *et al.*, 2005b), nutrient deficiency (Gonzalez-Meler *et al.*, 2001), and excess light (Noguchi *et al.*, 2001; Yoshida *et al.*, 2007). It is possible that the AP acts to reduce stress is through prevention of excess reactive oxygen species (ROS). ROS accumulate in plant mitochondria in response to low temperatures (Purvis & Shewfelt, 1993; Fiorani *et al.*, 2005; Sugie *et al.*, 2006) and other environmental stresses (Juszczuk, I *et al.*,

2001; Bartoli *et al.*, 2004) and can cause severe harm to the mitochondria (Rich & Bonner, 1978).

It is unknown how quickly changes in alternative respiration may affect total rates of respiration, and if this is sustained after a few days. Acclimation of dark respiration (R_d) to temperature has been found to occur quickly (2-10 days) in some species under controlled environment conditions (Rook, 1969; Rychter *et al.*, 1988; Atkin *et al.*, 2000b; Bolstad *et al.*, 2003; Armstrong *et al.*, 2008) and even under field conditions (Bolstad *et al.*, 2003), but this contrasts with the findings of Ow *et al.* (2008a, b). However, whereas changes in respiration brought about through acclimation are generally found to be sustained over long periods of constant temperature (Larigauderie & Korner, 1995; Arnone & Korner, 1997; Armstrong *et al.*, 2008), changes in AP engagement (Armstrong *et al.*, 2008), protein (Vanlerberghe & McIntosh, 1992; Mizuno *et al.*, 2008) and transcript abundance (Ito *et al.*, 1997; Sugie *et al.*, 2006) are sometimes found to be transitory on the timescale of days. Thus, it is unclear how AOX functions to alter metabolic function over multiple timescales, and how this is related to changes in respiration in variable temperature environments.

Alpine ecosystems provide a unique environment in which to study the natural response of plants to environmental stress. Alpine plants are regularly exposed to harsh conditions including low temperature, wind, high sunlight and drought. Day-to-day fluctuations in temperature may be rapid and extreme. Additionally, altitudinal gradients can be used as natural environmental gradients of temperature and other variables. Alpine areas are of high ecological importance, covering roughly one fifth of total terrestrial area and featuring high plant diversity (Korner, 2004). Lastly, high-altitude ecosystems will be particularly responsive to climate change in coming decades and may serve as a "bellwether of change" (Wookey *et al.*, 2009).

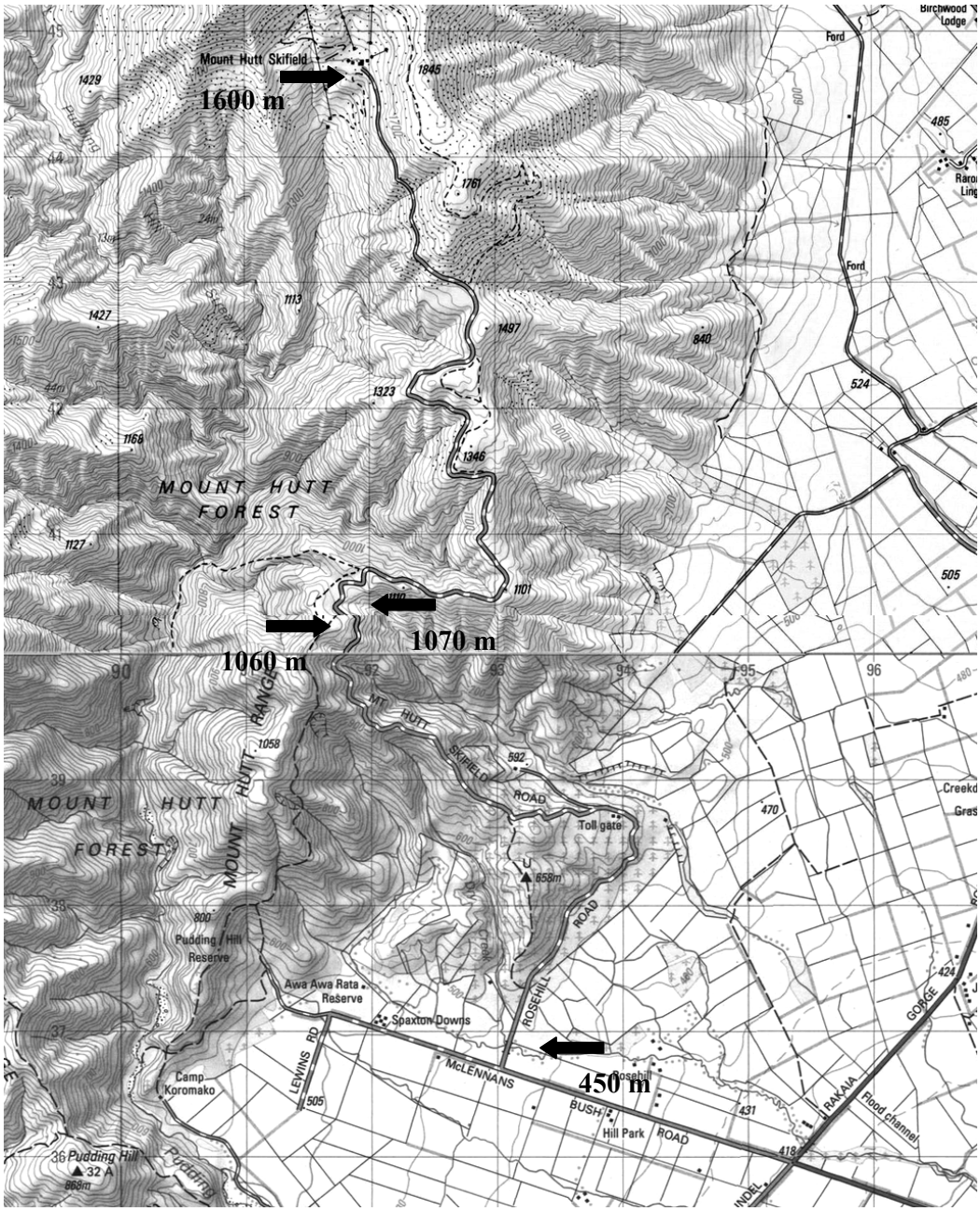
Here, I investigated the responses of R_d and AP engagement in field-grown *Chionochloa pallens* and *C. rubra* (evergreen perennial tussock grasses) to daily and seasonal changes in ambient temperature. Four sites were utilized along an altitudinal gradient on Mt. Hutt, New Zealand in order to extend the range of natural temperature variability experienced by these grasses. The following questions are addressed:

1. On what timescale does acclimation of leaf respiration occur in field-grown alpine grasses?
2. Does the oxygen isotope discrimination of respiration change in response to cold stress in the field? Is this mediated through changes in AOX protein abundance?
3. Does AOX contribute towards the seasonal acclimation of *R* in these species?

3.2 MATERIALS AND METHODS

3.2.1 Field sites

All grasses studied were found growing *in situ* on Mt. Hutt, Canterbury, New Zealand (43º32´S, 171º33´E), which is located on the eastern edge of the central Southern Alps, approximately 100 km west of Christchurch. Four previously established field sites at varying altitudes and densely populated with two species of *Chionochloa* were utilized. *Chionochloa rubra* Zotov was sampled at low- (450m a.s.l.) and mid-altitude (1070m) sites; *Chionochloa pallens* Zotov was sampled at mid- (1070m) and high-altitude (1600m) sites. Arrows point to field sites in Map 3.1.



Map 3.1: Arrows indicate the location of the four field sites on Mt. Hutt. Image produced by MapToaster Topo/NZ and published in Clifford (Clifford, 2007).

Weather stations were present at the low- and high-altitude sites, and at one of the mid-altitude sites (1070m). Measurements of ambient temperature at each altitude were taken at 1.4 m above the ground (YSI thermistors in a Stevenson screen) every

30 seconds, with daily mean, min, and max stored in a datalogger (Campbell Scientific CR21X; Logan, Utah). Measurements of photosynthetically active radiation (PAR) were made with a LiCor 190SB quantum sensor taking measurements every 5 seconds. Precipitation was measured at the low- and mid-altitude stations using 34 cm tipping buckets (Environmental Measurements ARG100, Osney Mead, UK). Mean daily air temperature and precipitation at the mid-altitude site are shown in Figure 3.1.

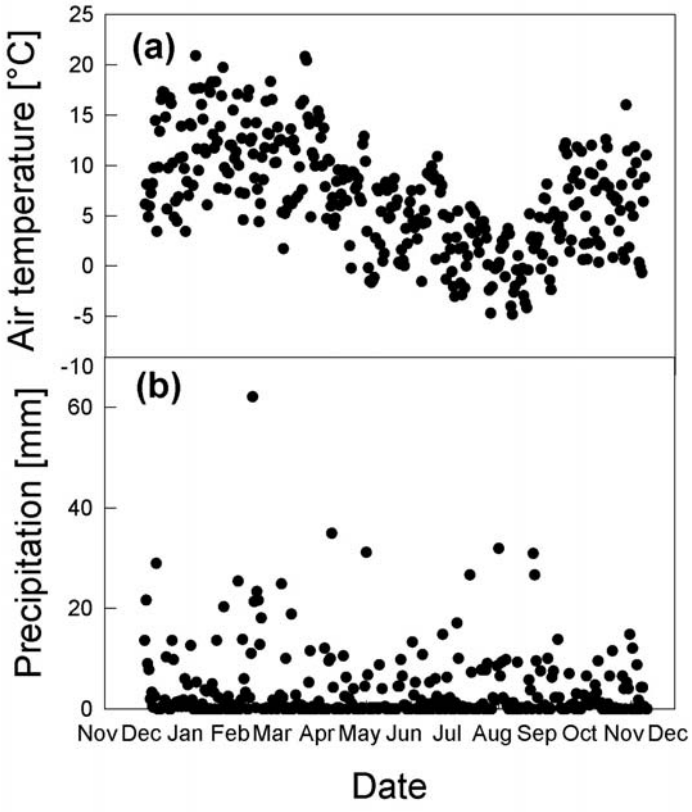


Figure 3.1: (a) Daily mean temperature (ºC) and **(b)** daily precipitation (mm) from the weather station at the middle field site on Mt. Hutt (1070m a.s.l.) from November 29 2007 – November 12 2008 (the course of the seasonal field experiment). Precipitation did not differ significantly between months.

3.2.2 Botanical description of study species

These herbaceous perennials, both commonly known as "snow tussock," are wild and endemic to New Zealand. *Chionochloa* belongs in the family Poaceae.

C. pallens Zotov

This species is sometimes known as "mid-ribbed snow tussock." It is widely distributed in New Zealand and is typically found growing in herbfields in alpine and sub-alpine zones. Leaves are V-shaped with a long sheath and smooth margin and lack prickle teeth. The leaf blade is persistent on the sheath. Leaves are typically about 9 mm wide and can be up to 65 cm long. The plant can reach up to 1 m in height. Leaf sheaths emerge from tillers and are evergreen. The inflorescence is glaborous, or can be glaborous below with scabrous or stiff hairy above, and some long hairs at branch axils. Spikelets have up to nine lightly purpled or straw-coloured florets that are wind-pollinated. Seeds are approximately 3.5 mm long.

C. rubra Zotov

This species is sometimes known as "red tussock" in addition to "snow tussock." Its habitat ranges from subalpine to warm temperate lowland and it is locally common throughout New Zealand. It grows in open fields and tussocklands. Leaves are rolled, crowded, erect, and stiff. The leaf-sheath has a scabrid or smooth margin and prickle teeth on the adaxial leaf blade. Leaf blades are 1 – 2 mm in diameter, and reach 1m in length. The mean plant height is 1.25 m, but can reach 1.5 m. Leaf sheaths emerge from tillers and are evergreen. The inflorescence is an open panicle that can reach 45 cm. It is glaborous except for long hairs at branch axils. Spikelets have up to 9 florets.

Florets are wind-pollinated. Seeds are 3.5 mm in length and width, and are dispersed by wind or attachment.

All information on Chionochloa spp. was obtained from the Plants Databases at Landcare Research (http://www.landcareresearch.co.nz/databases/db_category.asp?DB_Coll_Category_ID=2).

3.2.3 Sampling

Two field studies were conducted in order to determine the timescale of physiological changes in *Chionochloa* spp.: a seasonal study with approximately monthly measurements at all altitudes and a short-term study on *Chionochloa* at only the mid-altitude site (1070m). Measurements for the seasonal study took place at all altitudes at seven times in December 2007, and January, February, March, April, October, and November 2008 (respiration was also measured in November 2007 and is presented here). Measurements were not taken between April and October 2008, and *C. pallens* (upper site) was not sampled in October 2008 due to deep snow cover. All results from the seasonal experiment have been pooled between altitudinal sites for each species (i.e. data from *C. rubra* at 450m and 1070m have been pooled, and data from *C. pallens* at 1070m and 1600m have been pooled), as I did not detect an altitudinal effect in the results. Measurements for the short-term experiment took place on seven occasions between December 27, 2008 and January 9, 2009 at only the middle site. Different plants were sampled each day of both experiments. At mid-morning on each sampling day, grasses were cut at the base of the shoot, wrapped in damp paper towels, and transported back to the laboratory in darkness for measurements. N = 6 for all measurements.

Please see Chapter 2: Methods for details on measurements of respiration, oxygen isotope discrimination, immunoblotting, carbohydrates, nitrogen, specific leaf area, chlorophyll fluorescence, and statistical analysis.

3.3 RESULTS

3.3.1 Seasonal study

Temperature and precipitation at the middle field site (1070 m) over the course of the study are shown in Figure 3.1. Precipitation did not differ significantly between the lower and middle field sites ($p = 0.553$), or between months at the lower ($p = 0.426$) or middle sites ($p = 0.667$).

The appropriate time window explaining the extent and rate of acclimation of respiration to changes in ambient temperature was determined by evaluating the strength (R^2 value) of the relationship between R_{10} and ambient temperature averaged over a range of time periods (Fig. 3.2). The relationship was similar between E_0 and ambient temperature (data not shown). The R^2 value increased to a transient peak at 3-5 days, after which it decreased and then increased to a maximum for each species and site at around 120 days; thus, 120 days was chosen as the time window for calculating the ambient temperature to which *R* in *C. rubra* and *C. pallens* acclimated.

R_{10} was significantly and negatively correlated with 120d averaged temperature in both species (Fig 3.3a,b), while E_0 was significantly and positively correlated with 120d averaged temperature in *C. pallens* only. Respiration at the field growth temperature (R_g) was calculated using the R_{10} and E_0 values measured on each

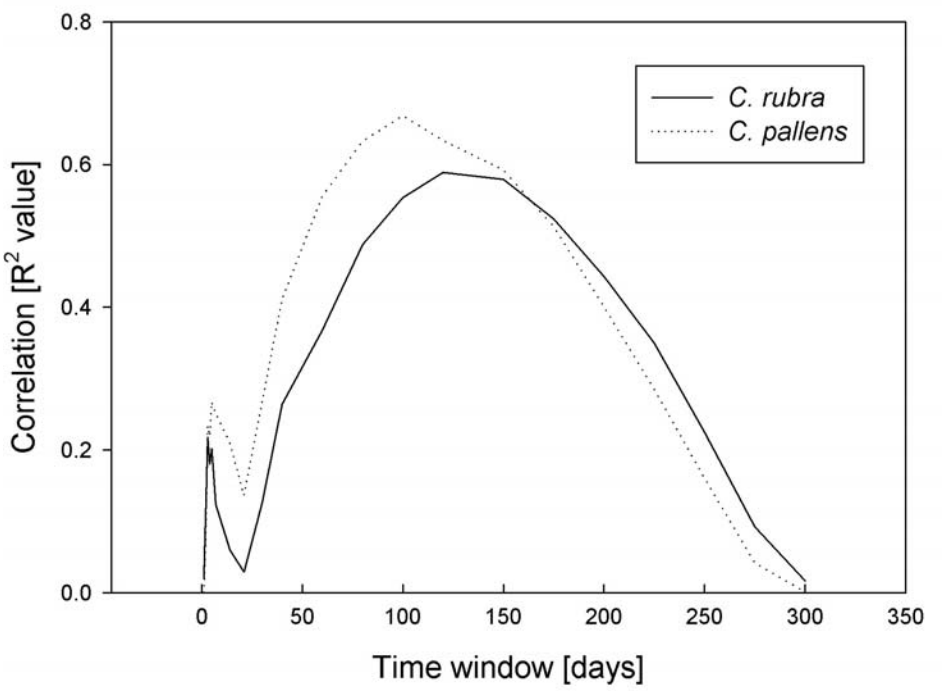


Figure 3.2: Correlation (R^2 value) for the linear regression between R_{10} and temperature averaged over an increasing time window preceding the day of measurements (in days) for *C. rubra* and *C. pallens* in the seasonal study.

sampling date and the minimum temperature the night prior to sampling. In both species, R_g shows a scattered response to the previous night's minimum temperature, but there is no evidence that (acclimated) R_g increases with temperature (Fig 3.3e,f). Neither R_{10} nor E_0 was significantly correlated with % sugar or % starch (see Table 3.1 for data). Leaf nitrogen (N) was positively correlated with R_{10} and with growth temperature in *C. pallens* ($R^2 = 0.373$; $p = 0.032$) but not in *C. rubra* (Table 3.1). N was not correlated with E_0.

The relationships between R_{10} and the AOX/COX protein ratio and between the AOX/COX protein ratio and 120d averaged temperature are shown in Fig 3.4. Neither 120d averaged temperature nor R_{10} in *C. rubra* was correlated with the AOX/COX protein ratio (Fig 3.4a,c; $p = 0.610$, 0.892 respectively). In *C. pallens*, the

Table 3.1: Values ± SEM for R_{10}, E_0, R_g, D, AOX/COX protein abundance ratio, soluble sugar, starch, nitrogen, and F_V/F_M in *Chionochloa rubra* and *C. pallens* during the course of the seasonal study.

		R_{10} (umol CO_2 kg^{-1} s^{-1})	E_0 (kJ mol^{-1})	R_g (umol CO_2 kg^{-1} s^{-1})	Discrimination (‰)	AOX/COX protein	Sugar (%)	Starch (%)	Leaf nitrogen (%)	F_V/F_M
Lower site	Nov, 2007	1.00 ± 0.09	50.1 ± 5.1	0.60 ± 0.08						
C. rubra	Dec, 2007	0.95 ± 0.04	43.4 ± 0.7	0.71 ± 0.03	21.68 ± 0.05	0.80 ± 0.23	4.58 ± 0.24	11.64 ± 0.84	1.15 ± 0.05	0.863 ± 0.006
	Jan, 2008	1.14 ± 0.14	40.2 ± 4.0	1.06 ± 0.13	17.77 ± 0.97	0.68 ± 0.30	5.67 ± 0.75	11.37 ± 0.66	1.05 ± 0.05	0.829 ± 0.007
	Feb, 2008	0.70 ± 0.10	60.6 ± 8.8	0.46 ± 0.08	16.85 ± 0.64	0.97 ± 0.43	4.75 ± 0.40	20.40 ± 1.38	1.00 ± 0.06	0.823 ± 0.004
	March, 2008	0.63 ± 0.13	64.3 ± 9.2	0.36 ± 0.10	19.56 ± 0.91		6.15 ± 0.94	11.93 ± 0.46	1.16 ± 0.03	0.821 ± 0.005
	April, 2008	0.36 ± 0.06	59.7 ± 1.5	0.33 ± 0.05	20.73 ± 0.97	0.81 ± 0.26	9.22 ± 1.01	15.28 ± 1.30	1.16 ± 0.07	0.834 ± 0.001
	Oct, 2008	1.92 ± 0.30	42.0 ± 5.5	1.01 ± 0.23	19.73 ± 0.50	0.98 ± 0.12	11.30 ± 1.21	12.75 ± 0.34	1.36 ± 0.04	0.837 ± 0.002
	Nov, 2008	1.48 ± 0.11	45.6 ± 4.0	1.42 ± 0.11	21.01 ± 1.34	1.24 ± 0.16	6.57 ± 0.92	12.11 ± 0.97	1.12 ± 0.06	0.840 ± 0.003
Middle site	Nov, 2007	0.97 ± 0.12	46.0 ± 5.2	0.59 ± 0.10						
C. rubra	Dec, 2007	1.16 ± 0.14	32.4 ± 6.1	0.91 ± 0.15	23.03 ± 0.57	1.76 ± 0.26	5.12 ± 0.32	13.38 ± 0.70	1.17 ± 0.05	0.856 ± 0.003
	Jan, 2008	1.07 ± 0.17	33.2 ± 5.0	0.94 ± 0.17	21.70 ± 1.19	0.72 ± 0.12	5.47 ± 0.86	13.56 ± 0.69	1.20 ± 0.06	0.834 ± 0.006
	Feb, 2008	0.91 ± 0.07	31.0 ± 3.0	0.56 ± 0.06	20.27 ± 0.55	2.28 ± 0.05	5.33 ± 0.27	23.55 ± 1.58	1.07 ± 0.10	0.823 ± 0.005
	March, 2008	0.50 ± 0.07	62.1 ± 6.6	0.29 ± 0.05	21.63 ± 1.13		6.36 ± 0.47	12.96 ± 0.50	1.00 ± 0.08	0.806 ± 0.007
	April, 2008	0.30 ± 0.10	74.6 ± 19.3	0.22 ± 0.08	21.06 ± 0.72	1.27 ± 2.06	13.94 ± 2.25	19.62 ± 1.69	0.89 ± 0.05	0.828 ± 0.002
	Oct, 2008	1.78 ± 0.23	40.1 ± 5.2	0.89 ± 0.17	20.53 ± 0.45	0.91 ± 0.28	10.3 ± 0.79	16.46 ± 0.74	1.00 ± 0.11	0.825 ± 0.004
	Nov, 2008	1.32 ± 0.08	42.1 ± 3.7	0.97 ± 0.08	22.01 ± 0.71	1.38 ± 0.30	10.38 ± 1.59	11.53 ± 0.84	0.93 ± 0.07	0.813 ± 0.004
Middle site	Nov, 2007	1.80 ± 0.18	38.0 ± 5.6	1.17 ± 0.15						
C. pallens	Dec, 2007	1.40 ± 0.16	50.2 ± 6.2	0.94 ± 0.14	23.42 ± 0.93	0.79 ± 0.17	7.86 ± 1.00	13.03 ± 1.18	0.97 ± 0.06	0.840 ± 0.005
	Jan, 2008	0.92 ± 0.15	60.0 ± 5.1	0.72 ± 0.13	22.85 ± 0.87	0.84 ± 0.06	6.76 ± 0.47	14.54 ± 0.96	0.94 ± 0.05	0.846 ± 0.004
	Feb, 2008	1.00 ± 0.10	61.0 ± 2.6	0.38 ± 0.03	21.22 ± 0.99	2.17 ± 0.22	6.97 ± 0.34	20.67 ± 1.22	0.93 ± 0.04	0.824 ± 0.003
	March, 2008	0.67 ± 0.05	73.7 ± 7.5	0.35 ± 0.05	20.16 ± 1.00	0.80 ± 0.04	8.21 ± 1.66	18.97 ± 0.59	0.95 ± 0.06	0.830 ± 0.004
	April, 2008	0.52 ± 0.08	75.8 ± 8.5	0.33 ± 0.07	20.21 ± 0.75	0.76 ± 0.11	13.18 ± 0.48	22.27 ± 2.43	0.96 ± 0.04	0.827 ± 0.002
	Oct, 2008	2.88 ± 0.28	43.9 ± 4.8	1.33 ± 0.25	20.42 ± 0.88	2.58 ± 0.39	14.2 ± 0.89	17.40 ± 1.41	1.05 ± 0.04	0.811 ± 0.005
	Nov, 2008	1.73 ± 0.13	44.3 ± 2.1	1.24 ± 0.10	20.63 ± 1.44	1.41 ± 0.08	17.94 ± 3.26	12.43 ± 1.00	0.94 ± 0.07	0.829 ± 0.004
Upper site	Nov, 2007	1.13 ± 0.27	60.2 ± 9.9	0.62 ± 0.19						
C. pallens	Dec, 2007	1.43 ± 0.18	60.9 ± 4.6	0.81 ± 0.13	23.80 ± 1.26	1.44 ± 0.12	8.41 ± 0.43	15.77 ± 0.92	1.09 ± 0.03	0.846 ± 0.008
	Jan, 2008	0.86 ± 0.10	63.9 ± 3.5	0.59 ± 0.08		1.08 ± 0.11	8.52 ± 0.60	18.46 ± 1.03	1.00 ± 0.04	0.833 ± 0.003
	Feb, 2008	1.06 ± 0.05	57.2 ± 2.7	0.38 ± 0.03	22.16 ± 0.43	0.81 ± 0.19	8.60 ± 0.64	23.36 ± 2.10	0.90 ± 0.05	0.810 ± 0.002
	March, 2008	0.80 ± 0.04	68.0 ± 3.9	0.33 ± 0.03	22.08 ± 0.83	1.38 ± 0.26	9.01 ± 0.32	18.76 ± 1.09	0.95 ± 0.05	0.816 ± 0.005
	April, 2008	0.47 ± 0.07	74.2 ± 7.5	0.28 ± 0.05	21.05 ± 0.54	0.57 ± 0.04	15.53 ± 1.06	35.05 ± 4.46	0.83 ± 0.05	0.809 ± 0.003
	Nov, 2008	1.97 ± 0.17	40.0 ± 2.6	1.36 ± 0.12	21.77 ± 0.51	5.16 ± 0.67	8.94 ± 0.64	15.93 ± 1.60	1.02 ± 0.04	0.804 ± 0.003

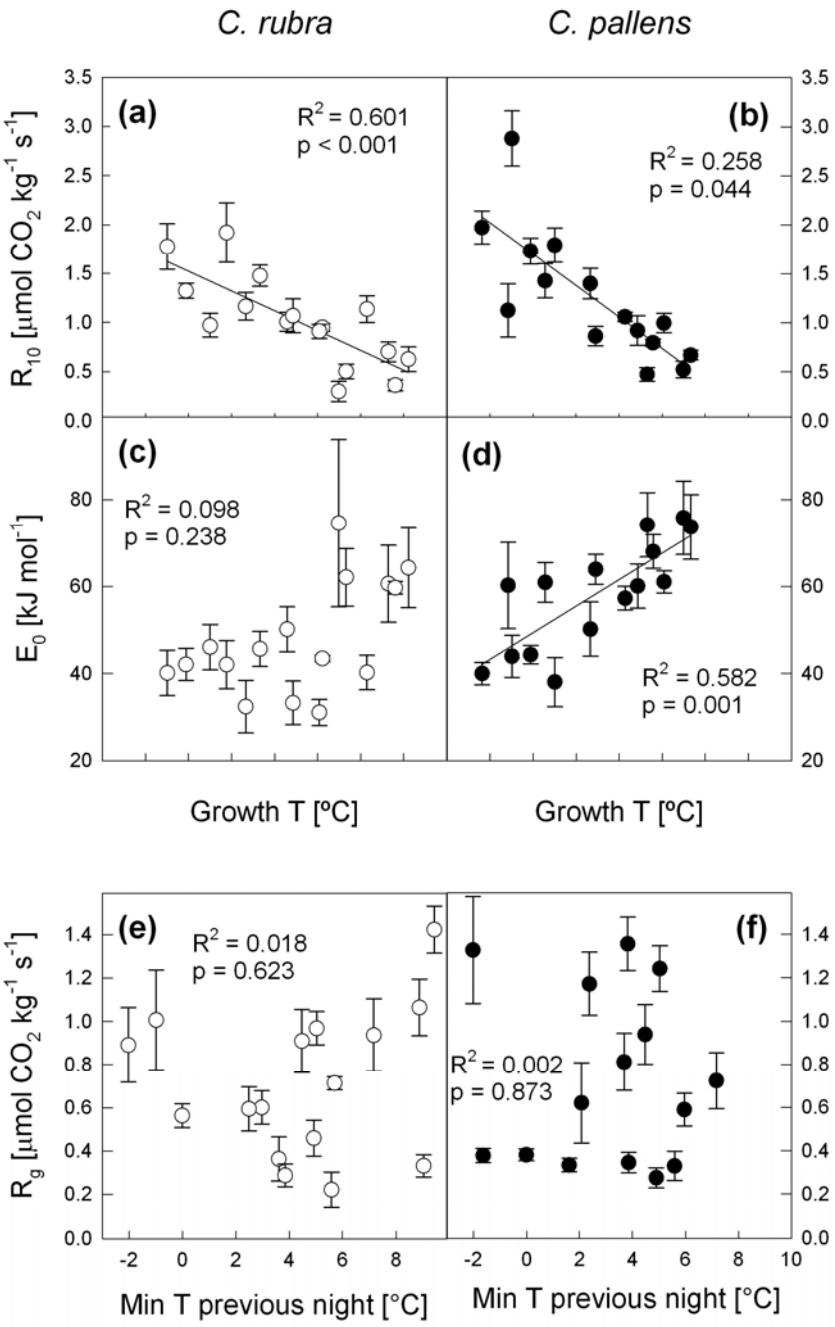


Figure 3.3: Respiratory parameters for *C. rubra* and *C. pallens* in the seasonal study. Values shown are mean ± SEM. **(a & b):** R_{10} [respiration at a set-point temperature of 10 ºC (µmol CO_2 kg^{-1} s^{-1})] plotted against growth temperature (ºC) (field temperature averaged over 120 d prior to measurement) **(c & d):** E_0 [the instantaneous temperature sensitivity of respiration (kJ mol^{-1})] plotted against growth temperature (ºC) **(e & f):** R_g = respiration at the ambient growth temperature (minimum temperature the night prior to sampling) (µmol CO_2 kg^{-1} s^{-1}) against the minimum temperature the night prior to sampling.

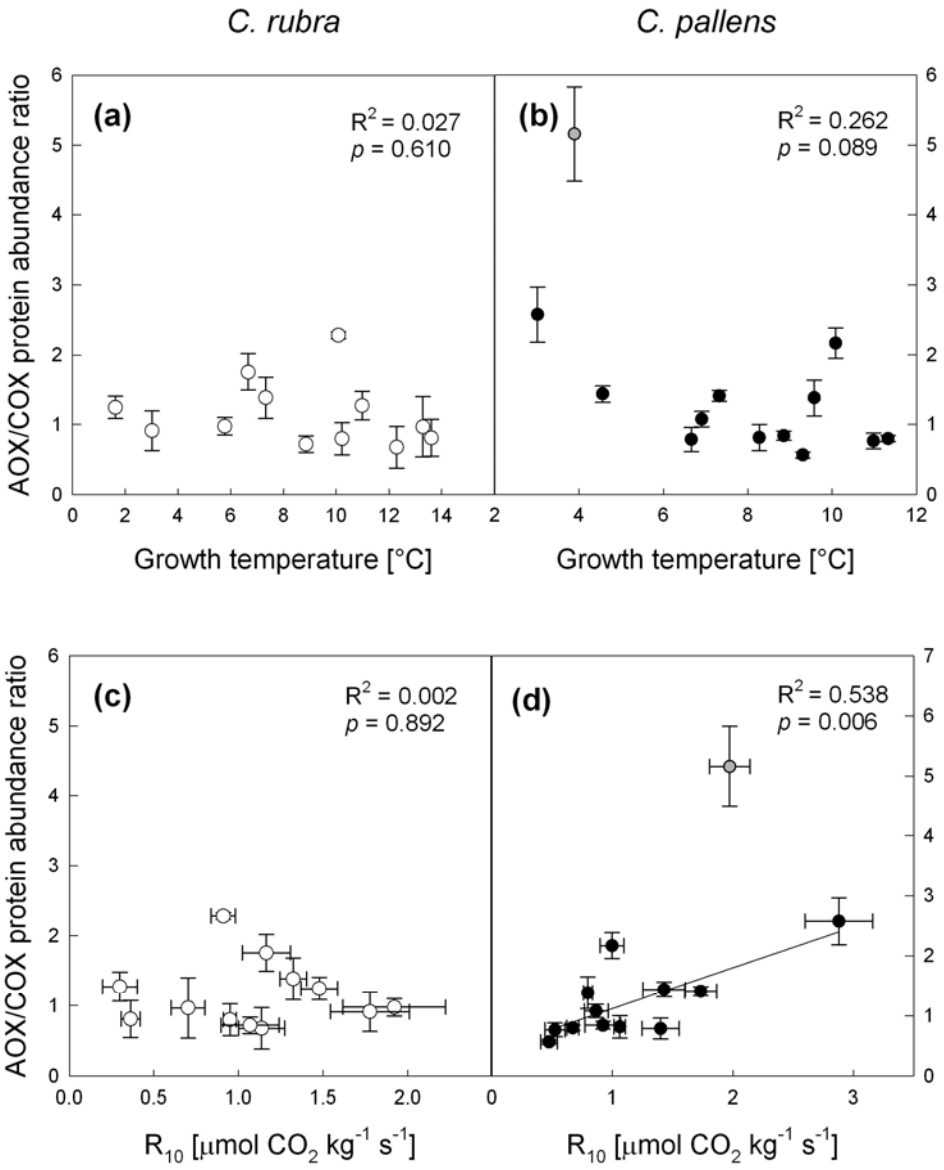


Figure 3.4: The ratio of alternative oxidase (AOX) and cytochrome c oxidase (COX) protein abundances in the seasonal study against growth temperature (field temperature averaged over 120 d prior to measurement) in *C. rubra* and *C. pallens* in **(a)** and **(b)**, and against R_{10} in **(c)** and **(d)**. In **(b)** and **(d)**, grey-colored symbols show outliers that were not included in the regression analysis. Values shown are mean ± SEM.

AOX/COX protein ratio showed a statistically insignificant negative relationship with 120d averaged temperature (Fig 3.4b; $R^2 = 0.262$; $p = 0.089$) and was significantly and positively correlated with R_{10} (Fig 3.4d; $R^2 = 0.538$, $p = 0.006$). Inclusion of an outlier in the regression analyses, shown in grey in Figure 3.4b,d, results in the statistics: $R^2 = 0.362$ and $p = 0.030$ for Figure 3.4b, and $R^2 = 0.393$ and $p = 0.022$ for Figure 3.4d.

Discrimination (D), as measured by oxygen isotope fractionation, was not correlated with ambient temperature, R_{10}, E_0, the AOX/COX protein ratio, or the contents of soluble sugars or starch. D was positively correlated with the previous day's integrated PAR in both species (Fig 3.5; *C. rubra*: $R^2 = 0.322$, $p = 0.034$; *C. pallens*: $R^2 = 0.525$, $p = 0.008$). Due to the difficulty in obtaining endpoints of discrimination for AOX and COX, values of AP engagement cannot be estimated, and discrimination numbers are interpreted as a measure of relative changes in electron partitioning between the two oxidases. Higher D indicates increased engagement of AOX, which discriminates more heavily against ^{18}O than does COX. Thus, I infer greater electron partitioning to the AP relative to the CP with high ambient light in these species.

Chlorophyll fluorescence yield (F_V/F_M) ranged from 0.803 to 0.863 (Table 3.1) and was not correlated with any measured parameters or environmental variables.

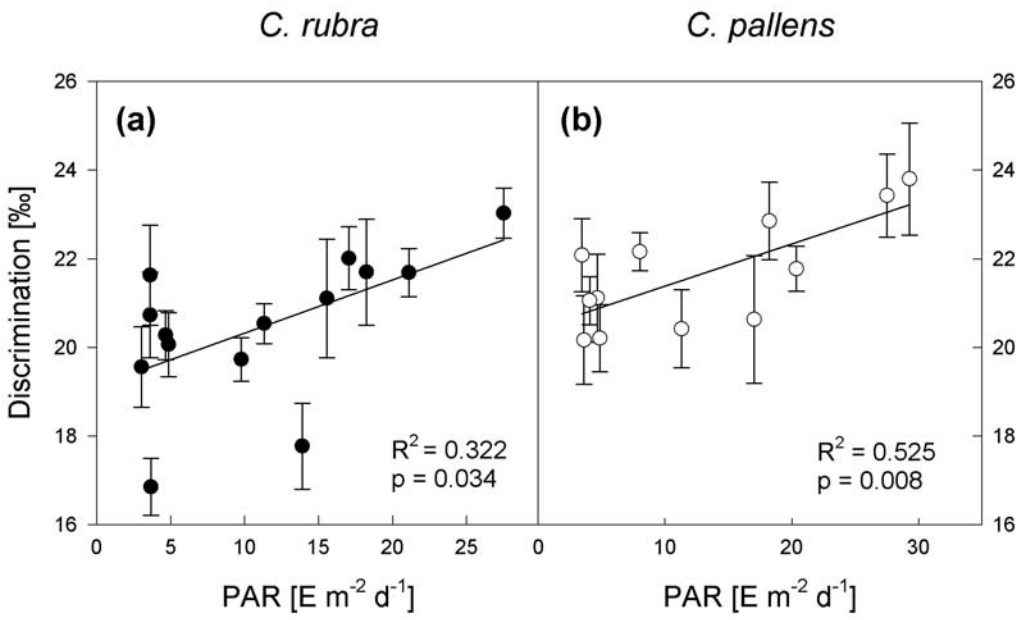


Fig 3.5: Oxygen isotope discrimination of respiration (*D*) of *C. rubra* and *C. pallens* in the seasonal study plotted against the previous day's integrated PAR. Values shown are mean ± standard error of regression.

3.3.2 Short-term study

Although a large variation in daily average temperature was observed at the middle site over the course of the short-term experiment (e.g. a drop from 19 to 7ºC; Fig 3.6a), there were no significant changes in R_{10} (p = 0.667; 0.526), E_0 (p = 0.177; 0.883), discrimination (F=0.263, df=43; Fig 3.6b - d), or AOX or COX protein abundance (Table 3.2) in either *C. rubra* or *C. pallens*. There was a significant increase in % TNC over the course of the experiment (Table 3.2; $p < 0.001$ in both species).

3.4 DISCUSSION

Strong acclimation of respiration occurred in *Chionochloa* spp. when measured over seasonal timescales: respiration at a basal reference temperature (R_{10}) decreased significantly with increases in ambient temperature (Fig 3.3a,b; Table 3.1). As a

result, respiration at the previous night's minimum field temperature (R_g) did not increase with temperature (Fig 3.3e,f), representing a reduction in long-term temperature sensitivity relative to the instantaneous response. The response of R_g to the previous night's minimum temperature was variable; thus the slow changes in respiratory parameters were clearly not sufficient to dampen the response of R to the large daily temperature fluctuations experienced in the field in the New Zealand alpine zone.

In field studies that incorporate an assessment of thermal acclimation of respiration, respiration at the growth temperature is typically calculated using a short time window (2-7 days) of averaged temperature (Atkin *et al.*, 2000b; Lee *et al.*, 2005; Xu & Griffin, 2006; Tjoelker *et al.*, 2008; Ow *et al.*, 2010). This has been appropriately based on experimental evidence suggesting that the thermal acclimation of R_d is a relatively rapid process in several previously studied species (Rook, 1969; Atkin *et al.*, 2000b; Bolstad *et al.*, 2003; Lee *et al.*, 2005; Armstrong *et al.*, 2008). Although there was a small peak in the correlation between respiratory parameters and field temperature averaged over 3-5 days, the strongest relationship occurred when temperature was averaged over a much longer period (110-120 days; Fig 3.2). Moreover, while there was no significant acclimation of R to seasonal changes in temperature, R_{10} and E_0 did not respond clearly to temperature variation on the timescale of days (Figs 3.3, 3.6). I speculate that seasonal changes in respiration in *Chionochloa* spp. may respond to long-term temperature changes as well as other seasonal environmental signals such as day length. Slow acclimation in these perennial alpine grasses may provide an adaptive physiological advantage in the face of constantly fluctuating ambient temperatures (Fig 3.1) – a relatively slow seasonal

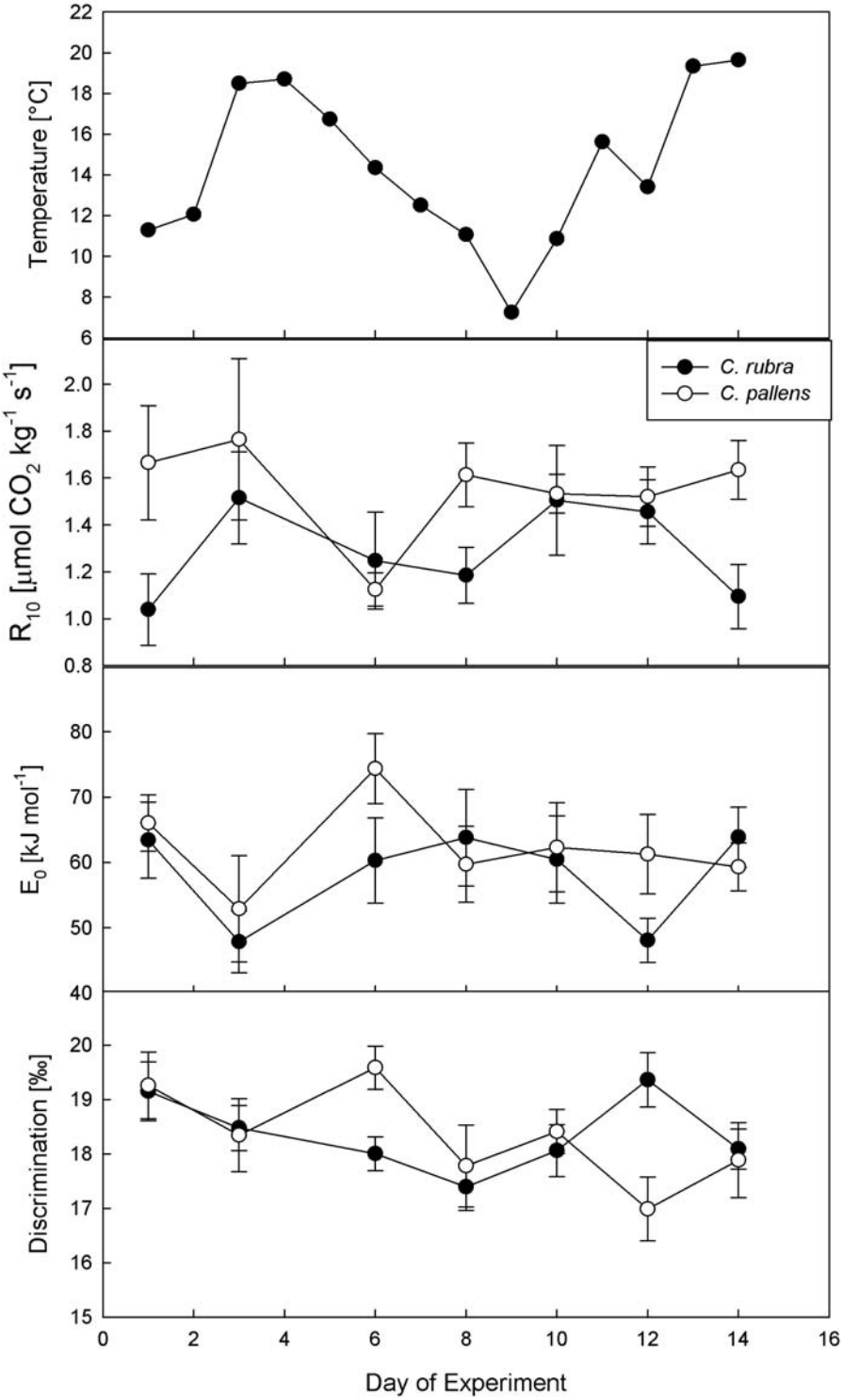


Figure 3.6: Short-term study. **(a):** mean daily field temperature for each day of the experiment. **(b – d)** show $R_{10,}$ E_0, and oxygen isotope discrimination (D) respectfully. Values shown are mean ± SEM.

Table 3.2: Percent TNC, AOX and COX protein abundance for each day of the short-term study. Values shown are mean ± SEM. P-values for one-way ANOVA are shown for each variable, with significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

C. rubra			
Day	**% TNC**	**AOX**	**COX**
1	34.7 ± 1.2		
3	20.5 ± 1.3	1.07 ± 0.14	0.88 ± 0.18
6	31.1 ± 1.9		
8	23.7 ± 1.6	1.23 ± 0.12	0.72 ± 0.07
10	14.5 ± 1.6	0.95 ± 0.3	1.11 ± 0.1
12	21.7 ± 0.7		
14	29.8 ± 1.6	1.44 ± 0.25	1.01 ± 0.18
***p*-value**	*** < 0.001	0.487	0.266

C. pallens			
Day	**% TNC**	**AOX**	**COX**
1	37 ± 0.5		
3	27.2 ± 4.2	0.76 ± 0.12	0.99 ± 0.25
6	37.5 ± 2.2		
8	28.5 ± 1.6	0.53 ± 0.06	0.73 ± 0.16
10	21 ± 2	1.34 ± 0.41	1.25 ± 0.21
12	29.3 ± 1.9		
14	40.5 ± 1.8	1.03 ± 0.21	1 ± 0.07
***p*-value**	*** < 0.001	0.134	0.359

response would avoid frequent metabolic changes and associated construction and/or maintenance costs.

Acclimation of respiration in pre-existing leaves subjected to new temperatures has been proposed to result primarily from changes in the instantaneous temperature sensitivity (E_0 or Q_{10}) and not R_{10} ("type I" acclimation; Atkin & Tjoelker, 2003; Atkin *et al.*, 2005). However, in this seasonal study of two perennial grasses with leaves that live multiple years, acclimation resulted from large changes in R_{10} ("type II" acclimation), with higher R_{10} in winter-acclimated leaves than in summer. Thermal acclimation via changes in R_{10} have been reported in seasonal field studies (Atkin *et al.*, 2000b; Xu & Griffin, 2006; Ow *et al.*, 2010) as well as in plants subjected to long-term temperature manipulations (Tjoelker *et al.*, 1999; Bruhn *et al.*,

2007). The results presented here, along with these studies, support the idea that acclimation in pre-existing leaves can result from changes in R_{10} (respiratory capacity). Additionally, whereas other studies have found that seasonal or long-term acclimation is accompanied by either no change in the instantaneous temperature sensitivity (E_0) of R_d (Tjoelker *et al.*, 1999; Bruhn *et al.*, 2007) or an increase in E_0 with cold (Atkin *et al.*, 2000b; Ow *et al.*, 2010), in this study there was a positive correlation between E_0 and 120d averaged temperature, with higher values of E_0 occurring in the summer and the lowest E_0 in the winter. Although it may seem counter-intuitive to observe a decrease in E_0 when an up-regulation of R_d is required, low E_0 during cold months would tend to reduce R_d primarily at high temperatures. This response would have little impact on *in situ* R_d at the low prevailing growth temperatures in winter, but would prevent an overcompensation of R_d during short periods of warm temperatures that are not uncommon in the variable climate of the New Zealand alpine zone.

I questioned whether acclimation of R_d to temperature in *Chionochloa* spp. would be underpinned by changes in nonstructural carbohydrates, leaf nitrogen, and abundance and engagement of AOX and COX. In contrast to a number of studies that link R to carbohydrate status (Tjoelker *et al.*, 1999; Whitehead *et al.*, 2004; Lee *et al.*, 2005; Tjoelker *et al.*, 2008; Ow *et al.*, 2010), here there was no relationship between concentrations of soluble sugars, starches, or TNC and respiratory parameters. There was a significant relationship between R_{10} and leaf N in *C. pallens* in the seasonal study, but not in *C. rubra* (Table 3.1). However, the change in N in both species was small (0.89-1.35% in *C. rubra*; 0.83-1.05% in *C. pallens*), so it is not clear that N exerts any real control over seasonal acclimation of R_d. Seasonal acclimation of R_d was linked with AOX and COX protein abundances in *C. pallens*. There was a

negative relationship between the AOX/COX protein balance and 120d averaged temperature in the seasonal study in *C. pallens* (Fig 3.4b), supporting previous laboratory studies reporting increased AOX protein in response to low treatment temperatures under controlled conditions (Vanlerberghe & McIntosh, 1992; Gonzalez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000a; Armstrong *et al.*, 2008; Mizuno *et al.*, 2008). There was also a positive relationship between the AOX/COX protein balance and R_{10} in *C. pallens* (Fig 3.4d). These results suggest that changes in the AOX/COX protein balance underpin the response of respiration to seasonal temperature changes in this species. In contrast with *C. pallens*, the AOX/COX protein ratio did not range as greatly in *C. rubra*, the lower altitude species (0.68 – 2.28 in *C. rubra*; 0.57 – 5.16 in *C. pallens*), nor was it correlated with R_{10} or temperature. A possible ecological explanation of the differences between these related species could be that the high-altitude species experiences more extreme temperature fluctuations and lower temperatures; thus, a change in both engagement of AOX and protein abundance is required in order for total AP output to fully respond to cold stress. In contrast, it is possible that the low-altitude species may economize on protein construction costs and rely more heavily on changes in the activation state of existing proteins.

Due to the difficulty in obtaining isotopic endpoints for the two oxidases, I interpret changes in oxygen isotope discrimination as relative changes in engagement of AOX and COX, with higher discrimination values indicating greater AOX contribution to respiration. This is based on consistent findings in the literature that AOX discriminates more heavily against ^{18}O than does COX (Ribas-Carbo *et al.*, 2005a). The range in *D* (i.e. the difference between AOX and COX endpoints) for leaves has been reported to be from 6 to 11‰, depending on species and the study

(Ribas-Carbo *et al.*, 2005a). Assuming a conservative range of 11‰ for *Chionochloa* spp., the findings presented here show that the contribution of AOX to *R* in *C. rubra* theoretically varies by up to 48%, and in *C. pallens* by 33% over the year. Interestingly, unlike the changes in AOX/COX protein balance, discrimination did not appear to respond to changes in growth temperature and was not related to changes in total respiration.

Similarly to results shown in several previous studies (Guy & Vanlerberghe, 2005; Ribas-Carbo *et al.*, 2005b; Vidal *et al.*, 2007), there was no clear correlation between AOX protein abundance and relative AP engagement in either species of *Chionochloa*. This is not surprising, as existing AOX protein in plant tissues is thought to be activated or engaged through changes in its redox state (Umbach & Siedow, 1993; Umbach *et al.*, 1994; Umbach *et al.*, 2006) or by signaling compounds such as pyruvate (Ribas-Carbo *et al.*, 1995; Millar *et al.*, 1996; Ribas-Carbo *et al.*, 1997; Rhoads *et al.*, 1998), salicylic acid (Rhoads & McIntosh, 1992; Lennon *et al.*, 1997) and ROS (Wagner, 1995; Vanlerberghe & McIntosh, 1996). One can therefore expect AP engagement to respond more rapidly to environmental triggers than would protein abundance.

Importantly, *D* was positively correlated with the previous day's integrated PAR in both species in the seasonal study (Fig. 3.5), but not with day length. This suggests that in *Chionochloa* spp., engagement of the AP is up-regulated in response to high ambient light in the field. An increase in AP engagement has been found in response to moderate increases in light in *Glycine max* cotyledons (Ribas-Carbo *et al.*, 2000b) and high light in *A. thaliana* (Noguchi *et al.*, 2001). AOX protein abundance has also been shown to increase in response to moderate light in isolated mitochondria of *Nicotiana sylvestris* (Dutilleul et al., 2003) and to high light in *A. thaliana*

(Yoshida *et al.*, 2007; Yoshida *et al.*, 2008). Yoshida *et al.* (2007) showed that AOX protein abundance increased concomitantly with an increase in reducing equivalents in the chloroplast and with a decrease in F_V/F_M (indicating chloroplastic stress) during high light exposure, demonstrating that AOX can dissipate excess reducing equivalents during photo-oxidative stress. As F_V/F_M remained relatively high (0.803 - 0.863) in these plants at all times throughout the year, it is unclear if a similar process occurred here as was reported in (Yoshida *et al.*, 2007). Providing a different perspective, Ribas-Carbo *et al.* (2008) found AP engagement to increase and CP engagement to decrease in soybean cotyledons exposed to red light under non-stressful conditions, indicating that phytochrome can control mitochondrial electron transport. Thus, the response of *D* to PAR in this study may simply be a direct result of phytochrome regulation.

It is interesting that *D* correlated best with the previous day's integrated PAR in the seasonal study, yet did not change on a timescale of days in the short-term study. However, the range of daily integrated PAR observed during the short-term study (12.8 – 27.8 E m^{-2} d^{-1}) was considerably smaller than that observed over the course of the seasonal study (3.03 – 29.3 E m^{-2} d^{-1}). Thus, it is possible that the changes in PAR over the short-term study were not sufficient to produce a significant change in *D*.

Few studies published to date have specifically compared the short- and long-term responses of AOX to temperature. Some studies have found transient (1-7 day) increases in AP engagement (Ribas-Carbo *et al.*, 2000a; Armstrong *et al.*, 2008), AOX protein abundance (Vanlerberghe & McIntosh, 1992; Mizuno *et al.*, 2008), and AOX transcript abundance in wheat and *A. thaliana* exposed to cold stress (Ito *et al.*, 1997; Sugie *et al.*, 2006); these observed increases in AOX typically reverted after a

few days. For instance, Armstrong *et al.* (2008) found AP engagement to peak at day 4 of cold treatment in *A. thaliana*, and to decrease by day 10. Here, I specifically investigated the timescale on which AP engagement and protein abundance respond to temperature changes in a perennial alpine grass and found that, like total R_d, AOX and COX protein abundances did not respond to daily changes in temperature (Fig. 3.6; Table 3.2), but only to long-term changes in temperature, indicating slow turnover of protein pools as part of the seasonal response of *R*. In contrast, *D* responded to daily changes in light, suggesting that the activation state of existing AOX and COX protein is regulated on shorter timescales.

The finding presented here that the AOX/COX protein balance was greater with low temperatures and that discrimination increased with high light provides field-based support for previous laboratory findings. However, there was a complex response in the field, with AOX/COX protein balance and discrimination responding to different abiotic variables and on different timescales. These findings highlight that an improved understanding of how the AP regulates respiration in the field is critical in our ability to connect carbon balance physiology with the ecology of plants in changing environments.

3.5 SUMMARY

Respiration in two species of *Chionochloa* acclimated slowly, responding to seasonal changes in temperature in the field, but not to short-term temperature fluctuations. Long-term changes in the balance of AOX to COX proteins were associated with respiratory acclimation in these grasses. However, discrimination, which reflects relative changes in alternative and cytochrome pathway partitioning, was not related to seasonal changes in temperature but instead to daily changes in light. This study

illustrates how respiratory acclimation can occur differently in field-grown grasses with a complicated thermal history than in other plants, and highlights the complex responses of alternative oxidase to constantly changing conditions in the field. The next chapter will attempt to better characterize the seasonal patterns in AP respiration and AOX protein abundances and how these relate to seasonal changes in respiration. Using both a deciduous angiosperm and an evergreen gymnosperm, Chapter 4 will also explore how seasonal changes in the instantaneous temperature response of discrimination affect engagement of the AP.

Chapter 4: Seasonal variation in respiration and alternative oxidase in a deciduous angiosperm and an evergreen conifer

A version of this chapter has published as an article in **Physiologia Plantarum** *and is included in Appendix B.*

4.1 INTRODUCTION

The degree of acclimation exhibited by different plant groups varies widely (Larigauderie & Korner, 1995; Tjoelker *et al.*, 1999; Loveys *et al.*, 2003) and is complicated by the temperature response of various respiratory components. Of particular interest are the activities of the two terminal oxidases in the electron transport chain of mitochondrial respiration in plants: cytochrome *c* oxidase (COX), and a second, cyanide-resistant terminal oxidase, alternative oxidase (AOX) (Vanlerberghe & McIntosh, 1997; Millenaar & Lambers, 2003). Whether or not there is an instantaneous temperature response of electron partitioning through the AP and CP (*D*; i.e. if the AP and CP have different temperature sensitivities) has been a matter of recent debate and has potential implications for the methodology of measuring *D*, especially in field studies where ambient temperature fluctuates frequently. The balance in electron partitioning through the AP and CP has been found to be highly sensitive to temperature in *Arabidopsis thaliana* (Armstrong *et al.,* 2008) and a small temperature dependence was found in leaves of *Vigna radiata* (Gonzalez-Meler *et al.*, 1999) and *Nicotiana tabacum* (Guy & Vanlerberghe, 2005). Each of these three studies found higher AP vs. CP contribution to respiration when leaves were measured at warm (i.e. 30 ºC) than at low (5 or 10 ºC) temperatures. However, Macfarlane *et al.*, (2009) found no temperature dependence of *D* in *Curcubita pepo, Nicotania sativa,* and *Vicia faba.* Interestingly, Gonzalez-Meler *et al.* (1999) found a decrease in the long-term temperature sensitivity of discrimination relative to the instantaneous response; i.e., there was an instantaneous temperature dependence of discrimination in warm-grown (28 ºC) *V. radiata* leaves, but discrimination was not different between warm and cool (19 ºC) grown leaves when measured at their respective growth temperature. Thus, overall, it is currently unclear

if the AP is more temperature sensitive than the CP, as is the extent to which these responses are affected by exposure to varying thermal regimes.

At present, the temperature responses of the AP and CP, and their role in the temperature response of total *R*, are difficult to define. Here, I investigated the AP in leaves of mature field-grown poplar trees (*Populus* x *canadensis*, a deciduous, continually-flushing angiosperm) as well as pine trees (*Pinus radiata*, an evergreen conifer) sampled at five points throughout the growing season. I sought to address whether there is an instantaneous temperature dependence of the AP/CP balance and how this changes with natural seasonal variation and plant functional group. The relationship between respiration and the abundance of AOX and COX proteins is also examined here.

4.2 MATERIALS AND METHODS

4.2.1 Study site

This study was carried out on large poplar (*Populus* x *canadensis* var. *italica* Moench, approx. 20 years old) and pine (*Pinus radiata* D. Don, approx. 10 years old) trees growing at two flat, free-draining sites two kilometers apart at Lincoln, New Zealand. The exact locations of the field sites were 43º64´S, 171º48´E (poplar) and 43º63´S, 171º48´E (pine). Annual precipitation at these sites is approximately 648 mm, most of which occurs in winter and spring. Maximum and minimum temperatures during the period of observation, measured at 1.3m above the ground using a standard weather station were in the range of 11.4 to 35 ^{o}C and -2 to 18.9 ^{o}C, respectively. Daily mean temperature over the course of this study is shown in Figure 4.1.

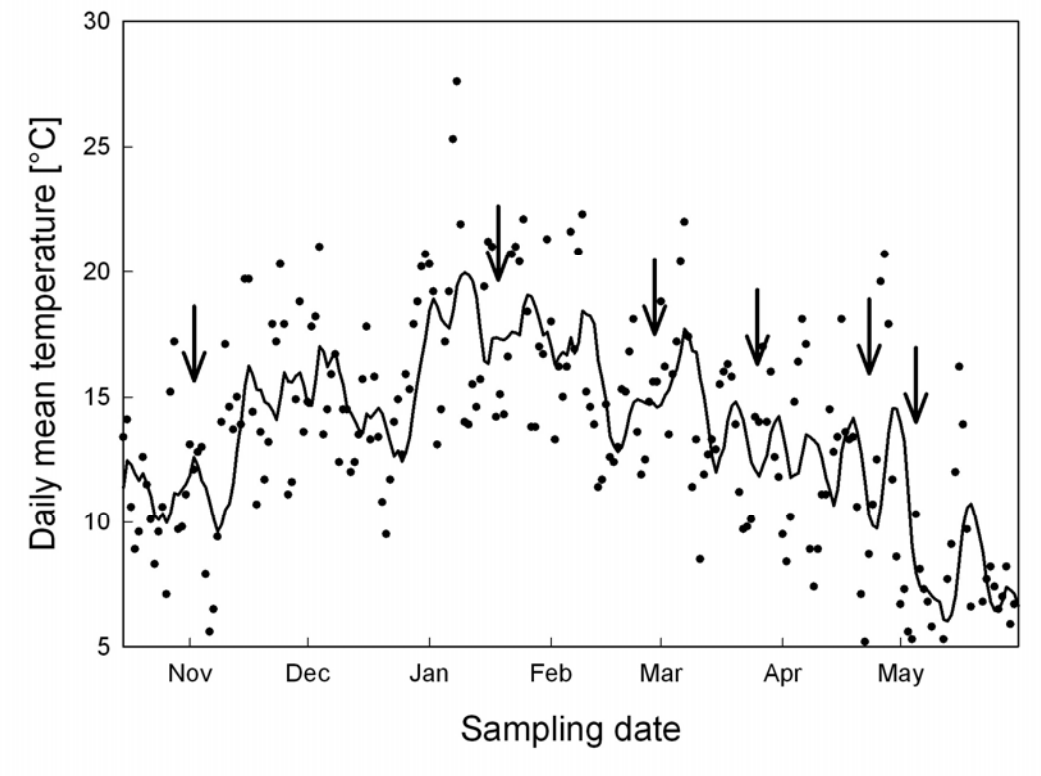


Figure 4.1: Mean daily temperature at the field site over the course of the study. The curve indicates a 7-day running mean. Arrows indicate the timing of field measurements.

4.2.2 Botanical description of study species

Pinus radiata D. Don

This evergreen perennial tree belongs to the family Pinaceae, and is native to California, USA. It has high economic value in New Zealand, Australia and Chile, where it is planted as a timber crop. It is commonly known as 'Monterey Pine'. The growth habit is erect with a single stem. Growth is rapid with mature trees reaching a maximum height of 39 m. Foliage is green and dense, with needle shape. Needles are typically three to a fascicle. Bark is dark grey-brown and deeply fissured. Male and female cones are separate but present on the same tree. Male cones are small and inconspicuous while female cones grow large to about 10 cm. Cones and seeds are brown and inconspicuous.

Populus x *canadensis* Moench [*deltoides* x *nigra*] var *italica*

This deciduous perennial tree belongs to the family Salicaceae and is native to the United States and to eastern Canada. It is commonly known as 'Carolina poplar'. The growth habit is erect with a single stem and narrow crown. Growth is rapid; with mature trees reaching a maximum height of 34 m. Leaves are continually flushing throughout the growing season of spring and summer, and are not retained over winter. Foliage is green and dense. Leaves are broad and triangular with pointed apices, and have a coarse texture and a finely toothed margin. Lifespan is typically short, but trees have re-sprouting ability and often form many clones. Flowers are yellow and fruits are white, both inconspicuous. Male and female flowers are on separate trees.

All information on Pinus radiata and Populus x *canadensis was obtained from the United States Department of Agriculture database: Natural Resources Conservation Service* (http://plants.usda.gov/java/nameSearch).

4.2.3 Sampling

Scaffolding was erected adjacent to both stands of trees, allowing sampling of the northern facing edge of each canopy. Approximately two hours after sunrise on each sampling day, small, fully sunlit branches at a height of 7-8m were cut from each of six *P.* x *canadensis* trees. Two fully sunlit braches at a height of 4-5m were cut from each of three *P. radiata* trees. Branches were immediately re-cut under water and transported in darkness to a laboratory at the University of Canterbury, Christchurch, New Zealand. Previous measurements have shown that leaf respiration remains stable

under these conditions for several hours (Turnbull *et al*., 2005). Three successive days were required to complete measurements: respiration was measured the first day, when samples were also taken for carbohydrates, nitrogen, and specific leaf area. Discrimination was measured and immunoblotting samples taken the second day for *P. radiata* and the third day for *P.* x *canadensis.*

4.2.4 Isotopic endpoints

Leaves of *P.* x *canadensis* were incubated in a solution of 10 mM HEPES and 10 mM MES pH 7.6 containing either 3 mM potassium cyanide or 30 mM salicyhydroxamic acid for 90 minutes to inhibit the CP and AP, respectively. These inhibitor concentrations were determined for this species by Ow (2009). Following inhibitor treatment, leaves were allowed to air-dry to original fresh mass before following the protocol for measurement of oxygen isotope discrimination as detailed in Chapter 2: Methods. The electron partitioning through the AP for *P.* x *canadensis* (τ_a; after Ribas Carbo *et al.,* 1995) was calculated as described by Guy *et al.,* (1989):

$$\tau_a = (D_{SAMPLE} - D_{SHAM}) / (D_{KCN} - D_{SHAM}) \qquad (4.1)$$

In a few cases, D_{SAMPLE} was measured to be slightly lower than D_{SHAM}; in these cases, τ_a was assumed to equal zero. The measured endpoints for *P. radiata* were almost identical (both 17.4‰) presumably due to diffusional issues in the thick needles. Therefore, I did not calculate τ_a for *P. radiata* and instead interpret changes in *D* as relative changes in electron partitioning through the AP and CP.

Please see Chapter 2: Methods for descriptions of respiration, oxygen isotope discrimination, immunoblotting, nonstructural carbohydrate and nitrogen measurements and statistical analysis.

4.3 RESULTS

4.3.1 Temperature

Mean daily temperatures over the growing season at the field site are shown in Figure 4.1. Temperatures peaked in January and February; however, large daily variation in ambient temperature was observed.

4.3.2 *Populus* x *canadensis*

Respiration at the reference temperature of 10 °C (R_{10}) generally declined from November to April (Fig. 4.2a). The temperature sensitivity of R (E_0) ranged between 40 and 60 kJ mol^{-1} but showed no significant trend over time (Fig. 4.2b). Respiration calculated at the growth temperature (temperature averaged over three days prior to measurement; R_g) was thus influenced most strongly by changes in R_{10} and generally declined from November to April (Fig. 4.2c). By April, R_g, which is indicative of actual respiration in the field, had dropped to one-third its value early in the growing season in November (i.e. a drop from 17.4 to 6.1 μmol CO_2 kg^{-1} s^{-1}). Changes in R_{10} ($F = 20.99$; $p < 0.001$), E_0 ($F = 10.59$; $p < 0.001$), and R_g ($F = 7.60$; $p < 0.001$) were significant over time.

Percent leaf soluble sugar and starch are shown in Table 4.1. Soluble sugar changed significantly over time ($F = 5.66$; $p = 0.002$), as did starch ($F = 8.14$;

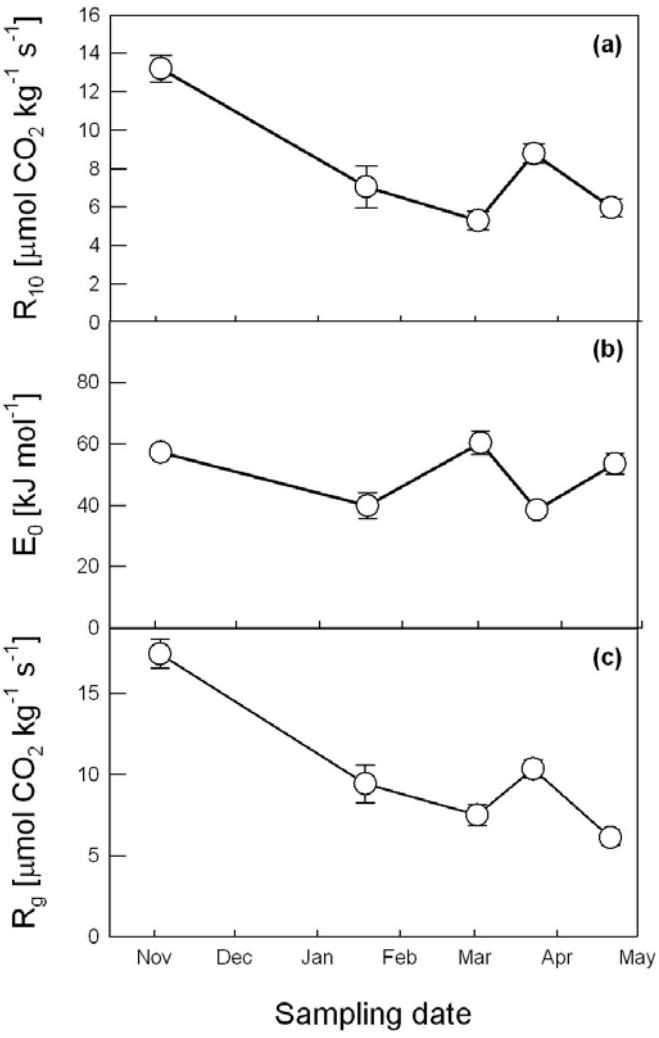


Figure 4.2: Respiratory parameters for *P.* x *canadensis* over the course of the study. **(a)** The basal rate of respiration at 10 ºC (R_{10}); **(b)** the instantaneous temperature sensitivity of respiration (E_0); and **(c)** the rate of respiration at the growth temperature (the temperature averaged over three days prior to sampling; R_g). Values shown are mean ± SEM, where n=6.

$p < 0.001$). Neither soluble sugar nor starch was correlated with R_{10}, E_0, or R_g. Percent leaf nitrogen (N) also changed significantly (F = 8.03; $p < 0.001$; Table 4.1), but was not related to respiratory parameters.

Table 4.1
Nonstructural carbohydrates (percent starch and sugar) and percent leaf nitrogen (*N*) over the growing season. Values shown are mean ± SEM, where n=6.

Measurement period	Date	% sugar	% starch	% N
P. x *canadensis*				
1	2-Nov-08	19.5 ± 0.8	10.1 ± 0.6	1.16 ± 0.07
2	19-Jan-09	21.6 ± 1.8	12.7 ± 0.5	0.89 ± 0.05
3	3-Mar-09	21.4 ± 0.9	9.8 ± 0.5	0.96 ± 0.04
4	23-Mar-09	17.7 ± 1.1	8.1 ± 1.0	0.84 ± 0.05
5	21-Apr-09	14.6 ± 0.6	9.7 ± 0.3	1.36 ± 0.04
P. radiata				
1	2-Nov-08	17.0 ± 0.6	10.7 ± 0.6	1.00 ± 0.11
2	19-Jan-09	18.4 ± 1.0	7.1 ± 0.5	1.05 ± 0.04
3	3-Mar-09	18.8 ± 1.8	7.3 ± 0.5	1.12 ± 0.05
4	23-Mar-09	18.4 ± 1.0	7.6 ± 1.0	0.93 ± 0.07
5	21-Apr-09	18.2 ± 0.7	6.8 ± 0.3	0.94 ± 0.07
6	4-May-09	17.3 ± 1.3	8.7 ± 0.4	1.02 ± 0.04

Both relative AOX and relative COX abundances rose to a peak in early March and declined through the end of the growing season (Fig. 4.3a). The balance of AOX/COX protein abundance showed a similar pattern (Fig. 4.3b). The change in AOX abundance was significant over time ($F = 4.60$; $p = 0.006$), but not in COX ($F = 2.03$; $p = 0.074$) or the AOX/COX balance ($F= 2.28$; $p = 0.089$).

Discrimination of respiratory oxygen consumption measured at 6, 12, 18, 24, and 30 °C at each sampling time is shown in Table 4.2. The isotopic endpoint for KCN treated leaves (assumed to be the endpoint for AOX) was 27.28 ± 0.48 (‰). The endpoint for SHAM treated leaves (COX) was 21.31 ± 0.38 (‰). The electron partitioning through the AP (τ_a, see Eqn 4.1) is shown for each measurement temperature and sampling date in Figure 4.4. τ_a generally rose from 6 to 24 °C at each sampling date and fell from 24 to 30 °C. τ_a was consistently higher on the third

sampling date than other sampling dates when measured at 12 – 30 °C. τ_a at the growth temperature (the average temperature 3 days prior to sampling) was calculated for each sampling date using the instantaneous temperature response; τ_a at the growth temperature increased from November to early March, and decreased thereafter (Fig. 4.5).

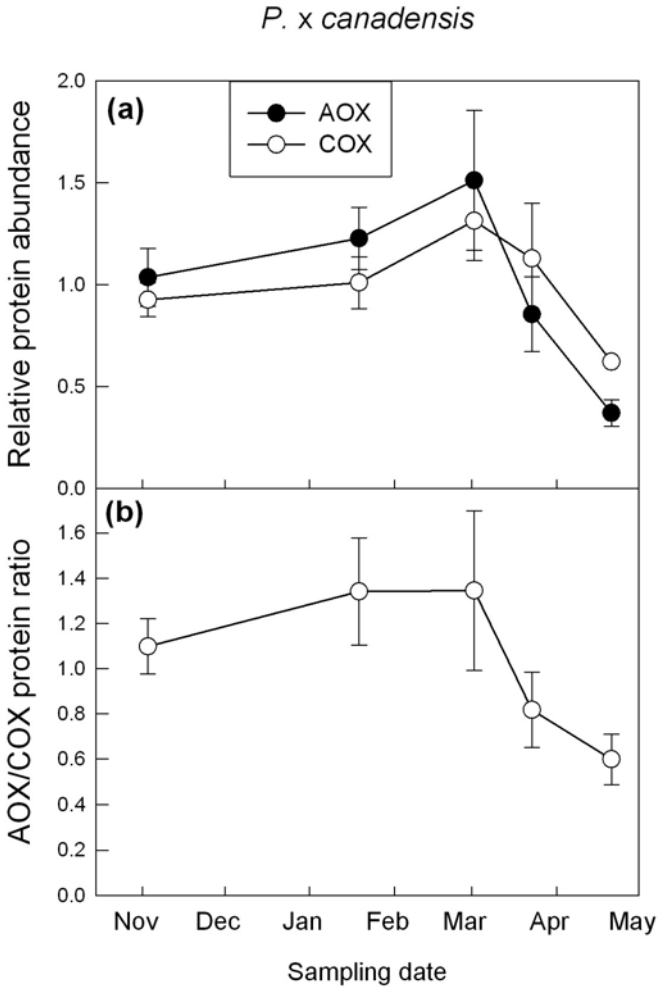


Figure 4.3: (a) Relative abundances of AOX and COX protein in *P.* x *canadensis* over time. **(b)** The ratio of AOX/COX protein abundances. Values shown are mean ± SEM, where n=6.

Table 4.2
Discrimination (*D*; ‰) at five measurement temperatures (6, 12, 18, 24, & 30 ºC) for each sampling date. Values shown are mean ± SEM. Significance is shown as F; *p*, where bold indicates statistically significant differences in *D* among measurement temperatures.

Sampling period	Date	**6**ºC	**12**ºC	**18**ºC	**24**ºC	**30**ºC
1	2-Nov-08	21.3 ± 0.7	21.6 ± 2.0	21.9 ± 1.8	23.0 ± 0.7	21.4 ± 0.2
2	19-Jan-09	21.4 ± 0.3	22.2 ± 0.3	22.3 ± 0.2	21.8 ± 0.3	22.1 ± 0.6
3	3-Mar-09	21.8 ± 0.4	22.9 ± 0.4	23.1 ± 0.3	23.5 ± 0.4	22.8 ± 0.4
4	23-Mar-09	21.4 ± 0.2	21.9 ± 0.5	22.7 ± 0.3	22.7 ± 0.4	22.9 ± 0.3
5	21-Apr-09	21.7 ± 0.4	22.0 ± 0.5	22.5 ± 0.3	22.7 ± 0.3	22.9 ± 0.6
	Significance	0.309; 0.586	0.201; 0.660	3.32; 0.082	**8.32; 0.008**	**6.02; 0.023**

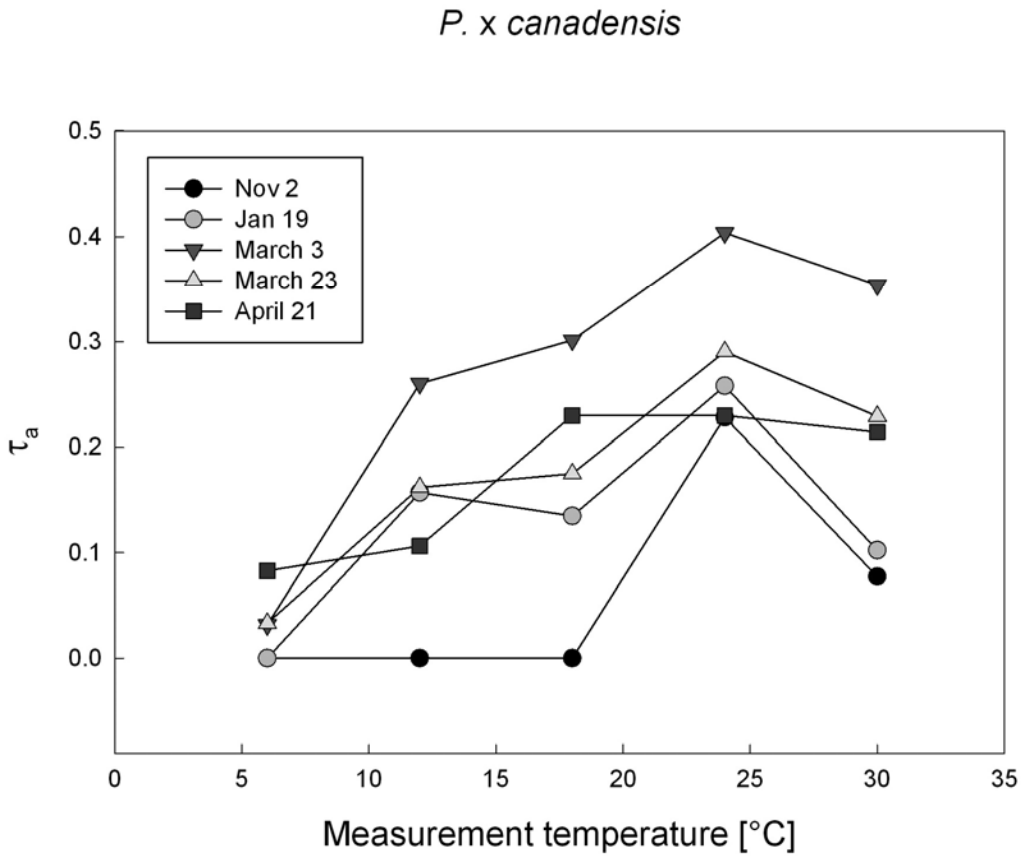


Figure 4.4: The instantaneous temperature response of the electron partitioning through the alternative pathway (τ_a) in *P.* x *canadensis* measured at different times during the growing season.

4.3.3 *Pinus radiata*

R_{10} decreased from November to early March by approximately 50% (from a rate of 2.4 to 1.3 μmol CO_2 kg^{-1} s^{-1}) and rose thereafter (Fig. 4.6a). By May, the rate of respiration at 10 °C was nearly equal to what it was in November. E_0 remained fairly constant from November to March, and decreased slightly thereafter (Fig. 4.6b). R_g decreased from November to March and then remained constant through May (Fig. 4.6c). Changes in R_{10} (F = 5.34, p = 0.003), E_0 (F = 8.24, p < 0.001) and R_g (F = 7.45, p < 0.001) were significant over time. Measurements of respiration made on

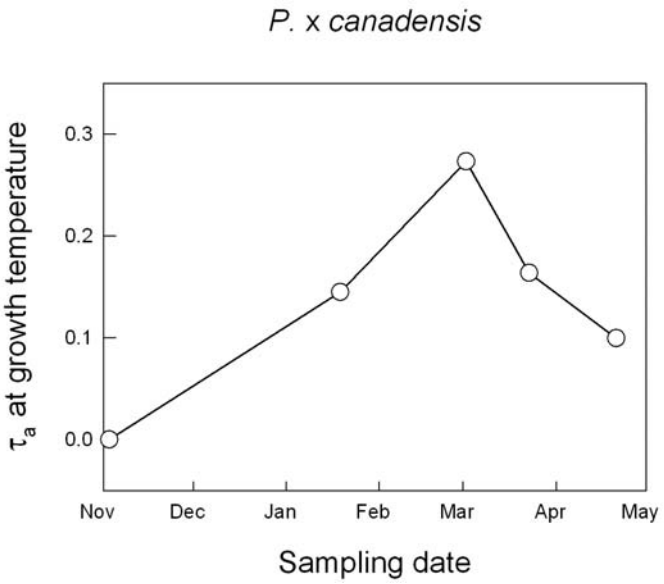


Figure 4.5: The electron partitioning through the alternative pathway (τ_a) at the growth temperature (the average temperature three days prior to sampling) in *P.* x *canadensis* plotted against time.

March 23 are not included as these data were not statistically robust.

Percent leaf soluble sugar and starch are shown in Table 4.1. Starch changed significantly over time (F = 6.06; p < 0.001), with values in November being significantly different from those in January, early March, and April. Neither sugar (F = 0.44, p = 0.82) nor N (F = 1.09, p = 0.38) changed significantly over time.

None of soluble sugar, starch and *N* was correlated with R_{10}, E_0, or R_g.

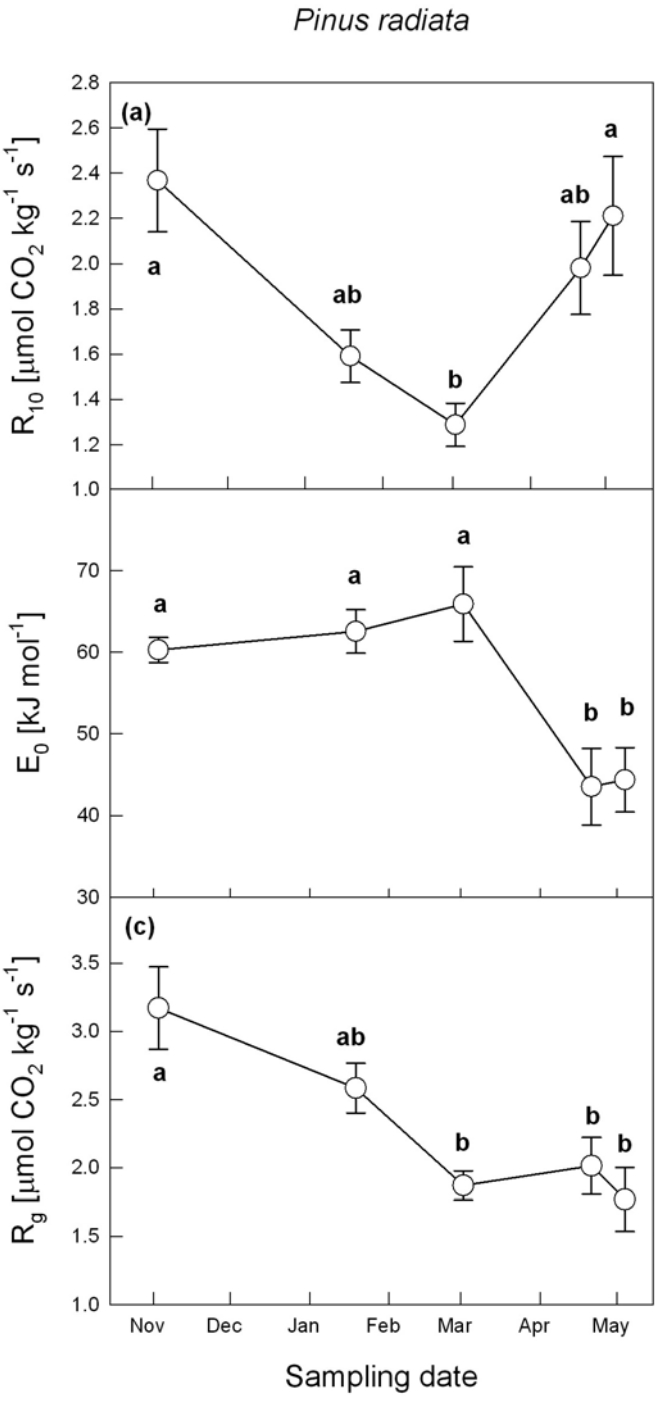


Figure 4.6: Respiratory parameters for *P. radiata* over the course of the study. **(a)** The basal rate of respiration at 10 ºC (R_{10}); **(b)** the instantaneous temperature sensitivity of respiration (E_0); and **(c)** the rate of respiration at the growth temperature (the average temperature three days prior to sampling; R_g). Values shown are mean ± SEM, where n=6. Letters show statistical differences between points.

Relative AOX and COX protein abundances exhibited a similar seasonal pattern, with protein abundances decreasing until late summer and sharply increasing thereafter (Fig. 4.7a). Changes in COX protein abundance was significant over time ($F = 7.23$, $p < 0.001$), although changes in AOX were not ($F = 1.60$, $p = 0.19$). The AOX/COX protein ratio did not change significantly over time ($F = 2.37$, $p = 0.06$; Fig. 4.7b). COX protein abundance was positively correlated with R_{10} (Fig. 4.8b). AOX protein abundance showed a similar trend with R_{10}, although it was not significant (Fig. 4.8a).

As isotopic endpoints were not obtained for *Pinus radiata*, I interpret changes in discrimination as relative changes in partitioning between the AP and CP. Discrimination measured on each sampling date as well as at varying measurement temperatures is shown in Figure 4.9. Changes in *D* with measurement temperature were not consistent between sampling dates. For instance, in May, *D* gradually decreased with increasing temperature, while in November and January, *D* increased then decreased and again increased with rising temperature. *D* at the growth temperature (the field temperature averaged over three days prior to measurement) was calculated by assuming a linear response of *D* to temperature between temperatures measured. *D* at the growth temperature remained fairly constant throughout the season except for a pronounced drop in April (Fig. 4.10).

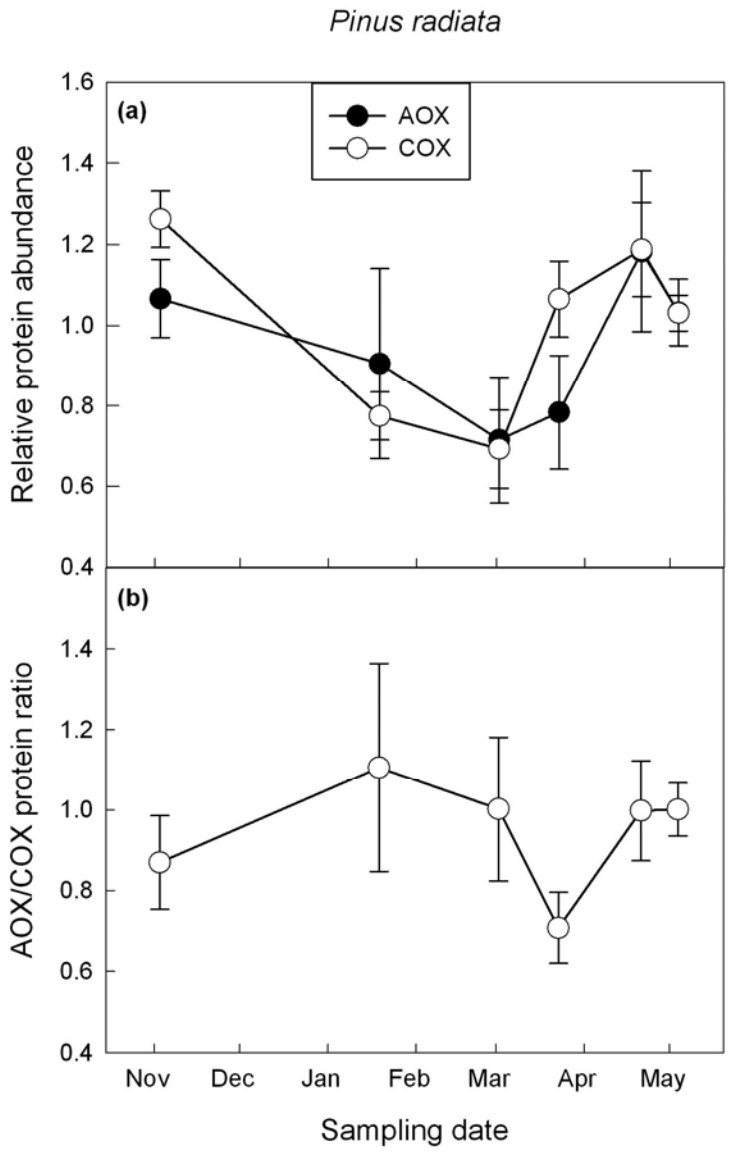


Figure 4.7: (a) Relative abundances of AOX and COX protein in *P. radiata* over time. **(b)** The ratio of AOX/COX protein abundances. Values shown are mean ± SEM, where n=6.

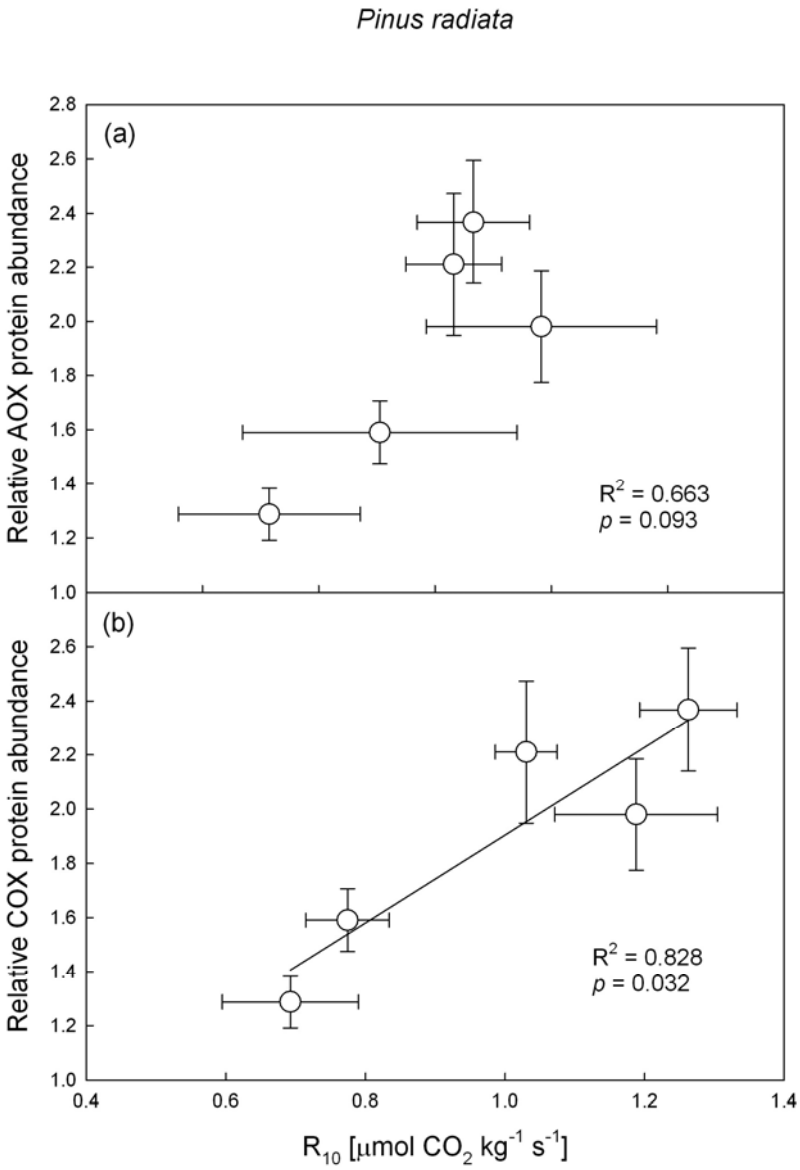


Figure 4.8: Relative abundances of **(a)** AOX and **(b)** COX protein in *P. radiata* plotted against R_{10}. Values shown are mean ± SEM, where n=6.

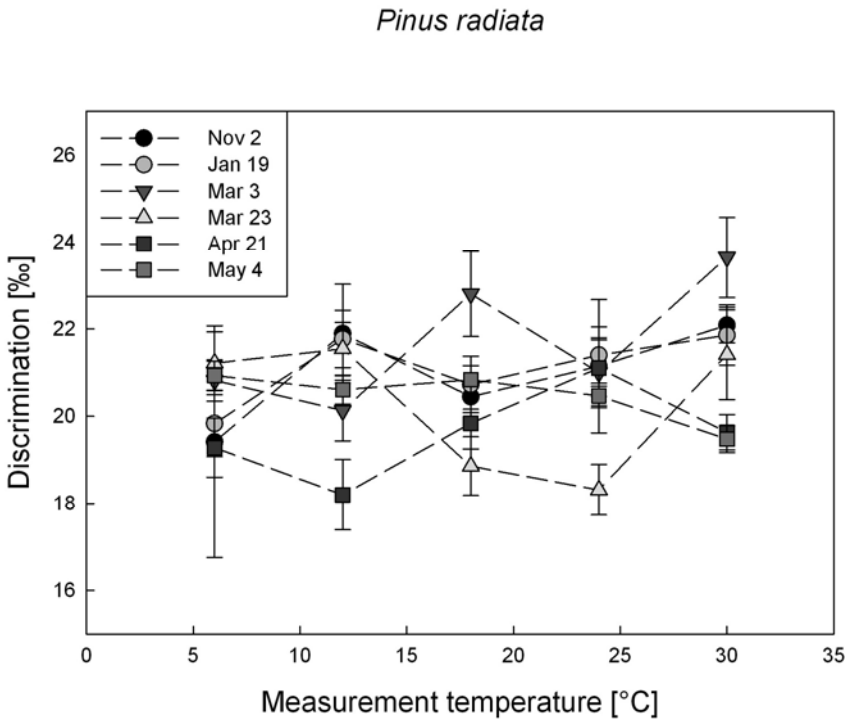


Figure 4.9: The instantaneous temperature response of respiratory oxygen isotope discrimination (*D*) in *P. radiata* measured at different times during the growing season.

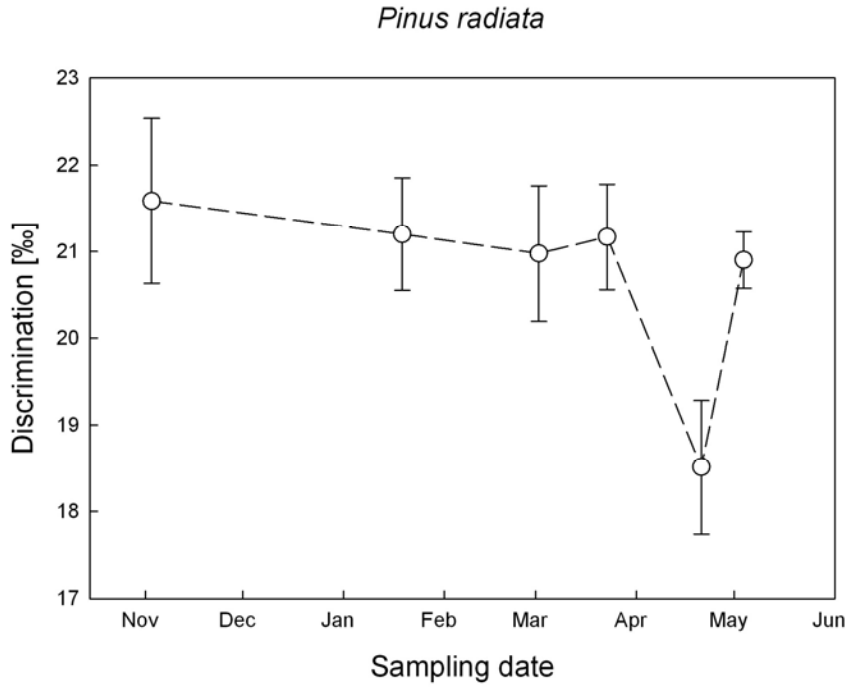


Figure 4.10: Respiratory oxygen isotope discrimination (*D*) at the growth temperature (the average temperature three days prior to sampling) in *P. radiata* plotted against time.

4.4 DISCUSSION

4.4.1 *Populus* x *canadensis*

Respiration at the growth temperature (the average temperature three days prior to sampling; R_g) declined throughout the growing season, mediated largely through changes in the basal capacity of respiration (R_{10}; Fig. 4.2). A decline in R_g throughout the growing season has previously been reported for leaves of deciduous trees (Xu & Griffin, 2006; Marra *et al.*, 2009; Ow *et al.*, 2010; Rodriguez-Calcerrada *et al.*, 2010). It is likely that some of this variation, particularly early in the season, was due to acclimation of R to warming temperatures. The decline in R_g later in the season may also have been mediated by age-related physiological leaf characteristics. The declines in R_{10} and R_g were not significantly correlated with nonstructural carbohydrates or leaf nitrogen (Table 4.1; see Results section for statistics), although a decline in both soluble sugar and starch from January to April may be partly responsible for the decrease in R_g over this period. The decline in R_g may also be linked with a decline in protein abundances; both AOX and COX relative protein abundance peaked on the third sampling date and declined sharply thereafter (Fig. 4.3a). AOX protein abundance declined faster than did COX, resulting in low AOX/COX protein ratios toward the end of the growing season (Fig. 4.3b). I had previously found the AOX/COX protein ratio to exert some control over changes in R_{10} in New Zealand alpine grasses (Chapter 3) as did Gonzalez-Meler et al. (1999) in mung bean hypocotyls and Armstrong et al. (2008) in *Arabidopsis thaliana* leaves when protein abundances were not normalized to porin. Thus, it is possible that the decline in R_g at the end of the growing season in *Populus* x *canadensis* was mediated through the decrease in protein abundances, particularly AOX.

Populus x *canadensis* displayed an instantaneous temperature dependency of discrimination (*D*) on each sampling date throughout the growing season. *D* increased with measurement temperature from 6 to 24 °C and decreased from 24 to 30 °C, with the exception of the last sampling date, when *D* was slightly higher when measured at 18 than at 24 °C (Table 4.2). The electron partitioning through the AP (τ_a) was calculated by comparing *D* to our measured isotopic endpoints of the AP and CP. τ_a showed a very similar pattern to measurement temperature as *D* (Fig. 4.4). These results are similar to those of Armstrong et al. (2008) and Gonzalez-Meler et al. (1999), who reported greater *D* with increasing measurement temperature in *A. thaliana* and *V. radiata* leaves, respectively. Guy and Vanlerberghe (2005) also reported an increase in *D* with measurement temperature from 10 – 30 °C in transgenic *N. tabacum*, with *D* decreasing from 35 – 40 °C. The results reported here provide field support for these previous laboratory findings that *D* increases with measurement temperature over the low to mid range of temperatures (10 – 26 ºC). The implication of this is that the AP must be more temperature sensitive than the CP when measured instantaneously. This contrasts with the findings of Atkin et al. (2002) that *Glycine max* cotyledons and isolated mitochondria inhibited with KCN or SHAM have similar temperature sensitivities, and also with those of Macfarlane et al. (2009), who found no temperature dependency of *D* in three species of herbaceous plants.

τ_a at the growth temperature (the temperature averaged over three days prior to sampling) increased from the first to second sampling date, peaked sharply at the third sampling date, and decreased thereafter (Fig. 4.5). Due to frequent variation in temperature (Fig. 4.1), it is likely that actual τ_a in the field fluctuated more than these data show; however, there appears to have been a seasonal trend with a peak in τ_a just after mid-summer. It is possible that the undetectable alternative respiration in

November was a result of leaf age; several studies have found discrimination to increase with age in developing leaves and roots (Millar *et al.*, 1998; Florez-Sarasa *et al.*, 2007; Priault *et al.*, 2007), presumably as a result of declining CP respiration. Although on all sampling dates we measured leaves that appeared to be mature, development may have influenced measured τ_a in the spring.

Some of the seasonal variation in τ_a may also be related to changes in AOX and COX protein levels. Both AOX and COX protein abundances peaked on March 4 and declined thereafter through to the end of the growing season (Fig. 4.3a). By April, AOX levels had declined more so than had COX, leading to a low AOX/COX protein ratio (Fig. 4.3b). Low protein levels, especially AOX, may explain why τ_a declined in autumn. However, AP activity has typically not been correlated with changes in AOX protein levels in previous studies (Millenaar *et al.*, 2000; Guy & Vanlerberghe, 2005; Ribas-Carbo *et al.*, 2005b; Florez-Sarasa *et al.*, 2007; Vidal *et al.*, 2007; Grant *et al.*, 2008), indicating that some post-translational control is exerted over AOX. The AOX protein exists in either an active reduced form or as an oxidized dimer, and it is thought that the activation state of the protein may regulate activity, although evidence for this in leaves is limited (Vidal et al., 2007). The AP has been found to be stimulated by addition of pyruvate (Ribas-Carbo et al., 1995; Ribas-Carbo et al., 1997) and salicylic acid (Liang & Liang, 2002), suggesting that these compounds can regulate AP activity. It is likely that one or more of these mechanisms regulated activity of existing AOX protein to produce the increase in τ_a on March 3 in this study (Fig. 4.6).

Curiously, the increase in τ_a in late summer did not produce a change in R_g. τ_a was not correlated with R_{10} or R_g, and respiration remained relatively low at the third measurement period despite a significant increase in τ_a. The electron partitioning

through the CP (τ_c) thus appears to have decreased significantly at this time. Previous studies on the AP have found it to rise with a concomitant decrease in the CP in response to environmental change, or vice versa (Gonzalez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000a; Noguchi *et al.*, 2001; Ribas-Carbo *et al.*, 2005b; Gomez-Casanovas *et al.*, 2007; Armstrong *et al.*, 2008; Ribas-Carbo *et al.*, 2008). The present findings, along with the studies listed above, suggest a balancing of the AP and CP in order to maintain R_d despite responses of the AP to environmental conditions. It has been suggested that electron flow via the CP decreases in response to various stresses, and AP activity increases in order to maintain respiration and possibly to prevent over-reduction of the ubiquinone pool (Millar *et al.*, 1998; Gonzalez-Meler *et al.*, 1999; Armstrong *et al.*, 2008).

4.4.2 *Pinus radiata*

Respiration in *P. radiata* partially acclimated to the seasonal change in temperatures. The basal rate of respiration at 10 °C (R_{10}) decreased from spring to late summer and increased thereafter (Fig. 4.6a). This result supports Ow *et al.* (2010) who found a similar trend in R_{10} in these same *P. radiata* trees measured in 2005 and 2006 and is illustrative of thermal acclimation (Atkin & Tjoelker, 2003; Atkin *et al.*, 2005). Respiration in evergreen tree needles has previously been shown to acclimate to seasonal changes in temperature (Stockfors & Linder, 1998; Atkin *et al.*, 2000b; Vose & Ryan, 2002; Ow *et al.*, 2010). E_0 decreased in autumn despite dropping temperatures; however, the low temperature sensitivity would not affect rates of respiration at the low ambient temperatures at this time. Respiration at the growth temperature (R_g) did not stay constant and decreased over the growing season (Fig. 4.6c). The decrease in R_g from November to March, despite an increase in ambient

temperature (Fig. 4.1) could be a result of leaf age (Azcòn-Bieto *et al.*, 1983b; Priault *et al.*, 2007), or of decreasing respiratory protein abundances over this period (Fig. 4.7a; see discussion above regarding protein abundances in *P.* x *canadensis*). In fact, the relative abundance of COX protein was positively correlated with R_{10} (Fig. 4.8b), and AOX proteins also showed a positive trend with R_{10}, although not significantly (Fig. 4.8a). While the ratio of AOX to COX protein in *P. radiata* remained constant throughout the season (Fig. 4.7b), it is clear that both AOX and COX protein levels underpin changes in respiration rates.

The unchanging balance between AOX and COX proteins was largely reflected in discrimination values, which indicate relative changes in electron partitioning through the AP vs. CP (Fig. 4.10). *D* remained constant through most of the growing season at approximately 21‰, except for a sudden drop in April to 18.5‰. Why *D* decreased in April is unknown. Environmental factors such as temperature, precipitation, solar radiation and wind were not unusual in the days before this sampling date (data not shown). Error in measurement is certainly possible. In fact, the response of *D* to measurement temperature was highly irregular in every month measured except for May (Fig. 4.9). The response of *D* to measurement temperature is typically a smooth positive trend, possibly decreasing at high temperature (Gonzalez-Meler *et al.*, 1999; Guy & Vanlerberghe, 2005; Armstrong *et al.*, 2008). To the best of my knowledge, there has been no previously published study reporting *D* in conifer needles. It is thus unknown, but highly possible, that the thick needles might create a long diffusional path from the mitochondria to the stomata, which could theoretically dampen the signal of oxygen isotope fractionation. Why this effect might change depending on measurement temperature and sampling date is unclear, but the diffusional issues in needles could

still be a significant source of error. Therefore, care must be taken in interpreting the *D* values presented here.

4.4.3 Comparison between taxa

Populus x *canadensis,* a deciduous angiosperm, and *Pinus radiata*, an evergreen conifer, exhibited several differences in their physiological responses to seasonal changes. Respiration at a basal temperature (R_{10}) decreased in warm summer months and increased in autumn in *P. radiata*, exhibiting acclimation to temperature, while in *P.* x *canadensis* it continued to decrease throughout the autumn. *P. radiata* has leaves that live multiple years and photosynthesize and respire throughout the winter; continuous respiration is thus needed to maintain physiological functioning in the needles over the winter and into the following spring. In contrast, leaves of *P.* x *canadensis* senesce in late autumn, and functionality decreases until this point. It is likely that photosynthesis and growth decreased throughout the growing season in this species, reducing the need for respiration.

The differences in seasonal respiration between these two taxa were mirrored in the seasonal patterns of respiratory proteins. In both tree species, the relative abundances of AOX and COX protein varied together over the growing season, and appeared to affect rates of respiration. However, in *P.* x *canadensis,* protein abundances decreased in the autumn, preceding senescence. In *P. radiata*, proteins accumulated again in the winter, aiding acclimation and the rise in the basal rate of respiration. Thus, the deciduous tree prepared for dormancy by stripping its resources before it dropped it leaves, while the evergreen conifer accumulated resources for the cold winter.

4.5 SUMMARY

This chapter illustrated some of the differences in the seasonal trends of respiration and alternative oxidase between deciduous and evergreen plants. In a deciduous poplar hybrid, respiration declined throughout the growing season, while respiration acclimated to low autumn temperatures in an evergreen pine species. Some of these trends in respiration were mirrored by AOX and COX protein abundances. However, partitioning to the AP exhibited very different trends, with τ_a peaking in early autumn in poplar, and staying relatively constant in pine. This study has highlighted seasonal differences in respiratory physiology in two contrasting plant types. It also showed that, at least in poplar, there is a consistent instantaneous temperature dependence of discrimination. The next chapter will examine seasonal trends in respiration and alternative oxidase in another deciduous angiosperm: northern red oak. Chapter 5 will build a case that discrimination responds to both high and low temperature extremes, not just low temperatures specifically.

Chapter 5: Alternative oxidase responds to both low and high temperature stress in *Quercus rubra* leaves along an urban-rural gradient in New York

A version of this chapter has been published as an article in **Functional Ecology** *and is included in Appendix C.*

5.1 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 as a "window into the future" 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 is expected to increase more at night than during the day (Easterling *et al.*, 1997; Alward *et al.*, 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 ppm 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 *et al.*, 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 *et al.*, 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.

An improved understanding of the physiological components of *R* and how they change with environment 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. Alternative oxidase (AOX), along with the cytochrome *c* oxidase (COX), catalyzes the reduction of oxygen to water in the electron transport chain of mitochondrial respiration but results in one-third the ATP production of COX. Alternative pathway (AP) respiration has been shown to respond to cold (Gonzalez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000a; 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). However, our understanding of how AOX affects the response of *R* to multiple environmental variables is extremely limited.

In this study, I investigated how plant respiration and respiratory metabolism in *Quercus rubra* L. (Northern red oak) differ along an urban-rural gradient originating in New York City. Branches were sampled from mature *Q. rubra* trees,

which is a 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 the natural growing season. Measurements included the temperature response of total *R*, relative changes in AP and CP electron partitioning, AOX and COX protein abundances, leaf nitrogen and nonstructural carbohydrates. I sought to understand how urbanization and its associated environmental conditions 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 (Larigauderie & Korner, 1995; Loveys *et al.*, 2003; Atkin *et al.*, 2005; Tjoelker *et al.*, 2008) I hypothesized that *R* would be similar between sites along this urban-rural gradient and throughout the growing season. The AP has been found to increase in response to low temperatures but also in response to heat (citations above). As a deciduous tree, *Q. rubra* leaves do not experience low winter temperatures; thus, I hypothesized that the AP in *Q. rubra* leaves would respond to high summer temperatures rather than cold. Finally, I hypothesized that changes in the AP and CP would be related to changes in AOX and COX protein levels, and that these changes would determine variation in total *R* along the urban-rural gradient and throughout the growing season.

5.2 MATERIALS AND METHODS

5.2.1 Study sites

I sampled at four sites along a gradient 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), the "suburban" site at Lamont-Doherty Earth Observatory in Palisades, NY (41.005 °N, 73.950 °W), the "rural" site at Black Rock Forest near Cornwall, NY (41.430 °N, 74.020 °W), and the "remote" site near the Ashokan Reservoir in the Catskill Range (41.925 °N, 74.248 °W). Each of the sites was exposed to full, direct sunlight, and was fenced to exclude large herbivores.

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 (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 minutes. Temperature at the rural site was measured by the Black Rock Forest Consortium every hour, and at the remote site a HOBO datalogger (Onset, Bourne MA) was installed to measure and record temperature every hour. A HOBO logger was also installed at 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 Figure 5.1.

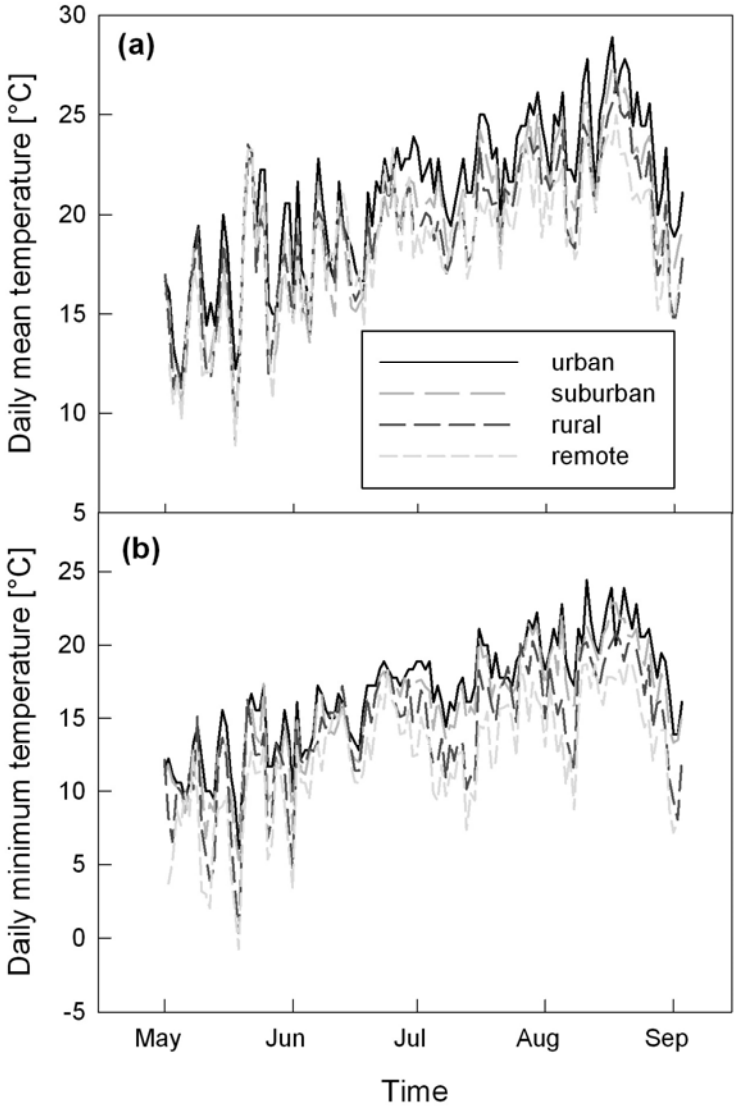


Figure 5.1: Daily **(a)** mean and **(b)** minimum temperature at all four sites over the growing season

5.2.2 Botanical description of *Quercus rubra* L.

This deciduous perennial tree belongs to the family Fagaceae and is native to the eastern United States and Canada. It is commonly known as 'northern red oak'. These trees have a long lifespan. Growth is moderate, forming erect single stems with a maximum height of 25 m. Trees are deciduous, flushing once in the spring and dropping leaves in autumn. Foliage is green and dense, with broad, lobed leaves and

bristle tips. Flowers are yellow and inconspicuous. Fruits form brown, conspicuous acorns that take two years to develop. This is a masting species, with mast years occurring on average every 3 years.

All information on Quercus rubra was obtained from the United States Department of Agriculture database: Natural Resources Conservation Service (http://plants.usda.gov/java/nameSearch).

5.2.3 Sampling

Sun-exposed branches with fully expanded leaves were cut from six mature *Q. rubra* trees in the early morning at each site six times throughout the growing season: May 19-20, June 9-12, June 30-July 1, July 22-23, August 9-10, and September 2-3. Branches were re-cut 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 (Chapter 2, Figure 2.2).

5.2.4 Growth cabinet experiment

The growth cabinet experiment was performed using six two-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 growth cabinets. Once in the growth cabinets, they were subjected to night/day temperatures of 15/25 ºC for one week before measurements began, to ensure acclimation to these relatively low

temperatures and moderate light levels (acclimation in *Q. rubra* has been shown to occur in as little as one day; Bolstad *et al.*, 2003). Measurements were then taken on days 0, 1, 4, and 7. On day 0, growth chamber temperatures were 15/25 ºC. Immediately following measurements, growth chamber temperature was increased to 25/35 ºC and remained at this temperature through day 7. Levels of photosynthetically active radiation were kept at 200 μmol photon m^{-2} and plants were watered daily throughout the experiment. Different leaves were measured each day.

Please see Chapter 2: Methods for details on measurements of respiration, oxygen isotope discrimination, immunoblotting, carbohydrates, nitrogen, specific leaf area, chlorophyll fluorescence, and statistical analysis.

5.3 RESULTS

5.3.1 Field study

Daily average and minimum field temperatures are shown for all four sites in Figure 5.1. 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 example, the maximum temperature at the rural site in 2009 was approximately 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 5.1. 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

Table 5.1: Results of two-way ANOVA for R_{10}, E_0, R_g, R_g/N, and the AOX/COX protein ratio. Values shown are *p*; F. Bold indicates statistically significant results.

	R_{10}	E_0	R_g	R_g/N	AOX/COX
Time	**< 0.001; 106.4**	0.617; 0.252	**< 0.001; 51.10**	**< 0.001; 48.34**	**0.003; 9.016**
Site	0.804; 0.062	0.764; 0.091	**< 0.001; 19.75**	**0.014; 6.162**	**0.041; 4.272**
Interaction	0.999; 0	0.723; 0.126	0.545; 0.363	0.500; 0.458	0.554; 0.3511

Table 5.2: Leaf nitrogen (*N*), nonstructural carbohydrates and specific leaf area (SLA) in the seasonal study.

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	q6.8 ± 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

of the growing season (Fig. 5.3a); the change in R_{10} over time was significant. There were no significant differences 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. 5.3b). E_0 was not significantly different between sites, nor did E_0 change significantly over time. Respiration at the

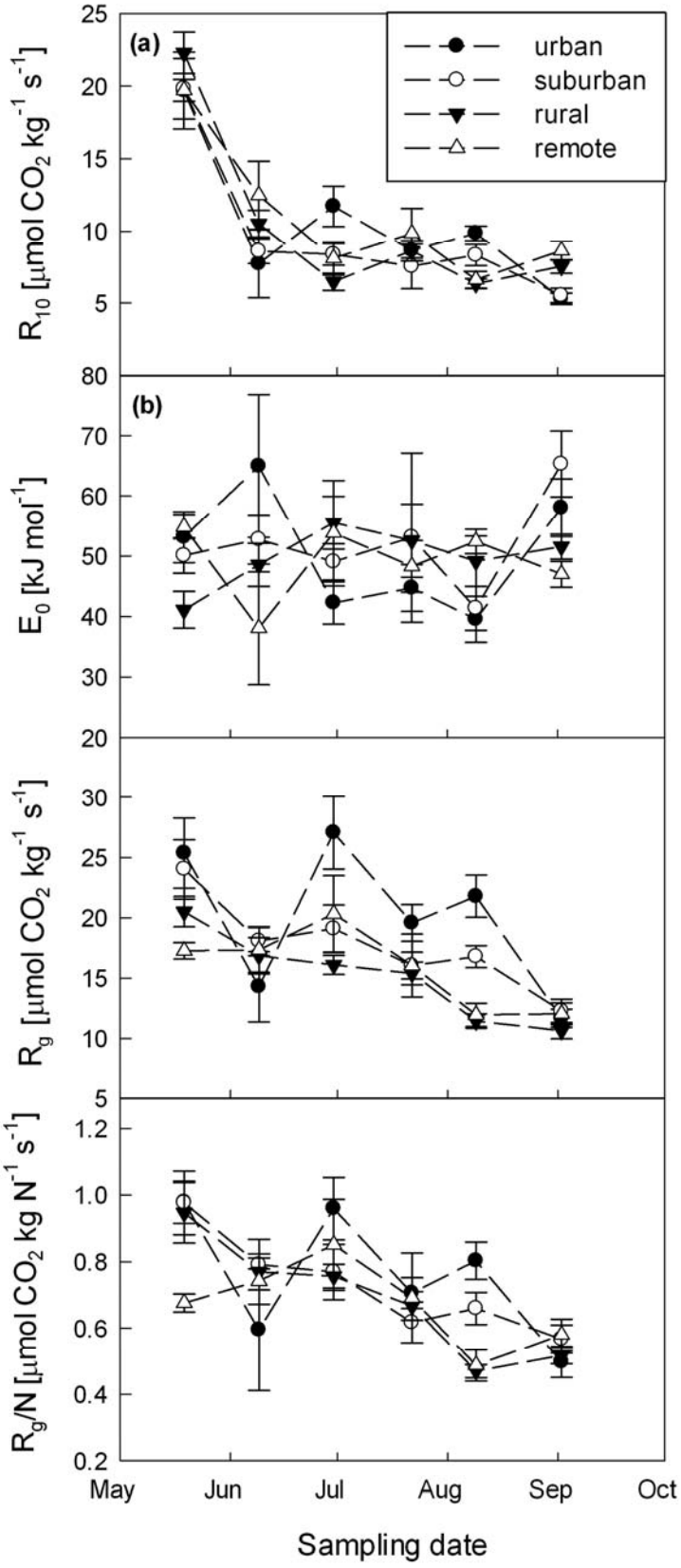

(a)
urban
suburban
rural
remote
R_{10} [μmol CO_2 kg^{-1} s^{-1}]
(b)
E_0 [kJ mol^{-1}]
R_g [μmol CO_2 kg^{-1} s^{-1}]
R_g/N [μmol CO_2 kg N^{-1} s^{-1}]
May
Jun
Jul
Aug
Sep
Oct
Sampling date

Figure 5.2: Respiratory parameters for each site over the growing season. **(a):** R_{10}; **b):** E_0; **(c):** Respiration at the growth temperature (R_g; the average temperature the day prior to measurement) over the growing season; **(d):** R_g on a nitrogen basis (R_g/N) over the growing season. Values shown are mean ± SEM, where n = 6.

growth temperature (R_g; the minimum temperature the day prior to measurement) generally declined throughout the growing season at all sites (Fig. 5.3c); 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. 5.3d) although the difference between sites was smaller than with R_g (Table 5.1). There was no significant interaction between site and time with R_{10}, E_0, R_g, or R_g/N.

R_g showed a significant positive correlation with leaf nitrogen (N) when all sites and sampling dates were pooled (Fig. 5.3; $R^2 = 0.222$, $p = 0.020$). Leaves at the urban site had significantly greater nitrogen concentrations than leaves at the other sites (Table 5.2). Soluble sugar, starch, and specific leaf area (SLA) are also shown in Table 5.2. With all sites pooled, there was a statistically significant but weak negative correlation between R_g and leaf starch ($R^2 = 0.191$, $p = 0.033$), 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 significant positive correlation between R_g and the AOX/COX protein ratio when all sites were pooled ($R^2 = 0.186$, $p = 0.035$), with a greater ratio of AOX to COX protein abundance being associated with higher rates of R_g (Fig. 5.4). The AOX/COX protein ratio changed significantly both over time and between sites, but there was no time-site interaction (Table 5.1). Discrimination is plotted against the minimum temperature the night prior to sampling for each site in Figure 5.5a. There was a significant linear relationship between

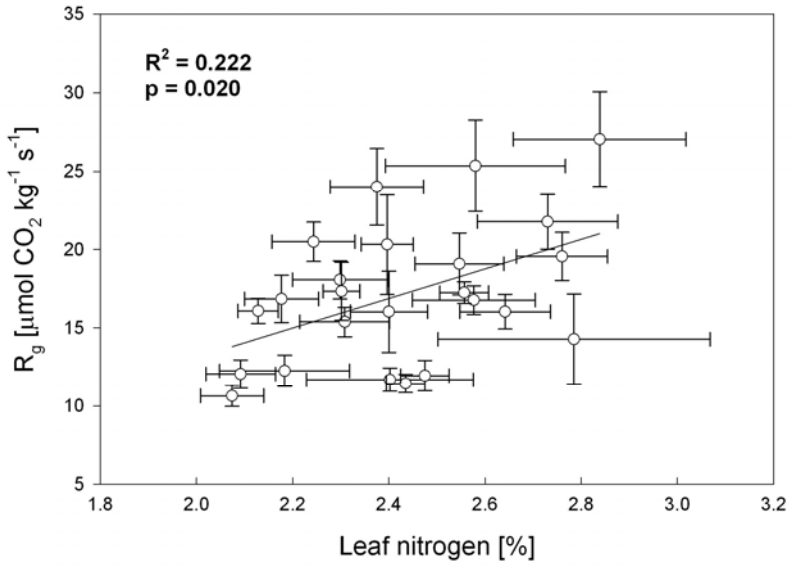


Figure 5.3: Respiration at the growth temperature (R_g) plotted against leaf nitrogen (N) in the seasonal study. Values shown are mean ± SEM, where n = 6.

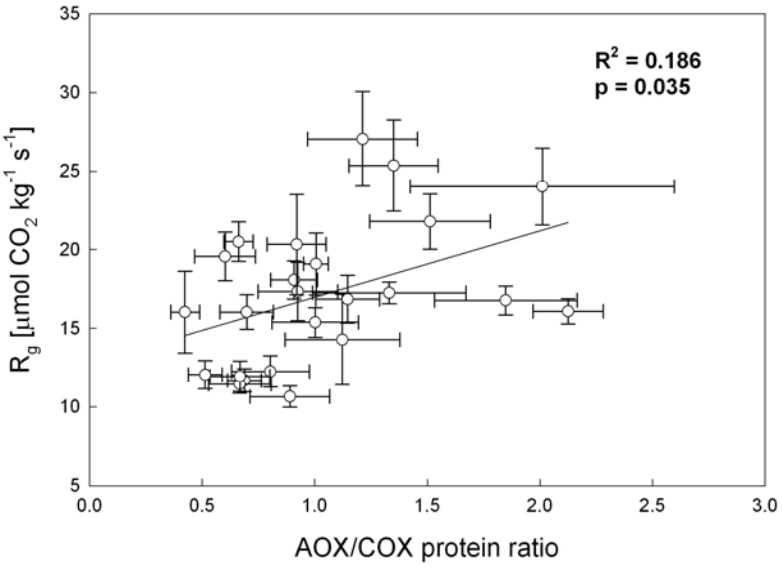


Figure 5.4: Respiration at the growth temperature (R_g) plotted against the AOX/COX protein ratio in the seasonal study. Values shown are mean ± SEM, where n = 6.

discrimination and temperature at the rural (R^2 = 0.836, p = 0.011) and remote (R^2 = 0.748, p = 0.026) sites, but not at the urban (R^2 = 0.006, p = 0.881) or suburban (R^2 = 0.002, p = 0.941) sites. Figure 5.5b shows D at each site over time.

5.3.2 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. 5.6a). R_{10} was significantly lower on days 4 and 7 than it had been on day 0 (p = 0.034). E_0 rose from day 0 to day 1, and declined from day 1 to day 7; however, these changes were not significant (p = 0.201; Fig. 5.6b). R_g rose from day 0 to day 1 with the increase in temperature, but declined again on days 4 and 7 (Fig. 5.6c). Figure 5.8c 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 = 0.042; 0.040), indicating acclimation to the warm temperatures.

Leaf soluble sugars were relatively constant throughout the growth cabinet experiment (p = 0.456; Fig. 5.6d). Leaf starch declined with the warming treatment, and was significantly lower on day 4 than on day 0 ($p < 0.001$; Fig. 5.6d). Discrimination was similar on days 0 and 1, but rose significantly on days 4 and 7 (p = 0.020; <0.001), indicating a greater contribution of the AP to respiration at higher growth temperatures (Fig. 5.7a). The AOX/COX protein balance declined with the warming treatment, and was significantly different between days 0 and 4 (p = 0.003; Fig. 5.7b).

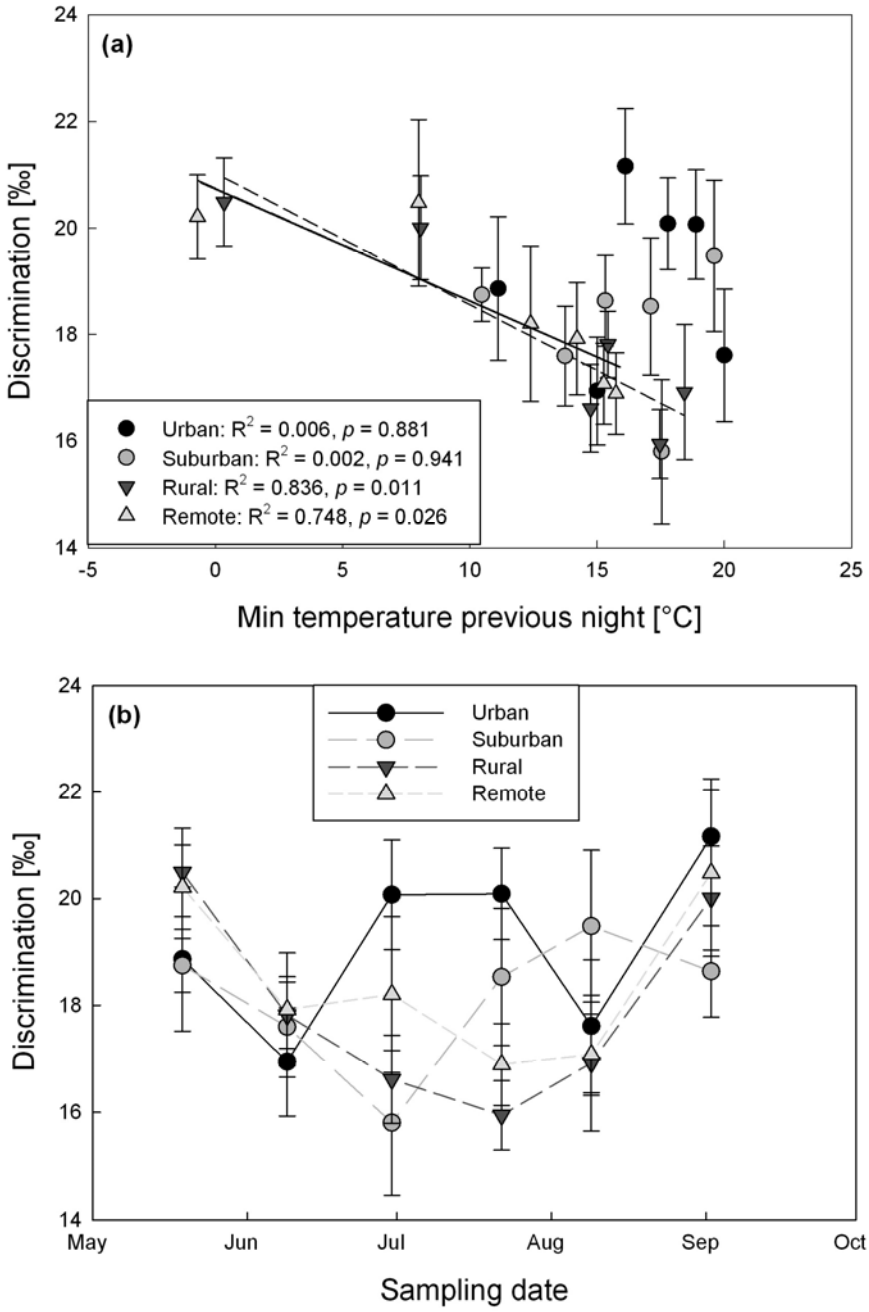


Figure 5.5 (a): Discrimination plotted against the minimum temperature the night prior to measurement in the seasonal study for each site. Higher discrimination values indicate greater relative contribution of the alternative pathway (AP) to respiration, and lower values indicate greater CP contribution. 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. Error bars show the standard error of regression.

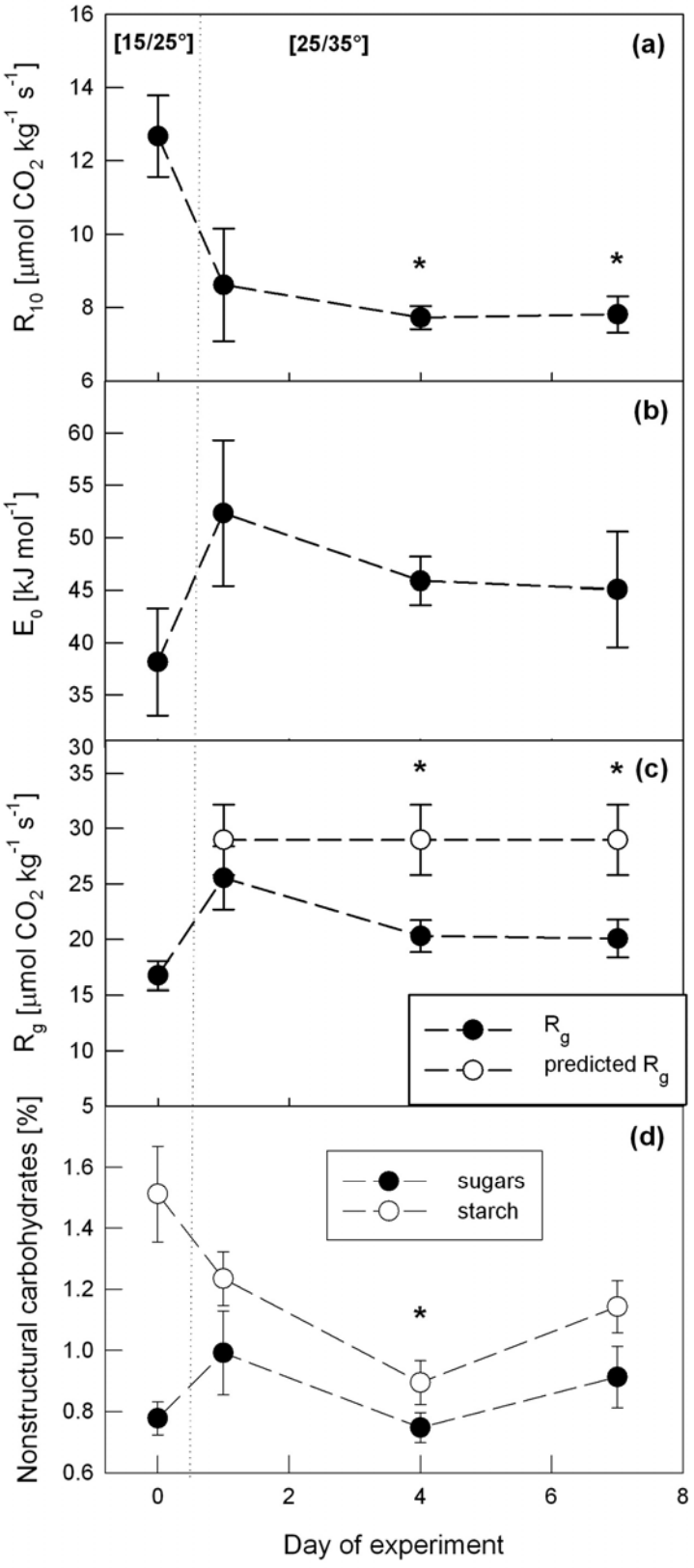

[15/25°]
[25/35°]
(a)
(b)
(c)
(d)
R_{10} [µmol CO_2 kg^{-1} s^{-1}]
E_0 [kJ mol^{-1}]
R_g [µmol CO_2 kg^{-1} s^{-1}]
Nonstructural carbohydrates [%]
R_g
predicted R_g
sugars
starch
Day of experiment

Figure 5.6: Respiratory parameters and leaf carbohydrates over time in 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.

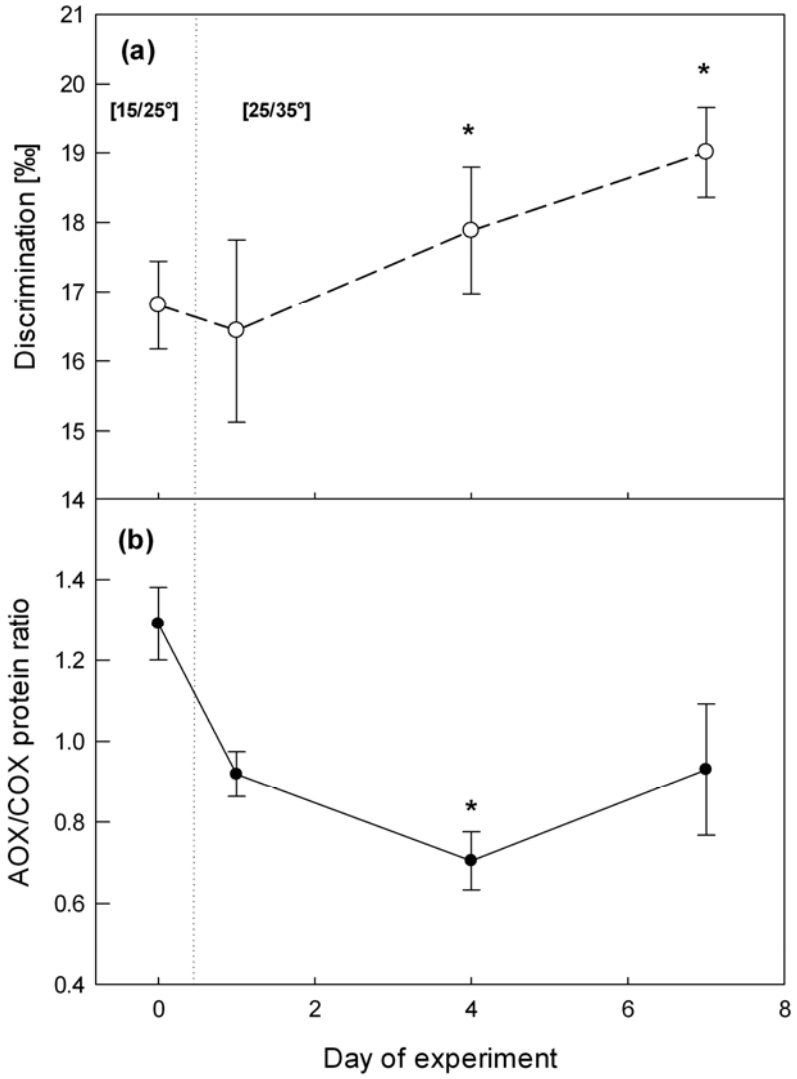


Figure 5.7 (a): Discrimination over time in the growth cabinet experiment. Higher discrimination values indicate greater relative contribution of the alternative pathway (AP) to respiration, and lower values indicate greater CP contribution. Error bars show the standard error of regression. **(b):** AOX/COX protein ratio over day of experiment. Values shown are mean ± SEM, where n = 6. 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.

5.4 DISCUSSION

The most important factor associated with differences in respiration along the urban-rural 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. 5.2c; Table 5.1). Urban-grown leaves had significantly higher *N* than leaves grown at other sites (Table 5.2), and respiration at the previous night's minimum temperature was positively correlated with *N* (Fig. 5.3). Nitrogen has been reported to be an important factor in determining respiration rates in a variety of species (Reich *et al.*, 1998; Tjoelker *et al.*, 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, the 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 support for the results of Bolstad *et al.* (2003), who found *R* in *Q. rubra* seedlings to acclimate 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 rising temperatures from May through August (Fig. 5.2c, Table 5.1). Although several studies have found evergreen leaf respiration to acclimate to seasonal changes in temperature (Stockfors & Linder, 1998; Atkin *et al.*, 2000b; 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 (Xu & Griffin, 2006; Marra *et al.*, 2009; Ow *et al.*, 2010; Rodriguez-Calcerrada *et al.*, 2010). Why R declines in deciduous leaves is not fully understood. Previous studies have found R to decrease during the first month of leaf development (Azcòn-Bieto *et al.*, 1983b; Priault *et al.*, 2007), but R typically stabilizes once leaves are mature and remains constant until near senescence (Hardwick *et al.*, 1968; Marron *et al.*, 2008). Thus, while the decline in R observed in the first month of this study may be due, in part, to leaf development, it is not likely that the continuing decrease in R over the remainder of the growing season was solely related to leaf age. It is possible that seasonal changes in R in these trees were a product of decreasing photosynthesis with leaf age and with decreasing day length after June; however, this effect was not clearly mediated by changes in carbohydrate availability in the present study, as carbohydrates were not positively linked with leaf R. The decline in R_g at the end of the growing season may also be related to a sharp decrease in N (Table 5.2). 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

northeastern US forests, which are currently considered a major carbon sink (Myneni *et al.*, 2001; Pacala *et al.*, 2001; Schuster *et al.*, 2008).

Another possible mechanism for the seasonal decline in R_g is change in key respiratory proteins. There was a significant positive correlation between R_g and the ratio of AOX to COX protein abundance when data were pooled between sites and sampling dates (Fig. 5.4). These field results are similar to those reported in Chapter 4 where a seasonal decline in R_g in leaves of *Populus* x *canadensis* was accompanied by a decline in the AOX/COX balance. The AOX/COX balance also contributed to the thermal acclimation of R_d in the alpine grass *Chionochloa pallens* in Chapter 3.

Contrary to my hypothesis, abundance of AOX and COX proteins in both the field and growth cabinet study was not correlated with discrimination (*D*), which here is interpreted as indicating relative changes in electron partitioning between the AP and CP. This is consistent with other studies, which have also not found a relationship between AOX protein abundance and discrimination or AP engagement (Lennon *et al.*, 1997; Guy & Vanlerberghe, 2005; Ribas-Carbo *et al.*, 2005b; Vidal *et al.*, 2007). The lack of correlation between protein abundance and discrimination may be partially due to the fact that the AOX protein exists in either an oxidized form or a reduced dimer and is thought to be active in only the oxidized state (Umbach & Siedow, 1993; Umbach *et al.*, 1994; Umbach *et al.*, 2005). Clearly, changes in activity and AP/CP partitioning may be more rapid 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. 5.5), indicating that at these colder sites, the AP was up-regulated relative to the CP following low temperatures. *D* was negatively correlated with

temperature in *Chionochloa rubra* and *C. pallens* in the field (Chapter 3) and discrimination has been found to increase in leaves exposed to low temperatures in controlled environment studies (Gonzalez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000a; Armstrong *et al.*, 2008). The results presented here provide additional field support for the notion that the AP responds to low temperatures.

In contrast, *D* did not respond linearly to temperature in leaves from the urban and suburban sites (Fig. 5.5a). Figure 5.5b 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 elicited a heat response of discrimination. These field results were clearly supported by those of the growth cabinet experiment. In this experiment, 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 days 4 and 7 (Fig. 5.7a), indicating a clear response to heat. Although this finding is consistent with my 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. The seemingly contradictory results presented here 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.*, 2000a; Fiorani *et al.*, 2005; Sugie *et al.*, 2006; Yoshida *et al.*, 2007).

There was no apparent 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 must have 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. Initially, I thought that an increase in the AP would result in greater respiration, as R via the AP must produce more CO_2 in order to meet the same energy demands as R via the CP. On the contrary, the balancing between the two oxidative pathways prevented an increase in R_d while up-regulating the AP in response to stress.

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 directly result in an increase in stand-level or regional plant respiration.

5.5 SUMMARY

This chapter explored responses of respiration and alternative oxidase in northern red oak to changes in temperature on three timescales: over a period of days in a growth cabinet experiment, over the course of the growing season in the field, and life-long differences in temperature between sites varying in proximity to New York City.

Differences in leaf nitrogen concentration along the gradient largely accounted for greater R_g in leaves grown at the urban site. Additionally, discrimination differed between field sites in its response to seasonal changes in temperature: discrimination in trees grown at the warmer, more urban sites increased with heat in mid-summer, while at the cooler, more rural sites, *D* responded to cold in the spring and autumn. This result supports the theory that AP respiration increases in response to stress, and not to specific environmental changes. Like the poplar trees described in Chapter 4, respiration in these deciduous oaks declined over the course of the growing season, contributing to the evidence that leaf respiration in deciduous trees does not acclimate seasonally as in evergreen plants. In the growth cabinet experiment, where oak seedlings were subjected to high temperatures for one week, respiration acclimated to the increased temperatures within four days, while discrimination rose significantly during this time. This experiment showed that, unlike for the tussock species in Chapter 3, respiratory parameters and alternative oxidase can change quite rapidly in plants with a more stable thermal history. Lastly, results from the growth cabinet experiment show that the AP and CP can have mirrored responses to temperature changes, with the CP decreasing concomitantly with increases in the AP. This chapter provides an improved understanding of acclimation in deciduous trees, as well as evidence that AP respiration responds to stress.

Chapter 6: Discussion and future directions

6.1 THESIS OBJECTIVES

This thesis investigated changes in plant respiration and in the respiratory enzyme alternative oxidase (AOX) with temperature. I have attempted to address the following questions:

1. How rapidly does respiration acclimate to natural changes in temperature in the field?
2. Does relative electron partitioning through the enzyme alternative oxidase respond to natural changes in temperature in the field?
3. Do changes in the electron partitioning through the alternative pathway (AP partitioning) contribute to the thermal acclimation of respiration?

6.2 SUMMARY OF KEY FINDINGS

- Leaf respiration acclimated to seasonal changes in temperature in evergreen plant species, but not in deciduous plants.
- Acclimation of respiration was rapid in red oak but unusually slow in an alpine grass with a highly variable thermal history.
- AP partitioning varied naturally in the field and increased in response to high and low temperatures, light, and measurement temperature.
- AP partitioning was not related to the abundance of the AOX protein.
- AP partitioning did not directly influence rates of respiration or the process of acclimation.

6.3 GENERAL DISCUSSION OF RESULTS

6.3.1 The rapidity of the thermal acclimation of respiration

Respiratory acclimation to temperature changes has been found to be rapid (i.e. occuring in less than one week) in various taxa under controlled environment conditions (Rook, 1969; Atkin *et al.*, 2000b; Bolstad *et al.*, 2003; Armstrong *et al.*, 2008). For example, Bolstad *et al.* (2003) found respiration in *Quercus rubra* and *Q. alba* to acclimate to controlled environment temperature changes in as little as one day; they also observed acclimation in response to a natural temperature change in the field in four days. However, few studies have specifically investigated how quickly acclimation occurs. Ow *et al.* (2008a) studied the rapidity of acclimation in *Populus deltoides* x *nigra* (*P.* x *canadensis*; a deciduous angiosperm), while Ow *et al.* (2008b) did the same in *Pinus radiata* (an evergreen conifer). In both species, partial acclimation occurred after one week at the new temperature, but full acclimation did not occur for several weeks and until the plants developed new leaves. In Chapter 3, I reported on the rapidity of acclimation in *Chionochloa rubra* and *C. pallens*, native New Zealand perennial alpine grasses, to natural temperature changes in the field. I found that acclimation occurred in response to broad, seasonal changes in temperature, but not to large temperature fluctuations over several days.

Why was acclimation in *Chionochloa* spp. slow when previous studies have shown acclimation to be rapid in various taxa? I speculate that the rapidity of acclimation is partially determined by either plant thermal history or the thermal history to which the species had adapted. In many studies that manipulate temperature in growth cabinets to study acclimation, study plants are raised either in growth cabinets or glasshouses, or place the plants in these environments for several weeks to months prior to the study (Rook, 1969; Larigauderie & Korner, 1995; Atkin *et al.*,

2000a; Bolstad *et al.*, 2003; Loveys *et al.*, 2003; Armstrong *et al.*, 2008). Indeed, in Chapter 5 I placed *Q. rubra* seedlings in a glasshouse and then a growth chamber for a total of one month prior to the experiment, and found acclimation to occur within days. In contrast, the snow tussocks I measured in Chapter 3 were found growing in the field in the alpine zone of New Zealand and were exposed to frequent, extreme temperature changes. Rapid acclimation to these frequent thermal fluctuations would presumably result in significant construction/maintenance costs without any clear benefit: as soon as the plant had acclimated to one temperature, the temperature would likely have changed again. Thus, I postulate that rapid acclimation may only occur following relatively stable temperature changes. The implications of this idea are vast: it is possible that acclimation in the field is greatly dampened compared to that observed in controlled environment studies, which would greatly alter predictions of future carbon uptake by the terrestrial biosphere.

6.3.2 The ontogenetic decline in respiration in deciduous plants

While I found respiration to acclimate to seasonal changes in temperature in two evergreen genera, *Chionochloa* and *Pinus*, respiration in two deciduous trees, *Populus* and *Quercus*, declined steadily throughout the growing season. Previous studies largely show the same trend, with evergreen trees acclimating seasonally (Stockfors & Linder, 1998; Atkin *et al.*, 2000b; Vose & Ryan, 2002; Ow *et al.*, 2010), and leaf respiration in deciduous trees steadily decreasing over the growing season (Xu & Griffin, 2006; Marra *et al.*, 2009; Ow *et al.*, 2010; Rodriguez-Calcerrada *et al.*, 2010).

Why respiration declines in deciduous leaves is not clear. Respiration typically declines during the first month of leaf development (Azcòn-Bieto *et al.*, 1983b; Priault *et al.*, 2007), but stabilizes once the leaves are mature (Hardwick *et al.*, 1968;

Marron *et al.*, 2008). Thus, continued decreases later in the growing season are not likely directly related to leaf age (until senescence). It is possible that this decline is indirectly related to photosynthesis: photosynthesis typically decreases with age in both deciduous (Wilson *et al.*, 2000; Onoda *et al.*, 2005; Ow *et al.*, 2010) and evergreen plants (Escudero & Mediavilla, 2003; Miyazawa *et al.*, 2004; Niinemets *et al.*, 2005). The age-related decline in photosynthesis could impact respiration through carbohydrate status and also through a decrease in maintenance costs. While this decline is rapid in short-lived deciduous leaves, it is more gradual and thus would impact physiology to a lesser degree in evergreen leaves that live several years.

6.3.3 The role of leaf nitrogen and carbohydrates in changes in respiration

Nitrogen has been reported to be an important factor in determining respiration rates in a variety of species (Reich *et al.*, 1998; Tjoelker *et al.*, 1999; Wright *et al.*, 2006; Xu & Griffin, 2006; Tjoelker *et al.*, 2008). In Chapter 5, I found that leaf nitrogen accounted for some differences in respiration in *Q. rubra* between sites along the urban-rural gradient and over the course of the growing season. Respiration positively correlated with leaf nitrogen when data from all sites and sampling periods were analyzed together. However, leaf nitrogen was not related to seasonal changes in respiration rates in *Chionochloa* spp., *Populus* x *canadensis*, or *Pinus radiata*. Thus, it is clear that leaf nitrogen is not always a determinant of respiration rates across taxa.

It is also not clear that nonstructural carbohydrates always underpin changes in respiration. A number of studies have linked carbohydrate status to changes in leaf and root respiration in a range of taxa (Azcòn-Bieto *et al.*, 1983a; Noguchi & Terashima, 1997; Tjoelker *et al.*, 1999; Covey-Crump *et al.*, 2002; Lee *et al.*, 2005; Tjoelker *et al.*, 2008; Ow *et al.*, 2010). I found a clear decrease in starch with

respiratory acclimation to high temperatures in *Q. rubra* in the growth cabinet experiment. Additionally, respiration in the field study declined over the growing season concomitantly with a decline in soluble sugars. Similarly, the seasonal decline in respiration in *P.* x *canadensis* was accompanied by a decrease in soluble sugars. However, seasonal changes in respiration in *P. radiata* and *Chionochloa* spp. were not related to carbohydrate status. Although Tjoelker *et al.* (1999; 2008) correlated carbohydrates with respiration in coniferous species, it is possible that respiration is more tightly coupled with carbohydrates in deciduous than it is in evergreen plants.

6.3.4 The relationship between AOX protein abundance and AP respiration

I hypothesized that electron partitioning through the AP would be dependent on the abundance of AOX proteins. In each of the experimental chapters presented in this thesis, there was no correlation between AOX protein abundance and discrimination. In the growth cabinet experiment with *Quercus rubra* (Chapter 5), discrimination actually increased while AOX protein abundances declined. The literature suggests that this is not an unusual result. Only five out of fourteen studies reviewed here that report both oxygen isotope discrimination and AOX protein abundance in leaves, roots, and isolated mitochondria found a link between the two (Ribas-Carbo *et al.*, 1997; Millar *et al.*, 1998; Gonzalez-Meler *et al.*, 1999; Liang & Liang, 2002; Rachmilevitch *et al.*, 2007), while nine did not (Lennon *et al.*, 1997; Millenaar *et al.*, 2000; Guy & Vanlerberghe, 2005; Ribas-Carbo *et al.*, 2005b; Florez-Sarasa *et al.*, 2007; Juszczuk *et al.*, 2007; Vidal *et al.*, 2007; Armstrong *et al.*, 2008; Grant *et al.*, 2008). In fact, two of the studies reporting a correlation between AOX engagement and protein did not find this link in a second species or treatment in the same study (Liang & Liang, 2002; Rachmilevitch *et al.*, 2007). One example is Guy &

Vanlerberghe (2005), who reported that changes in AOX protein levels in tobacco mutants over-expressing or deficient in AOX did not result in changes in actual AP engagement. Thus, it is abundantly clear that post-translational regulation of AP respiration takes place.

How is electron flow via the AP regulated in existing AOX protein? The AP has been found to be stimulated in isolated mitochondria from soybean cotyledons by the addition of pyruvate (Ribas-Carbo *et al.*, 1995; Ribas-Carbo *et al.*, 1997), supposedly by lowering the threshold level of ubiquinone pool reduction at which the AP becomes engaged (Umbach *et al.*, 1994). However, to the best of my knowledge, it is unknown how pyruvate might naturally accumulate in plant cells other than through rapid glycolysis due to high carbohydrate concentrations. Carbohydrate status has typically not been linked with AP partitioning (Millenaar *et al.*, 2000; Ribas-Carbo *et al.*, 2000a; Grant *et al.*, 2008), suggesting that stimulation of the AP by naturally produced pyruvate is likely not common *in vivo*. Liang & Liang (2002) found that addition of salicylic acid induced the AP in aged potato tubers. It is likely that naturally produced salicylic acid induces the AP during infection: infection has been shown to stimulate both the AP (Vidal *et al.*, 2007) and production of salicylic acid (Malamy *et al.*, 1990). It is highly possible that other stress-related compounds trigger AP respiration, but many of these have not been investigated. Reactive oxygen species (Wagner & Krab, 1995; Vanlerberghe & McIntosh, 1996), hydrogen peroxide (Ho *et al.*, 2008), nitric oxide (Millar & Day, 1996; Huang *et al.*, 2002), ethylene and jasmonic acid (Ederli *et al.*, 2006) have all been found to induce increases in AOX capacity, but their effects on oxygen isotope discrimination have not been examined.

It has also been suggested that activity of AOX is regulated by its redox state. AOX exists in monomeric form, but also as a dimer with a disulfide bond. It is

thought to be active in only the reduced, monomeric state. While the presence of both of these AOX forms has been found in isolated mitochondria (Umbach & Siedow, 1993; Ribas-Carbo *et al.*, 1997), detection of the AOX dimer in whole plant tissues has been relatively uncommon. Millar *et al.* (1998) and Rachmilevitch *et al.* (2007) have detected the AOX dimer in roots of soybean and *Agrostis*, respectively, and have linked reduction of the dimer to the active form with increased AP engagement. Vidal *et al.* (2007) detected the AOX dimer in leaves of tobacco, although its presence or absence was not linked with changes in *D*. So, while the redox state of the AOX protein does not always control changes in *D*, there is evidence that it may be an important regulatory feature and deserves further study. Lastly, AP respiration may be triggered by changes in the state of phytochrome (Ribas-Carbo *et al.*, 2000b; Ribas-Carbo *et al.*, 2008) and this is discussed in section 6.3.6.

6.3.5 Alternative oxidase responses to environmental change

As detailed in the introduction, there is evidence in the literature that alternative oxidase responds to temperature, light, nutrient availability, drought, and pathogen infection. The research presented in this thesis set out to study changes in alternative oxidase with temperature, although light levels were found to influence discrimination in Chapter 3. With regard to temperature, it had previously been unclear whether there is an instantaneous temperature dependence of the balance between AP and cytochrome pathway (CP) respiration. Armstrong *et al.* (2008) reported that discrimination (*D*) increased with measurement temperature in *Arabidopsis thaliana*, indicating that the AP was more temperature sensitive than the CP in this species (i.e. as temperature increases, the AP increases at a greater proportional rate than the CP). Gonzalez-Meler *et al.* (1999) and Guy & Vanlerberghe (2005) measured similar

temperature dependences of *Vigna radiata* and *Tabacum nicotiana*, respectively, although Guy & Vanlerberghe (2005) found discrimination to decrease above 35 °C. However, Macfarlane *et al.* (2009) measured no temperature dependence of discrimination in *Cucurbita pepo, Nicotania sativa,* or *Vicia faba*. In Chapter 4, I found a clear temperature dependence of discrimination in *Populus* x *canadensis* that was consistent at various times throughout the growing season. This finding mirrored that of the studies listed above, with discrimination rising with increasing temperature. In contrast, there was no consistent temperature dependence of discrimination in *Pinus radiata*. However, it is possible that the technique for measuring oxygen isotope fractionation in Chapter 2 was not sufficiently accurate when applied to a conifer with thick needles. In any case, it is apparent that the AP is more temperature sensitive than the CP in some species, but not in others.

Whether or not discrimination changes with measurement temperature, it is fairly clear that exposure to relatively low temperatures for a period of days or longer can elicit an increase in AP respiration. Armstrong *et al.* (2008) found discrimination to increase in *A. thaliana* over a period of days of exposure to 5 °C. Ribas-Carbo *et al.* (2000a) reported an increase in AP respiration relative to the CP in *Zea mays* after one day of exposure to 5 °C. Discrimination was also greater in leaves and hypocotyls of *Vigna radiata* grown at 19 °C relative to plants grown at 28 °C (Gonzalez-Meler *et al.*, 1999). In Chapter 5, I showed that discrimination in *Quercus rubra* responded to low temperatures: AP respiration in trees grown at the colder sites along the urban-rural gradient increased relative to CP respiration at seasonally cold times of the year. One question is - how quickly do these changes occur? The fact that the AP is often found to be lower at low measurement temperatures relative to warm, as discussed above, begs the question of how AP respiration can increase in plants exposed to low

temperatures over longer periods of time. Armstrong *et al.* (2008) even found *D* to be higher in cold-treated *A. thaliana* plants (after 4d) than in warm-treated plants when measured at their respective growth temperatures. It is possible that rapid increases in AOX protein levels account for this change, although Armstrong *et al.* (2008) and Ribas-Carbo *et al.* (2000a) both reported no change in AOX protein abundance with increases in discrimination. It is also possible that biochemical triggers of the AOX, such as pyruvate (Ribas-Carbo *et al.*, 1995; Ribas-Carbo *et al.*, 1997), salicylic acid (Liang & Liang, 2002), and reactive oxygen species accumulate in plant cells over several hours to days of cold exposure, overcoming the naturally slow activity of the AOX at low measurement temperatures. It is not clear how quickly AP respiration increases with cold exposure over longer periods of time, or whether this increase is transient. For instance, Armstrong *et al.* (2008) found that discrimination peaked on day 4 of cold exposure and decreased thereafter, and there was no difference in discrimination between cold-grown and warm-grown plants. However, Gonzalez-Meler *et al.* (1999) clearly showed that changes in discrimination with temperature can be prolonged in some species. In contrast to the findings of Armstrong *et al.* (2008), I showed that discrimination in *Chionochloa* spp. varied over seasonal timescales but not over a period of two weeks. On the other hand, changes in discrimination in *Q. rubra* occurred independently over both short and long timescales in one species. It is clear that AP respiration does not exhibit consistent temporal trends in response to high or low temperature stress, which, like the rapidity of respiratory acclimation, could possibly be related to thermal history.

Interestingly, in both the field and growth cabinet studies I also found a response of discrimination to high temperatures in *Q. rubra*. Trees grown at the warmer sites along the urban-rural gradient exhibited increases in discrimination in

mid-summer, when field temperatures were highest. Supporting this finding, when seedlings were subjected to high (35 °C) temperatures over one week in the growth cabinet experiment, discrimination increased. Until now, there has been limited evidence that the AP responds to heat (Rachmilevitch *et al.*, 2007). Theoretically, the increase in discrimination with heat could simply be a result of the instantaneous temperature dependence of *D*. The balance in electron partitioning to the AP increases relative to the CP with increasing measurement temperature; it is possible that this effect is prolonged over longer periods of time. It is perhaps more likely that the heat response of the AP reflects physiological stress at some level; this is further discussed in section 6.3.6.

I had hypothesized that discrimination would respond to temperature in *Chionochloa* spp. (Chapter 1). There was in fact no clear correlation between discrimination and temperature over any time window in both the seasonal and short-term studies. I did find that discrimination correlated strongly with photosynthetically active radiation (PAR) integrated over the day prior to measurement. High light has been found to increase AP respiration in *Glycine max* (Ribas-Carbo *et al.*, 2000b) and *Spinacea oleracea* (Noguchi *et al.*, 2001), but not in *Nicotiana tabacum* (Guy & Vanlerberghe, 2005) or *Cucumis sativa* (Florez-Sarasa *et al.*, 2009). Thus, in an environment where both temperature and light fluctuate greatly and can be extreme, it is not surprising that discrimination responded to light. It is curious that discrimination in *Chionochloa* spp. did not change significantly over the course of the short-term experiment. Changes in light were not as great during this two-week period as over the course of the year, so it is possible that these changes were not sufficient to produce a response in discrimination. It is also highly possible that discrimination in these species actually responded to changes in both light and temperature, and that

the relationship between *D* and field temperature was obscured by the interaction with light. In fact, *D* showed a negative but insignificant trend with temperature in both species of *Chionochloa*. This study in particular highlights the difficulties in discerning specific responses in a variable environment.

One remaining question is why the ratio of AOX/COX protein abundances correlated with both temperature and respiration in *Chionochloa* spp. when discrimination did not? While protein abundances do not directly account for variation in activity of AOX and COX, as discussed above, the amount of protein should theoretically determine the maximum possible activity of the AP and CP. It may be that synthesis of new AOX and COX protein responds directly to temperature, providing resources if up-regulation of the AP is required. However, as will be discussed in the next section, the AP likely responds to various biochemical triggers, many of them related to stress. Thus, the extent to which increased protein abundances in the cold are actually engaged may depend on stress responses to a combination of environmental factors.

6.3.6 Mechanisms of AP regulation and physiological function

There is mounting evidence that the AP increases in response to various types of stress, including low and high temperatures, drought, and excess light. For example, Ribas-Carbo *et al.* (2000a) measured an increase in discrimination in a cold-sensitive maize cultivar in response to cold stress, but not in a cold-tolerant cultivar; this result suggests that the AP responds to stress, and not specifically to low temperatures. Additionally, Chapter 5 clearly illustrates that the AP does not respond to specific environmental conditions, as discrimination in *Q. rubra* responded to both low and high temperatures.

Increases in the AP may function to prevent over-reduction of the ubiquinone pool when the CP is limited by stressful conditions. In Chapters 4 and 5, *P.* x *canadensis* and *Q. rubra* exhibited increases in discrimination with no concomitant change in respiration, showing that the CP must have decreased at these times. It may be that the AP is activated when the CP is limited, avoiding over-reduction of the ubiquinone pool, which could potentially result in the production of damaging reactive oxygen species. Previous results in the literature have supported this notion (Gonzalez-Meler *et al.*, 1999; Ribas-Carbo *et al.*, 2000a; Gonzalez-Meler *et al.*, 2001; Noguchi *et al.*, 2001; Ribas-Carbo *et al.*, 2005b; Gomez-Casanovas *et al.*, 2007; Armstrong *et al.*, 2008; Ribas-Carbo *et al.*, 2008). It has been proposed that excess carbohydrates lead to an increase in the AP by over-reducing the ubiquinone pool when the CP is fully engaged (Azcòn-Bieto *et al.*, 1983a). Although two studies measuring *D* in leaves have suggested that activity of the CP is related to carbohydrate status (Gonzalez-Meler *et al.*, 2001; Florez-Sarasa *et al.*, 2007), in each of the chapters presented here, along with other studies, there was no correlation between carbohydrates and the AP in leaves and cotyledons of a variety of species (Millenaar *et al.*, 2000; Ribas-Carbo *et al.*, 2000a; Grant *et al.*, 2008). It thus seems likely that limitations on CP engagement are induced by factors other than carbohydrates.

In Chapter 3, I reported that discrimination in *Chionochloa* spp. responded to changes in light. Previous studies have reported the AP to respond to high light, although it is unclear if this response is stress-related (Ribas-Carbo *et al.*, 2000b; Noguchi *et al.*, 2001; Guy & Vanlerberghe, 2005). It has been suggested that the AP functions to prevent damage to chloroplasts through over-reduction of the electron transport chain in photosynthesis (Noguchi & Yoshida, 2008). Yoshida *et al.* (2006)

found that inhibition of AOX with SHAM in the presence of light resulted in a reduction in photosynthesis and ΦPSII operating efficiency, while Yoshida *et al.* (2007) reported high light-induced increases in AOX protein abundance concomitant with accumulation of reducing equivalents in the chloroplasts (both studies performed on leaves of *A. thaliana*). While AOX capacity and protein abundance do not accurately reflect changes in AP engagement, these results do suggest that the AOX may serve to reduce excess NADPH produced in chloroplasts and maintain efficient photosynthesis. However, there is also evidence that this process is light-activated, rather that stress-induced. Ribas-Carbo *et al.* (2000b) subjected soybean cotyledons to darkness with 5 minutes of light every 12 hours; these plants maintained high levels of AP engagement relative to those grown in complete darkness. This increase in AP engagement in light-flashed plants is not likely related to photosynthesis, as little occurred, but suggests that light itself regulates the AP. Furthermore, Ribas-Carbo *et al.* (2008) found that the AP is stimulated by exposure to non-stressful red light, suggesting phytochrome regulation of AOX. Thus, it is possible that the increase in the AP increases with light prevents damage to photosynthetic machinery well before any such damage could actually occur. This notion is further supported in Chapter 3: at times when the AP increased in response to high ambient light, chlorophyll fluorescence, which detects stress in photosystem II, remained at healthy levels. This response indicates that increases in the AP in *Chionochloa* spp. prevent damage.

6.3.7 Alternative oxidase and changes in respiration

I hypothesized that the alternative pathway contributes to the acclimation of respiration to temperature according to the following logic: acclimation is partially achieved through increases in the basal capacity of respiration at low temperatures (Atkin & Tjoelker, 2003). AP partitioning has been shown to increase in the cold (discussed above). As the AP produces far less ATP than the CP, increases in the AP with cold would theoretically result in increases in respiration rates (i.e. acclimation) in order to meet the same energy demands. However, this hypothesis has not been supported by the work presented in this thesis. Discrimination was not correlated with respiration or basal respiratory capacity (R_{10}) in any of the experimental chapters. The clearest examples are for *P.* x *canadensis* in Chapter 4 and in the growth cabinet experiment with *Q. rubra* in Chapter 5: in both instances respiration remained constant during a surge in the AP. It is clear that the CP decreased during these times. As detailed in section 6.3.6, several studies have found concomitant decreases in the CP with increases in the AP. There are two possible explanations: (1) as discussed in the previous section, it is possible that CP activity decreases in response to stress, and at these times the AP increases in order to maintain respiration and continue oxidation of excess NADH. (2) The CP decreases in response to AP increases (irrespective of whether the stimulus is oxidative stress, chemical stimulation, or state of phytochrome) in order to prevent excessive carbon loss through respiration.

There is some evidence for the second explanation, that changes in the AP and CP may function to maintain a positive carbon balance in plants. Gomez-Casanovas *et al.* (2007) and Gonzalez-Meler *et al.* (2009) found that AP engagement increased in response to elevated CO_2 (700-780 ppm) in *Opuntia ficus-indica* (a CAM plant) and *A. thaliana*, respectively. In particular, Gonzalez-Meler *et al.* (2009) found that the

AP decreased in plants grown at 200 ppm relative to plants grown at ambient CO_2. There was no evidence of stress, and activity of the CP did not change in response to changes in CO_2 (Gonzalez-Meler *et al.*, 2009). Therefore it appears that reduction in the AP with lower concentrations of CO_2 functions to offset the CO_2-related decreases in photosynthesis that likely occurred, maintaining a positive carbon balance at all levels of growth CO_2.

6.4 RECOMMENDATIONS FOR FUTURE WORK

Although there is now substantial data regarding acclimation of various plant taxa, a deeper understanding of the process of acclimation is needed in order to accurately model respiration responses to climate change. Whether or not deciduous trees acclimate to seasonal changes in temperature needs to be better determined. This work would be relatively simple and would have important implications for understanding the carbon balance of deciduous forests. The results of Chapter 3 suggest that the speed of acclimation may be influenced by thermal history or by adaptation to a certain thermal regime. This issue could be resolved in growth cabinet studies by subjecting plants to various degrees of temperature fluctuation and with temperature differences.

Further work is also needed to understand how environmental stress induces the AP. Measurements of mitochondrial reducing equivalents and the redox state of the ubiquinone pool alongside AP engagement in stressed plants could ascertain whether stress-related AP increases function to prevent ROS production through over-reduction of the ubiquinone pool. With regard to light, oxygen isotope results confirming the link between the AP and chloroplast redox status would be particularly helpful.

Research is also needed in order to identify the mechanisms responsible for AP increases in non-stressful situations. Studies investigating the response of the AP to light have the potential to identify the function of the AOX as well as the mechanism regulating its activity. The experiment of Ribas-Carbo *et al.* (2008) finding that red light may potentially regulate the AP should be repeated in other plant types along with measurements of the signaling state of phytochrome. Additionally, more attention should be paid to plant carbon balance when studying changes in the AP.

Lastly, in order to fully understand the function of AOX in the areas detailed above, more field studies are needed to examine the AP in plants grown under naturally variable conditions with multiple stresses, such as a combination of low temperatures and high light. Additionally, most studies on AOX have been performed on herbaceous, annual plants; other functional groups should be included. As trees constitute the largest component of terrestrial plant respiration, a better understanding of AP functioning in this group could have far-reaching implications. In particular, little AOX research has been performed in gymnosperms, in which the physiological role of this enzyme may be different than in typically studied angiosperms. Field studies on the AP in plants that are relevant both ecologically and to the global carbon budget would allow connecting AOX to respiration on a larger scale.

6.5 CONCLUDING STATEMENT

This thesis marks a milestone in alternative oxidase research, being some of the first work investigating AOX in the field. In a variety of plant functional groups and in geographical and environmental settings, the work presented here highlights the complexity of alternative oxidase responses to environmental changes and supports the theory that AOX is part of a general stress response. It makes clear that, like respiration, the response of alternative oxidase to temperature is different on longer and shorter timescales. The results presented here also raise important issues of how deciduous and evergreen plants differ in seasonal patterns of respiration, and how the rapidity of respiration responses may depend on the thermal history of the plant.

By introducing protocols to measure AP respiration and AOX protein abundance in wild plants and by providing a framework for the design of field experiments, this thesis sets the stage for future work on alternative oxidase in the field.

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Appendix A

Leaf respiration and alternative oxidase in fieldgrown alpine grasses respond to natural changes in temperature and light

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Key words: acclimation, alternative oxidase (AOX), Chionochloa, cytochrome oxidase, leaf respiration, oxygen isotope discrimination, photosynthetically active radiation (PAR), seasonal variation.

Summary

• We report the first investigation of changes in electron partitioning via the alternative respiratory pathway (AP) and alternative oxidase (AOX) protein abundance in field-grown plants and their role in seasonal acclimation of respiration.

• We sampled two alpine grasses native to New Zealand, Chionochloa rubra and Chionochloa pallens, from field sites of different altitudes, over 1 yr and also intensively over a 2-wk period.

• In both species, respiration acclimated to seasonal changes in temperature through changes in basal capacity (R_{10}) but not temperature sensitivity (E_0). In C. pallens, acclimation of respiration may be associated with a higher AOX : cytochrome c oxidase (COX) protein abundance ratio. Oxygen isotope discrimination (D), which reflects relative changes in AP electron partitioning, correlated positively with daily integrated photosynthetically active radiation (PAR) in both species over seasonal timescales. Respiratory parameters, the AOX : COX protein ratio and D were stable over a 2-wk period, during which significant temperature changes were experienced in the field.

• We conclude that respiration in Chionochloa spp. acclimates strongly to seasonal, but not to short-term, temperature variation. Alternative oxidase appears to be involved in the plant response to both seasonal changes in temperature and daily changes in light, highlighting the complexity of the function of AOX in the field.

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Appendix B

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Seasonal variation of leaf respiration and the alternative pathway in field-grown *Populus* × *canadensis*

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The temperature response of plant respiration varies between species and can acclimate to changing temperatures. Mitochondrial respiration in plants has two terminal oxidases: the cytochrome c oxidase (COX) and the cyanideresistant alternative oxidase (AOX). In Populus × canadensis var. italica, a deciduous tree species, we investigated the temperature response of leaf respiration via the alternative and cytochrome pathways, as well as seasonal changes in these pathways, using the oxygen isotope fractionation technique. The electron partitioning through the alternative pathway (!a) increased from 0 to 30–40% with measurement temperatures from 6 to 30#C at all times measured throughout the growing season. !a at the growth temperature (the average temperature during 3 days prior to sampling) increased from 12 to 29% from spring until late summer and decreased thereafter. Total respiration declined throughout the growing season by 50%, concomitantly with decreases in both AOX (64%) and COX (32%) protein abundances. Our results provide new insight into the natural variability of AOX protein abundances and alternative respiration electron partitioning over immediate and seasonal timescales.

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Appendix C

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

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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

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