MECHANISMS REGULATING THE THERMAL ACCLIMATION OF DARK RESPIRATION IN SNOW TUSSOCK AND RYEGRASS

A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science in Biochemistry

(2007)

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Acknowledgements

I’m extremely grateful for all the assistance I’ve had over the course of my thesis. My thanks go out to all my friends and family that have supported and encouraged me to make this thesis a success.

My greatest thanks go out to Professor Mathew Turnbull (Biological Sciences Department, University of Canterbury) for his supervision and support of this study. Your outstanding enthusiasm, constant encouragement and guidance helped me persevere.

I’d like to express my gratitude to both my secondary supervisors: Professor Kevin Griffin (Department of Earth and Environmental Sciences, Columbia University) and Professor Juliet Gerrard (Biological Sciences Department, University of Canterbury). I am very grateful for Kevin’s support, enthusiasm and discussions. I wish to thank Juliet Gerrard also for her support and supervision. Thank you for letting me use your laboratory facilities extensively, and most of all for making me feel so welcome whenever I have been in your lab meetings for discussions on my progress.

I acknowledge Manfred Ingerfeld’s guidance and excellent technical assistance in microscopy and the endless hours spent taking EM images for me. I am also very appreciative of the endless help from Dave Conder, Maggie Tisch, Craig Galilee and Jackie Healy throughout my research.

In particular, I gratefully acknowledge the Marsden Fund of the Royal Society of New Zealand, for providing the financial support for this research. I appreciate and thank DOC for permission to carry out my field work at Mount Hutt. Many thanks also to PGG Wrightson (Christchurch) for the donation of ryegrass seeds.
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Abbreviations

\( R_d \)  Dark respiration
\( R \)  Respiration
\( R_{10} \)  Respiration rate at 10°C
\( Q_{10} \)  Proportional change in \( R_d \) with a 10°C increase in temperature
\( N \)  Nitrogen
\( Na \)  Nitrogen content on a area-basis
\( N_m \)  Nitrogen content on a mass-basis
\( SLA \)  Specific leaf area
\( SDH \)  Succinate dehydrogenase

\( ATP \)  Adenosine 5'-triphosphate
\( ADP \)  Adenosine diphosphate
\( BSA \)  Bovine serum albumin
\( DCIP \)  Dichlorophenol indophenol
\( DTT \)  Dithiothreitol
\( EGTA \)  Ethylene glycol tetraacetic acid
\( EDTA \)  Ethylenediamine tetraacetic acid
\( HEPES \)  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
\( KCN \)  Potassium cyanide
\( KCl \)  Potassium chloride
\( KH_2PO_4 \)  Potassium dihydrogenphosphate (monobasic potassium phosphate)
\( K_2HPO_4 \)  Di-potassium monohydrogen phosphate (dibasic potassium phosphate)
\( MES \)  2-Morpholinoethanesulfonic acid
\( MOPS \)  Morpholinopropane sulfonic acid
\( NaCl \)  Sodium chloride
\( MgCl_2 \)  Magnesium chloride
\( MgSO_4 \)  Magnesium sulphate
\( NAD \)  Nicotinamide adenine dinucleotide (NAD^+)
\( NADH \)  Nicotinamide adenine dinucleotide (NADH is the reduced form of NAD^+)
\( TES \)  N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
\( PMS \)  Phenazine methosulfate
\( PMSF \)  Phenylmethysulfonyl fluoride
\( PVPP \)  Polyvinylpolypyrrolidine
Abstract

The aim of this research was to identify the mechanisms that underpin changes in respiratory capacity during acclimation to temperature. Dark respiration, enzyme activities and leaf ultrastructure were measured from ryegrass (*Lolium perenne*) in controlled environmental chambers and two species of native grass (*Chionochloa rubra* & *C. pallens*) growing at different altitudinal ranges on Mount Hutt, Canterbury, New Zealand. The overall hypothesis was that the changes in both mitochondrial numbers and enzyme activity underpin the greater respiratory capacity observed in response to decreasing temperatures.

Gas exchange measurements were carried out to measure rates of dark respiration (*Rd*) in leaves of both ryegrass and tussocks. Respiratory homeostasis (full acclimation) was achieved in ryegrass leaves but only partial acclimation in both species of tussock plants. Dark respiration rates for warm-grown ryegrass were greatly reduced compared to cool-grown grasses. *Rd* was lower for *C. rubra* growing at the base of the mountain (450m) compared to plants at a higher altitude (1060m). The dark respiration rates were also lower for *C. pallens* growing at 1070m than at 1600m. When comparing *Rd* between high and low altitude plants, it was significantly lower in low altitude plants at 450m than at 1600m.

Oxygen consumption was measured in intact leaves and roots, crude mitochondria and isolated mitochondria from ryegrass to investigate whether a change in respiratory capacity was involved with changes in *Rd*. Mitochondrial respiratory capacity was slightly reduced in warm leaves and roots (not significantly). The respiratory capacity results from isolated mitochondria for *C. rubra* (at 450m and 1060m) and *C. pallens* (at 1070m and 1600m) were consistent with the hypothesis that plants from warm sites have lower respiratory capacity in comparison to plants from cool sites. Based on these results and those of previous studies, it was concluded that respiratory flux for any given temperature is not simply determined by maximal capacities of the respiratory apparatus but rather a combination of the availability of substrate supply, the demand for respiratory products (i.e. ATP) and/or the maximal capacity of respiratory enzymes.
Abstract

Utilizing transmission electron micrographs, it was found that mitochondria were significantly less abundant in warm-grown than cool-grown ryegrass mesophyll cells. Mitochondria dimensions increased slightly between the cool and warm treatment. At lower altitudes (C. rubra), there was a significant decrease in mitochondria numbers with decreasing elevation. At higher altitudes (C. pallens), there was no noticeable change in mitochondria numbers between 1070m and 1600m. It was concluded that mitochondrial abundance for the controlled and field experiments, and mitochondrial sizes in the field, were associated with changes in $R_d$.

The maximal activities of fumarase and succinate dehydrogenase (SDH) in isolated mitochondria from leaves of ryegrass and tussock were measured spectrophotometrically. The results in the controlled experiment indicate that enzymes other than fumarase and SDH could be responsible for the increased respiratory capacity observed in cold acclimated leaves of ryegrass. However, fumarase maximal activity was significantly reduced in C. rubra at low altitude compared with C. pallens growing at high altitude - this suggests that it may be involved in the differences in respiratory capacity and $R_d$ between the two sites. Succinate dehydrogenase did not differ significantly in response to altitude. The large difference between the two field sites for fumarase activity is comparable to the large difference in $R_d$ and reduction in mitochondrial abundance and dimensions seen between the two sites. This supports the overall hypothesis that cool-grown plants keep up with energy demands at low temperatures by increasing enzyme concentrations/capacity.

The results of this study are supportive of the hypothesis that growth in low altitudes and warm conditions will result in the reduction of $R_d$ as a consequence of: (1) temperature sensitivity of the respiratory apparatus, resulting in the reduction of the respiratory capacities of mitochondria; (2) a reduction in mitochondria size and numbers; and as a consequence of this (3) a reduction in the activities of mitochondrial enzymes. However, these responses are species specific and vary according to the range of temperatures experienced by plants in the field and controlled environments.
CHAPTER ONE
INTRODUCTION AND RATIONAL
1.1 Introduction

1.1.1 Global climate change and carbon balance

Initially, global climate change was a part in history that did not attract much attention. Today, climate change is attracting huge scientific and political interest. Evidence gathered from gas trapped in ice cores, shows that in the past CO$_2$ levels have fluctuated with the glacial and interglacial periods (Neftel et al., 1994; 1998; Fischer et al., 1999; Monnin et al., 2004), and since 1958 there has been continuous monitoring of CO$_2$ levels at the Mauna Loa observatory in Hawaii (Keeling and Whorf, 2004). Figure 1 illustrates the evidence reported on variations in CO$_2$ in the atmosphere during the last 400,000 years. Throughout most of the record, the largest changes in CO$_2$ can be related to glacial/interglacial cycles. The past atmospheric CO$_2$ levels fluctuated between 180 and 280 parts per million depending on the glacial and interglacial cycles. These cycles are most likely caused by changes in the Earth's orbit, which affects the earth’s temperature and in turn influences the level of carbon dioxide in the atmosphere (Muller and Mac Donald, 1997). With the onset of the industrial revolution (~ 1800s), the burning of fossil fuels has created a dramatic increase in CO$_2$ in to the atmosphere, and today CO$_2$ has clearly reached levels that have not been seen in the last 400,000 years. In little as 100 years ago, the level elevated up to almost 350 ppm and is expected to continue to rise rapidly.

Human activity has been implicated as a primary cause of the astounding change in CO$_2$ in such short amount of time (IPCC, 2001). Accelerated fossil fuel burning, cement production and large-scale land transformation (deforestation and agricultural activities)
have been associated with increasing CO₂ and other greenhouse gases into the
atmosphere (IPCC, 2001). A number of other gases (i.e. water vapor, methane, nitrous
oxide, ozone, chlorofluorocarbons and hydrofluorocarbons) are also implicated to
stimulate global warming. The absorption and re-emission of infrared radiation by these
gases warms the earth’s surface and atmosphere, greater than the climate system’s
sensitivity to solar forcing (Stott et al., 2003). A lot of controversy still remains about
global warming, but what is clear from numerous studies published in the Inter-
governmental Panel on Climate Change reports (IPCC, 2001, , 2007), and current
monitoring of CO₂ and surface temperatures, is that CO₂ and temperature are increasing
today and predicted to increase in the future (Figures 1.2, 1.3, 1.4).

![Figure 1.1. Illustrates fluctuations in temperature (blue) and the atmospheric concentration of carbon dioxide (red) over the past 400,000 years as inferred from Antarctic ice-core records. The vertical red bar is the increase in atmospheric carbon dioxide levels over the past two centuries and before 2006. Figure reproduced from Fedorov et al. (2006).]
Figure 1.1. Illustrates the instrumental record of surface temperatures over the past 1,100 years. This figure is a reconstruction of (Northern Hemisphere average or global average) surface temperature variations from six research teams (in different color shades) along with the instrumental record of global average surface temperature (in black). Each curve illustrates a somewhat different history of temperature changes, with a range of uncertainties that tend to increase backward in time (as indicated by the shading). Reference: NRC (2006).

Figure 1.2. Illustrates the history of CO₂ atmospheric concentrations (1958-2007) measured from Mauna Loa, Hawaii. The curve represents the average monthly CO₂ concentrations in parts per million (ppm). Figure reproduced from Scripps CO₂ Program, 2007 (http://scrippscoc.ucsd.edu.)
Scientists have been producing long term trend data and sophisticated computer models that reveal and predict the present and future relationship between temperature and CO₂ levels (IPCC, 2001 & 2007). Mathematical models utilizing past and present data can predict the future atmospheric CO₂ concentration. The most common class of models used for understanding and predicting climate change are the General Circulation Models (GCMs). The 2001 IPCC Third Assessment Report, utilizing several different models, has forecasted that continued increments of gas emissions could result in global CO₂ levels and temperatures increasing at rates faster than previously experienced (Figure 1.4). Global temperatures are predicted to increase about 1.4 - 5.8°C due to global warming between 1990-2100 (IPCC, 2001).

Today, the potential for climate change in response to gas emissions has been taken more seriously by policy makers as it is now considered a major environmental concern. Temperatures today are about 0.6°C higher than they were in the early 1900s (IPCC, 2001). There is a strong possibility that the climate of New Zealand will change over the next 100 years; inevitably impacting biodiversity and ecosystem functioning (McGlone,
2001) along with economical impacts on agriculture, horticulture, forestry, or environment and nature conservation for tourism. It will be important to reduce greenhouse emissions to prevent further changes to the climate. New Zealand is one of the many countries that have ratified the Kyoto protocol and must provide a precise inventory of carbon sinks and sources. The aim of the Kyoto protocol is to reduce levels of greenhouse gas emissions that are responsible for current global warming (Schulze et al., 2002). Nations are allowed to create carbon sinks (storage of carbon in plant biomass) in order to offset and reduce their emissions (Schulze et al., 2002). The concern about global changes in climate has led to the exploration of the potential for forests to reduce carbon build up in the atmosphere. This process is known as carbon sequestration, in which CO₂ is withdrawn from the atmosphere by plants and used in photosynthesis and stored in biomass. Absorbing CO₂ from the atmosphere by growing large areas of forests (and other vegetation) will allow a country to achieve the desired net emissions levels set by the Kyoto Protocol. However, the balance between plant respiration and photosynthesis in an ecosystem will determine whether it is a source or sink of atmospheric CO₂. Predicting how rising temperatures, elevated CO₂ and changes in precipitation affects carbon balance in plants is of importance for global carbon budgets and understanding plant growth.

### 1.1.2 Global carbon balance

The global carbon cycle (Fig. 1.5) is the movement of carbon by several processes (i.e. biological and geological) (Campbell et al., 1999). The major sources of carbon are the atmosphere, terrestrial biosphere, and the ocean. Carbon in the atmosphere mainly exists as a gas, carbon dioxide (CO₂). Carbon dioxide can be removed from the atmosphere into sinks in several ways, such as: (1) photosynthesis converting CO₂ into carbohydrates; (2) CO₂ absorption by cool oceans near the poles, and circulation of currents causing carbon to build up in sediments; (3) phytoplankton in oceans absorbing CO₂, and when these die the exoskeletons fall to the ocean floor. Carbon can also be released back into the atmosphere from sources in many ways, such as: (1) respiration by plants and animals; (2) breakdown of plant and animal matter; (3) warm oceans releasing
dissolved carbon dioxide. Usually, these fluxes between the atmosphere, terrestrial biosphere and the ocean are balanced. However, imbalances can affect the global carbon cycle - fossil fuel burning and large scale changes in land (i.e. deforestation) cause such imbalances. Deforestation removes a carbon sink, preventing absorption of CO₂, and burning of forests releases further CO₂. Most of the CO₂ released by human activities is taken up by the land or the ocean. However, climate change (such as increases in temperatures) has been linked to elevated concentrations of CO₂ and other greenhouse gases into the atmosphere, and will most likely impact the carbon cycle (IPCC, 2001 & 2007).

Figure 1.4. Illustrates the main components of the global carbon cycle: storages (PgC) and fluxes (PgC/yr) estimated for the 1980s. The thick arrows denote the most important fluxes from the point of view of the contemporary CO₂ balance of the atmosphere: gross primary production and respiration by the land biosphere, and physical air-sea exchange. Image was reproduced from IPCC (2001).
1.1.3 Ecosystem carbon balance

Increases in atmospheric CO₂ and temperature and altered precipitation directly affects biological and chemical processes, causing a cascade of effects in individual organisms up to the ecosystem scale (Beier, 2004), which in turn has an effect on the climate system. There has been much recent research interest on the effects of global warming on plant growth and the global carbon balance. The study of the relationships between vegetation, global carbon cycle, climate, CO₂ and other greenhouse gases in the atmosphere is crucial to ensure that we have a better understanding and anticipate how climates and ecosystems might respond in the future. The distribution and functioning of vegetation depends essentially on the climate (Betts et al., 1997; Walther, 2003). The climate itself can be influence by the distribution and functioning of vegetation as carbon and water are exchanged with the atmosphere (Meir et al., 2006; Betts et al., 1997).

Forests account for more than 75% of carbon stored in terrestrial ecosystems and approximately 40% of the carbon exchanged with the atmosphere (Hamilton et al., 2002). At the ecosystem level, increased activity of northern hemisphere vegetation has been detected by observed seasonal changes in atmospheric CO₂ concentration (Keeling et al., 1996). Growth during summer results in the reduction in atmospheric CO₂ concentration by photosynthesis. Increases in atmospheric CO₂ during winter are observed as a result of respiration and decaying vegetation. The amplitude of these seasonal changes in CO₂ has increased during the same period of increased temperatures. It has thus been suggested that plant growth and respiration at the ecosystem and global scales are sensitive to temperature (Keeling et al., 1996).

The role of temperature in mediating a positive feedback on respiratory CO₂ flux to the atmosphere is of concern for its effects on global atmospheric CO₂ concentrations and its contribution to further greenhouse warming (Atkin and Tjoelker, 2003). Environmental warming is likely to have significant effects on plant carbon relations via its effect on photosynthesis and respiration (Turnbull et al., 2002; Griffin et al., 2004). Up to two thirds of photosynthetic carbon gain is released back into the atmosphere by plants, and at a global scale plant respiration represent a much greater flux (~ 60 gigatonnes of C per
year) compared to the release of CO$_2$ by combustion of fossil fuels, cement production and land use changes (~ 7.1 Gt C year$^{-1}$ in total) (Schimel, 1995). The balance between photosynthesis and respiration will determine whether an ecosystem is a net source or sink of CO$_2$ from the atmosphere. The responses of forests to changes in climate requires the use of models to forecast ecosystem response to environmental change (Beier et al., 2004). Research is necessary to understand the effects that changes in temperature, precipitation and CO$_2$ concentrations have on the structural and physiological dynamics of terrestrial ecosystems.

### 1.1.4 Responses of plants to global changes in environment

Temperature, CO$_2$, and water are the main drivers for many biological and chemical processes, and thus, ecosystem functioning (Meir et al., 2006; Betts et al., 1997; Walther, 2003). Plants must respond to changing environments diurnally and seasonally. Elevated CO$_2$, temperatures, nutrient availability and changes in rainfall patterns (affecting soil moisture) will influence plant competitiveness and growth, ultimately affecting species richness and biodiversity (McCarty, 2001; Walther, 2003; Zavaleta et al., 2003). Changes in environmental conditions can alter the diversity of plant communities by changing resource availability, affecting an individual species’ performance and, in turn, the outcomes of competition (Zavaleta et al., 2003). At a global scale, climatic parameters will affect species ranges and densities - these will largely determine the composition and distribution of ecosystems (McCarty, 2001; Walther, 2003).

Phenological studies (study of changes in biological events throughout the year) indicate that the spring initiation of growth has been happening earlier in recent decades for a wide range of taxa and across many geographic locations (Walther, 2003). Phenological data also indicate that growing seasons are lengthening, contributing to changes in the distribution and abundance of species (Walther, 2003). A number of studies have reported changes in species compositions and shifting species ranges with climate change (especially increased temperatures) (Read and Hill, 1985; Walther, 1997; Penuelas and
Boada, 2003). A climatic shift, such as milder winters, provides favourable conditions for the survival of dense stands of evergreen broad-leaved forests on sites with former deciduous forests (Walther, 2000). Danby and Hik (2007), used tree rings to measure changes in the density and altitude of spruce trees over the past 300 years, from six sites in the south western Yukon. Treeline (the boundary between forest and tundra zones) and stand density increased considerably over the 21st century; spruce advanced 65-85m in elevation, on the warm, south-facing slopes. On the cooler, north-facing slopes, only tree density increased 45-65%. It was suggested that mast seeding (high seed production) triggered by hot, dry summers, followed by years of warm temperatures would favour seedling growth and survival. This in turn would have impacts on tundra habitats, forcing them upwards. Vegetation changes have also been reported during the past 50 years by Strum et al. (2001) and in experimental field studies (Chapin et al., 1995). Shrubs have been observed expanding into areas that were once shrub-free in the Arctic, and species richness has also declined. Clearly, changes in temperature may benefit some species but not others.

In addition to temperature-induced changes, rainfall-driven changes in vegetation compositions must also be considered (Walther, 2003). It is speculated that an altered climatic regime with increased winter precipitation has resulted in greater shrub numbers and total cover of woody shrubs in a grassland-shrubland region in south eastern Arizona (Brown et al., 1997). Another example where water stress is affecting the distribution of different vegetation types, is the observation of retreatment drought intolerant species (Pinus ponderosa) and an increase in drought tolerant species (Pinus edulis & Juniperus monosperma) in New Mexico (Allen and Breshears, 1998). Elevated CO2 has also been shown to stimulate tree growth in forest ecosystems (DeLucia et al., 1999; Norby et al., 2001). In one study, lobolly pine plots were exposed to elevated CO2 for four years, and displayed considerable stimulation of growth during the first three years - during the fourth year the stimulation declined as a likely result of growth not been sustained by nitrogen limitation (Hamilton et al., 2002).
The above studies illustrate the effects of long term global climate change on vegetation growth and ecosystem function. To understand and predict climate-driven changes to plants requires studies involving combinations of temperature, water and CO₂. This is because CO₂, water and temperature are the major drivers affecting plant functioning and ultimately success in an environment. It is also essential to understand the physiological mechanisms regulating plant growth and development within ecosystems in response to changes in climate.

1.1.5 Role of photosynthesis and respiration in ecosystem carbon balance

The effects of climate change on carbon cycles will depend on the balance between photosynthesis and respiration. An imbalance between CO₂ uptake and release could modify the upward trend in increasing CO₂ concentrations (Keeling et al., 1995). CO₂ uptake by plants via photosynthesis is roughly balanced by plant and soil respiration. Plant photosynthesis represents an important flux of CO₂ out of the atmosphere (Griffin and Seemann, 1996). Photosynthesis incorporates carbon to provide plants with energy and structural building blocks (Pearcy et al., 1987). Respiration plays an important role in plant carbon exchange; it is a major carbon flux into the atmosphere. Respiration is a process in which plants release carbon dioxide into the atmosphere when they metabolize carbon substrates to provide carbon precursors and energy for cellular use and new plant biomass (Pearcy et al., 1987; Atkin and Tjoelker, 2003). In higher plants, approximately 50% of photosynthetic carbon uptake is returned to the atmosphere by leaf, stem and root respiration (Ryan, 1991; Griffin, 2001; Atkin and Tjoelker, 2003; González-Meler et al., 2004).

Temperature is one of the most important factors affecting both photosynthesis and respiration of plants, and their responses will play significant roles in future cycling of carbon. Both photosynthesis and respiration are sensitive to temperature, and changes in the climate are likely to affect carbon storage in plants and ultimately an ecosystem’s
carbon balance. Increases in temperature and CO$_2$ have been shown to stimulate photosynthesis, resulting in the increase of carbon being stored both below-ground and above-ground (Reddy et al., 1998; Centritto et al., 2002). It is predicted that stimulation of photosynthesis by CO$_2$ and temperature would increase ecosystem carbon uptake (Drake et al., 1997; Norby et al., 2001). The effects on ecosystem carbon storage will depend on the response of respiration to changes in climate (Ryan, 1991). Higher rates of respiration in a warmer climate could lead to the release of more CO$_2$ to the atmosphere from terrestrial ecosystems. However, numerous studies have reported that respiration can acclimate to changes in temperatures (Larigaurderie and Körner, 1995; Atkin et al., 2000b; Xiong et al., 2000; Browse and Xin, 2001; Atkin and Tjoelker, 2003; Bolstad et al., 2003; Loveys et al., 2003; Kurimoto et al., 2004c; Talts et al., 2004; Atkin et al., 2005; Armstrong et al., 2006b). Acclimation is defined as a change in respiratory activity that compensates for a change in temperature. This phenomenon can be observed when plants growing at contrasting temperatures (cool and warm) have the same rate of respiration when measured at their growth temperatures (respiratory homeostasis). It is suggested that respiratory acclimation to continued warming would improve plant carbon balance by reducing carbon losses (Xiong et al., 2000). Acclimation of respiration could reduce the predicted increase in annual respiratory CO$_2$ flux into the atmosphere (Atkin et al., 2000b), and failure to take acclimation into account will result in an over-estimation of the effects of global warming on CO$_2$ release (Griffin and Luo, 1999; King et al., 2006). Clearly, the response of plant respiration to temperature is an important link for predictions of carbon balance. If temperature changes as a result of greenhouse warming, then understanding the effects of temperature on plant respiratory flux will be a prerequisite for predicting future plant growth and CO$_2$ fluxes into the atmosphere (Atkin and Tjoelker, 2003).

1.1.6 Environmental effects on respiration

Many environmental factors can alter respiration rates, such as water, light, nitrogen availability and temperature. There have been a number of recent studies on the response
of respiration to temperature and how it changes under environmental conditions. Increased cloudiness and aerosols can result in night temperatures increasing more rapidly than day temperatures (as clouds reduce night cooling) (Turnbull et al., 2002; Turnbull et al., 2004; Huang et al., 2006). An increase in respiration occurs with increased temperature (Ryan, 1991), especially night temperatures (Turnbull et al., 2002; Griffin et al., 2004b). Drought conditions (water stress) lead to a reduction in both leaf and root respiration, especially in warmer soils (Ryan, 1991; Atkin et al., 2005). However, respiration rates may also decline due to nutrient limitations as growth and repair cannot be sustained over long periods of time (Hamilton et al., 2002; Atkin et al., 2005). Atkin et al. (2005) reviewed numerous studies, and suggest that growth in elevated CO$_2$ concentrations on average does not affect the temperature sensitivity of dark respiration in roots, leaves or shoots. They also indicate that regardless of growth temperature, the $Q_{10}$ (proportional change in respiration with a 10°C rise in temperature) of leaf respiration is often lower in the light than in darkness. In some cases, the reduction in $Q_{10}$ by light did not happen to all species. Changes in growth temperatures can affect the $Q_{10}$ in both leaves and roots of plants. In some cases the change in $Q_{10}$ is due to changes in the availability of respiratory substrates and ADP (Atkin and Tjoelker, 2003). An increase in soluble sugars may also result in increases in respiration rates (Tjoelker et al., 1999b; Atkin et al., 2000b). Exposure to elevated CO$_2$ (which increases photosynthesis and thus carbohydrate concentrations) has also been shown to increase the $Q_{10}$ of leaf respiration (Shapiro et al., 2004). It has been suggested that an increase in respiratory capacity is needed at very low temperatures, to keep up with the demands for energy (ATP synthesis) (Atkin and Tjoelker, 2003; Atkin et al., 2005).

### 1.1.7 Responses of respiration to temperature

Temperature is one of the most important environmental factors that control the rate of respiration in plants (Atkin et al., 2005). Respiration rates typically increases in response to an increase in temperature (Taiz and Zeiger, 2002). Between 0 and 30°C, the increase in respiration rate for every 10°C in ambient temperature (referred to as $Q_{10}$), is about 2.0
(Taiz and Zeiger, 2002). Most models assume that $Q_{10}$ is fixed at 2.0 and that respiration will respond to short and long term changes in temperature in a predictable manner (Atkin et al., 2005). However, as shown in the previous section, $Q_{10}$ values can change depending on the environmental situation and are not constant or always near 2.0 (Atkin and Tjoelker, 2003). There is growing evidence that respiration can acclimate to changes in temperature and that the degree of acclimation can vary between tissues and species (Larigauderie and Körner, 1995; Tjoelker et al., 1999a; Atkin et al., 2000b; Bolstad et al., 2003; Loveys et al., 2003; Armstrong et al., 2006b). Acclimation can be defined as the adjustment of the rate of respiration to compensate for a change in temperature. This can be seen when plants grown at contrasting temperatures maintain similar respiration rates (when measured at their respective growth temperature). Atkin et al. (2005) has proposed two types of mechanisms underpin observed responses (Figure 1.6 a & b): either (type I) a biochemical change that can occur within 1-2 days (i.e. change in gene expression as a result of substrate and/or ADP availability); or (type II) a change in respiratory capacity (i.e. changes in the amounts of specific enzymes and increased mitochondria density). It was suggested that type II acclimation is likely associated with changes in respiratory capacity that can be realized only through growth of new tissues. Any plants that are capable of thermal respiratory acclimation will contribute to a smaller increase in annual respiratory CO$_2$ release into the atmosphere with increasing temperature (Figure 1.6 c). It is speculated that acclimation of respiration to elevated temperatures has clear implications for predictions of higher plant respiration in a warmer world (King et al., 2006). To successfully predict future rates of respiration and impacts on ecosystem fluxes of CO$_2$, it is necessary to establish what determines the degree of acclimation (Atkin, 2005).
Figure 1.5. Two proposed examples of respiratory acclimation: (a) type I and (b) type II; and (c) the effects of respiration on atmospheric CO2 concentrations and global warming. Figure was reproduced from the review by Atkin et al. (2003).
1.1.8 Cellular and biochemical response

Acclimation of respiration to temperature can be defined as an up- or down-regulation of the respiratory machinery (i.e. amount of respiratory enzymes and numbers of mitochondria). It has been suggested that differences in mitochondrial respiratory capacity or the number of mitochondria are responsible for changes in the responses of respiration to temperature (Miroslavov and Kravkina, 1991; Atkin and Tjoelker, 2003). Evidence also indicates that respiratory CO₂ is also controlled by maximum enzyme activity (Atkin et al., 2002). At lower temperatures, activity of enzymes is generally reduced as they are not functioning at optimum temperatures. At moderately higher temperatures, the respiratory flux will no longer be limited by enzyme activity but most likely due to substrate and ADP concentrations (Atkin and Tjoelker, 2003).

Leaf mitochondria (Figure 1.7) are the sites of respiration and carry it out by a three step process (Taiz and Zeiger, 2002): (1) glycolysis converts sugars into organic acids in the cytosol by a series of reactions; (2) the tricarboxylic acid cycle (TCA cycle) oxidizes the organic acids and creates NADH and FADH₂ for ATP synthesis (Figure 1.8); (3) oxidative phosphorylation transfers electrons from NADH and FADH₂ (created by glycolysis, pentose phosphate pathway and TCA cycle) along an electron transport chain onto oxygen while synthesizing ATP (Figure 1.9). The TCA cycle it is often called the Citric acid cycle, the Krebs cycle (after the discoverer, Hans Krebs), or dark respiration (because it does not require light) (Taiz and Zeiger, 2002). The respiratory chain of plant mitochondria contains the standard four enzyme complexes (I-IV), plus five other proteins that are considered energy wasteful.

Succinate dehydrogenase (complex II) is an inner membrane-bound protein involved in both the respiratory electron transport chain and the TCA cycle. It couples the reduction of ubiquinone to the oxidation of succinate. This duality suggests a potentially important role in the control of energy and metabolism (Affourtit et al., 2001). Another enzyme, fumarase, located in the mitochondrial matrix, is also involved in the TCA cycle, catalyzing the conversion of fumarate into malate. This enzyme, and succinate dehydrogenase, has been considered as an excellent mitochondrial markers because they
are specific to mitochondria. These two key mitochondrial enzymes (and many others i.e. alternative oxidase and cytochrome c oxidase) can be used in measuring respiratory capacity under changing environmental conditions (Atkin et al., 2002; Noguchi et al., 2005).

Figure 1.7. The structure of plant mitochondria: (a) representative drawing of a mitochondrion and (b) an electron micrograph of mitochondria. Figure reproduced from Taiz & Zeiger, 2002.
Figure 1.8. The reactions of the TCA cycle. Figure reproduced from Taiz & Zeiger (2002).

Figure 1.9. The electron transport chain in the inner membrane of plant mitochondria. Figure reproduced from Taiz & Zeiger (2002).
1.2 Rational for Research

The aim of this study was to investigate what is regulating the response of plant respiration to thermal acclimation (acclimation of respiration in relation to temperature). This will be done by measuring dark respiration ($R_d$) at the whole plant level (leaves and roots), then at a cellular level via transmission electron microscopy looking at the size and numbers of mitochondria. Then further investigation at a biochemical level, by analysing enzyme activity, to see if mitochondrial enzymes underpin the change in respiration. It is expected that a decline in $R_d$ with warm-acclimated plants compared to those grown in the cold. Based on previous research (Larigauderie and Körner, 1995; Noguchi et al., 1996; Griffin, 2001; Atkin et al., 2002; Atkin et al., 2005; Noguchi et al., 2005) it was hypothesized that changes in dark respiration were associated with: (1) changes in mitochondrial respiratory capacity; (2) changes in mitochondria number & sizes; or (3) changes in the activity of two key mitochondrial enzymes (fumarase and succinate dehydrogenase).

In this study dark respiration, enzyme activities and leaf ultrastructure were measured from ryegrass (*Lolium perenne*) in growth chambers and two species of native grass (*Chionochloa rubra* & *C. pallens*) growing over different altitudinal ranges on Mount Hutt, Canterbury, New Zealand. Ryegrass was chosen because it is fast growing, easy to homogenise and found in many agricultural fields of New Zealand. Snow tussock grasses were chosen because they dominate the slopes of many mountainous terrains of New Zealand and the mechanisms of thermal respiratory acclimation are unclear for plants in the field. Investigating the mechanisms behind the responses of respiration to changing temperature is important for two reasons: (1) Understanding of likely plant responses to climate change; (2) calculating/modelling of carbon exchange between terrestrial ecosystems and the atmosphere.
1.3 Overview of Chapters

Chapter two
This chapter describes the experiment which set out to identify the mechanisms that underpin changes in respiratory capacity during acclimation to temperature in a controlled environment. The first step was to investigate the response of ryegrass respiration to changes in temperature. The second step was to investigate the mechanisms underpinning the differences in rates of dark respiration \( (R_d) \) between cool- and warm-grown ryegrass. Several hypotheses were tested: changes in dark respiration rate are associated: (1) a change in respiratory capacity, which could come about by either: (2) changes in mitochondria number & sizes; or (3) changes in the activity of key mitochondrial enzymes. The experimental results and future investigations are discussed and compared with previous literature.

Chapter three
Many studies have examined the influence temperature has on plants in laboratory conditions. The aim of the field-based experiments described in this chapter was to identify any differences in respiratory acclimation attributable to temperature by comparing plant species of different altitudes. The same hypotheses as described in chapter two were tested. In this study dark respiration rates \( (R_d) \), enzyme activities and leaf ultrastructure were measured from two species of native grass, *Chionochloa rubra* & *C. pallens* growing over different altitudinal ranges on Mount Hutt, New Zealand. These results are important for understanding alpine plant responses to climate change and for calculating/modelling carbon exchanges in New Zealand. The findings from the field results and future investigations are discussed.

Chapter four
The final chapter briefly discusses the results of the experimental and field chapters and puts them in perspective with results and findings from earlier investigations. The implications of the both experiments are discussed in a global context and the direction of further research is considered.
CHAPTER TWO
MECHANISMS REGULATING THE THERMAL ACCLIMATION OF DARK RESPIRATION IN PERRENIAL RYEGRASS
CHAPTER TWO
MECHANISMS REGULATING THE THERMAL
ACCLIMATION OF DARK RESPIRATION IN
PERRENIAL RYEGRASS

2.1 Introduction

This chapter examines the mechanisms that underpin changes in respiratory capacity
during acclimation to temperature in a controlled environment. Dark respiration \((R\text{d})\),
activities of two mitochondrial enzymes and leaf ultrastructure were measured from
ryegrass \((Lolium perenne)\) from a warm or cool treatment. Several hypotheses were
tested: changes in dark respiration rate are associated: (1) a change in respiratory
capacity, which could come about by either: (2) changes in mitochondria number & sizes;
or (3) changes in the activity of key mitochondrial enzymes.

Ryegrass was chosen as the control/model experimental plant as it grows fast, is in cheap
supply and easy to homogenise. Ryegrass was also selected because it is the most world
wide used grass in agriculture. Perennial ryegrass is a cool-season perennial bunchgrass
native to Europe, temperate Asia, and North Africa. It is widely distributed throughout
the world, including North and South America, Europe, New Zealand, and Australia
(Hannaway et al., 1999). Because grasslands account for a large area of the globe, a
change in climate could mean contributing significantly to the carbon cycle. Therefore,
the results of this experiment will be useful for understanding the responses of plants to a
changing climate, such as increased temperatures, in a controlled environment. These
results were then used as a foundation for the next chapter on plants from the field.
2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

*Lolium perenne* L. (perennial ryegrass) plants were grown from seed in 6 replicate trays per treatment. Trays contained vermiculite, were watered daily and fertilizers supplemented prior to sowing of seeds. The plants were kept in controlled environment cabinets under a warm (25/15°C day/night) or a cool (15/8°C) treatment. Both growth chambers were set for relative humidity of 60 & 80% (day & night), a photoperiod of 16hrs light and 8hrs dark with a light intensity of 650 μmol photons m⁻²s⁻¹. While the ryegrass was growing from seed, the growth chambers were programmed to be night time during the day for ease of measuring respiration. Only healthy leaf or root material was selected for harvesting.

2.2.2 Monitoring soil and air temperatures

Thermocouples were placed in each growth chamber to monitor the soil and air temperature that the treatment plants experienced. Two probes were used per chamber. One was situated above the plants to monitor changes in air temperature that leaves experienced. The second probe was inserted diagonally into the soil (of an approximate depth of 3-6cm) to monitor the temperature experienced by roots. The recorded temperatures were an average of three measurements recorded every 30 minutes over the course of two days and two nights. Figure 2.1 shows that Ryegrass leaves experienced the temperature set points intended. But the buffering effect of soil resulted in roots experiencing a more limited range of temperatures.
Figure 2.1. Monitoring soil and air temperatures that ryegrass experienced in the (a) cool and (b) warm treatments over two days and two nights. Each value is an average of three replicate recordings every 30 minutes.

### 2.2.3 Specific leaf area and fresh & dry mass

Fully expanded leaves were collected from well established healthy plants. Senescent or damaged leaves were avoided. Four leaves of 10 cm in length were cut and the width of each leaf was measured with digital vernier callipers. Leaf area was then calculated. Leaves were then weighed to determine their fresh weight. The samples were then oven-dried at 60°C for three days and their dry weights determined. Specific leaf area (SLA) was expressed as the ratio of leaf area to leaf dry mass (m² kg⁻¹) and can be located in Tables 2.1 & 2.2.

### 2.2.4 Nitrogen analysis

Nitrogen (N) concentrations of healthy ryegrass leaves and roots were measured. Total nitrogen content was determined from dried and ground leaf material from each replicate plant. Six to ten leaves of 10cm in length from each plant were dried overnight in a 60°C
oven then ground to powder with a ball mill. The samples were placed in small sealable vials, labelled and sent for analysis. The percentage $N$ content ($\%N$), i.e. g of nitrogen per 100 g tissue, were measured from each sample. Nitrogen content was then calculated on a mass-basis ($N_m$) and area basis ($N_a$). These values are shown in Tables 2.1 & 2.2. Table 2.1 represent $N$ content of one month old plant material that was firstly used to measure the temperature response of leaf respiration, and then harvested to measure mitochondria capacity in the oxygen electrodes. Clearly growth temperature influences $N$ content on a mass basis, especially in leaf material. In Table 2.1, $N_m$ is significantly greater in the warm leaves compared to the cool-grown leaves ($P = 0.0027$). When $N$ content is expressed on an area-basis the results are not significantly different. Table 2.2 shows the amount of $N$ measured from two month old leaf material that was grown for ultrastructural analysis and optimisation experiments. Table 2.2 results reinforce that temperature influences $N$ content in cool and warm-grown ryegrass leaf material. Warm grown leaves had a significantly greater $N$ content than cool leaves ($P_{N_m} = 0.0002$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>SLA (m² kg⁻¹)</th>
<th>$N_m$ (mmol N g⁻¹)</th>
<th>$N_a$ (mmol N m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cool</td>
<td>leaf</td>
<td>21.90 ± 1.47</td>
<td>0.83 ± 0.05</td>
<td>37.04 ± 3.3</td>
</tr>
<tr>
<td>Warm</td>
<td>leaf</td>
<td>28.56 ± 3.93</td>
<td>1.14 ± 0.07</td>
<td>43.52 ± 4.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ns</td>
<td>SIG (0.0027)</td>
<td>ns</td>
</tr>
<tr>
<td>Cool</td>
<td>root</td>
<td></td>
<td>0.74 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Warm</td>
<td>root</td>
<td></td>
<td>0.90 ± 0.09</td>
<td>ns (0.1297)</td>
</tr>
</tbody>
</table>
Table 2.2. Leaf characteristics for 2 month old cool- and warm-grown ryegrass (± SEM) grown for ultrastructural analysis and optimisation experiments. Values are based on specific leaf area (SLA), N on a mass-basis ($N_m$), N on a SLA-basis ($N_{SLA}$) and N on an area basis ($N_a$). Values represent means, ± SE, n = 6 samples per treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>SLA</th>
<th>$N_m$</th>
<th>$N_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cool</td>
<td>leaf</td>
<td>19.71 ± 2.02</td>
<td>1.08 ± 0.09</td>
<td>57.67 ± 8.09</td>
</tr>
<tr>
<td>Warm</td>
<td>leaf</td>
<td>24.98 ± 2.8</td>
<td>1.77 ± 0.09</td>
<td>73.09 ± 5.55</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>SIG (0.0002)</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

2.2.5 Ultrastructure analysis

Physiologically similar sections of leaf material were sampled from the middle of the leaf. Following the methods of Griffin et al. (2001), six small segments of leaf material from each of the six plants in both treatments were fixed in a buffered (pH 7.2) 2% glutaraldehyde fixative in the field. The samples were then vacuumed infiltrated to ensure the leaf drew in the fixative and there were no airspaces. The samples were then post-fixed in an osmium tetroxide solution (4%), dehydrated via a series of acetone gradients and finally embedded in a basic resin. Ultra thin transverse cross sections were cut in order to determine the number and sizes of mitochondria present in mesophyll cells near the upper and lower epidermal areas. Mesophyll cells were observed with a transmission electron microscope (TEM) and randomly selected in the region below the upper and lower epidermal layers of each leaf. Electron micrographs (6 per replicate plant in both treatments) were taken to assess the numbers of mitochondria and chloroplasts per cell. When looking at the digital images, care was taken not to count microbodies (that have one outer membrane and no cristae) or small plastids (matrix is denser than mitochondria). This was important so that error was avoided while counting mitochondria.

To obtain an estimate of the number of mitochondria per unit area of cytoplasm, the number of mitochondria and chloroplasts within the area enclosing the cytoplasm were
counted. Then the area of each cytoplasm was measured using image analysis software (Adobe Photoshop 7.0). This was done by measuring the pixel counts then converting the pixel area of the cytoplasm to cm\(^2\) which then was divided by the magnification of the image to get the area in \(\mu\text{m}^2\). Finally, mitochondria and chloroplasts were expressed per 100 um\(^2\) of cytoplasmic area.

The length (major axis) and width (minor axis) of mitochondria and chloroplasts were measured in microns (\(\mu\)) using the digital images in Image Pro Plus (Media Cybernetics). This was done to determine if the sizes of the mitochondria or chloroplast were similar between treatments. Every mitochondrion and chloroplast in all the mesophyll cells had their dimensions recorded.

### 2.2.6 Gas exchange measurements

The temperature response of dark respiration (\(R_d\)) at night was determined using healthy mature leaf material from 6 plants in the cool and warm treatments. \(R_d\) was measured using a portable infra-red gas analysis system (Li-Cor model 6400; Li-Cor, Lincoln, NE, USA). Four fully expanded leaves were selected and enclosed in the gas exchange cuvette. \(R_d\) at night was measured over a range of air temperatures of 5, 10, 15, 20, 25 & 30 °C. Each respiration measurement was the average of 15 values logged during 5 minutes of the leaf material enclosed in the cuvette. Prior to taking measurements, plants were given 30 minutes to acclimate to the change of each experimental temperature. The reference and sample IRGAs were matched frequently, usually before each replicate measurement at each temperature. The \(\text{CO}_2\) concentration inside the cuvette was controlled at 370 ppm. Gas exchange measurements were calculated on the basis of the area of the leaves enclosed in the cuvette, which was determined using calipers.

### 2.2.7 Liquid phase oxygen electrode

Dark respiration was measured in intact leaf and root material and crude and pure mitochondrial extracts (see section 2.2.8 below) using a liquid phase oxygen electrode
(Rank Brothers, Cambridge, UK). Six replicate plants from the cool and warm treatments were used. Optimisation experiments were conducted to determine an appropriate volume of crude or pure extract to use. 300 μL of crude and pure extracts was used after the isolation procedure in the afternoon/evening of the same day.

Oxygen consumption measurements for intact roots, crude and pure mitochondria were carried out following a combination of the methods of Noguchi et al. (2005); Vincente et al. (2005) & Werhahn et al. (2001). Four wells containing 3 mLs of reaction buffer were simultaneously used for each replicate plant. The depletion of oxygen was measured at 20°C after incubating the leaf material or extracts in buffer for 10 minutes before recording. Oxygen consumption rates were calculated assuming the concentration of oxygen in the air-saturated buffer at 20°C is 276 μmol L⁻¹ (Noguchi et al., 2005). Results using extracts are calculated on a protein basis. For leaf or root material, results were based on a dry weight basis.

The air-saturated reaction buffer (pH 7.5) for leaf and root material contained 50 mM HEPES, 10 mM MES and 0.2 mM CaCl₂. The reaction buffer for crude and pure mitochondria contained: 0.3 M sucrose, 10 mM TES-KOH (pH 7.5), 5 mM KH₂PO₄, 10 mM NaCl, 2 mM MgSO₄, and 1% (w/v) BSA. Glycine (10 mM), pyruvate (5 mM), malate (1 mM), 2-oxoglutarate (5 mM), succinate (5 mM), NADH (1 mM), NAD (0.5 mM), ATP (0.5 mM), ADP (0.1-1 mM), DTT (1.5 mM), n-propylgallate (30 μM), myxothiazol (1.5 μM) were also added.

### 2.2.8 Biochemical assays

#### 2.2.8.1 Extraction protocol

Several papers were consulted to form a modified protocol to obtain crude and pure mitochondrial extracts. Only healthy leaf and root material was harvested for extraction purposes. Dead or unhealthy leaves and roots were discarded. Mitochondria were isolated from 100g leaf material analogous to the methods of Douce et al. (1987); Focke et al. (2003); Hájek et al. (2004); Kech et al. (2005); Vincente et al. (2005) & Werhahn
et al. (2001). The ice cold medium contained: 0.4 M mannitol, 1 mM EGTA, 25 mM morpholinopropane sulfonic acid (MOPS), 5 mM dithiothreitol (DTT), 0.6 % [w/v] polyvinylpyrrolidone 40, 0.2 mM phenylmethanesulfonyl fluoride (PMSF) and 1% [w/v] bovine serum albumin, pH 7.8. All operations were preformed at 0-4 °C. Extracts were kept on ice and in the refrigerator; and when not in use, they were frozen in a -80°C freezer.

60 - 100g of leaf material was finely chopped (approx 2mm) with scissors. Half the tissue was then was placed into two 1000mL plastic beakers that contained 350mL ice cold extraction medium each (7mL per 1g tissue). Each beaker was kept on ice during homogenisation. Tissue was disrupted using an ultra-turrax with several short (30 second) bursts each time with increasing voltage. To remove larger tissue fragments the homogenate was passed through four layers of muslin/nylon net. A small amount (5mL) was saved as a crude leaf extract.

Purification of mitochondria involved differential centrifugation (a five step process). Firstly, the filtered homogenate was spun at 3,500g for 10 minutes, and then the pellet was discarded to remove cell debris. The supernatant was spun again for 10 minutes at 3,500g. Again the pellet was discarded and supernatant was spun at 6,000g for 10 minutes. The pellet was again discarded and the supernatant was finally spun at 18,000g for 30 minutes (or 13800g for 45 minutes) where the mitochondria are sedimented and the supernatant was discarded.

The crude mitochondrial pellet was resuspended in a few mLs of “re-suspension buffer” containing: 0.4 M mannitol, 1 mM EGTA, 0.2 mM PMSF, 1% [w/v] bovine serum albumin, and 10 mM KH₂PO₄, pH 7.2. It was then centrifuged again at 18,000g for 10 minutes. The suspension was discarded and the pellet was gently re-suspended in buffer.

The intact crude mitochondria was further separated from contaminants and enriched by a three-step density Percoll gradient centrifugation. The three step discontinuous Percoll gradient consisted of 2.5mL 45% Percoll, 4mL 23% Percoll, 4mL 14% Percoll and
2.5mL extract. The extract was then spun in an ultra-centrifuge at 50,000g for 75mins at 4°C in a swing bucket rotor. After centrifugation the mitochondria form a distinctive band (yellow/brown band) between the 23% and 45% layer. The isolated mitochondrial layer was further washed free from Percoll by diluting with the re-suspension buffer and centrifuged for 20 minutes. The supernatant was discarded. The pellet was diluted again and spun in the centrifuge for a second time. The purified mitochondrial pellet was then re-suspended and the extract was either used immediately or frozen at -80 °C until required.

### 2.2.8.2 Enzyme analysis

Triplicate assays were performed for each extract, with or without the substrate to establish that the change in absorbance was due to the enzyme in question. A second control (where the mitochondria were heat destroyed) acted as a negative control to ensure any rate was due to the mitochondrial enzyme. Extracts in all assays were kept on ice to minimise enzyme degradation. Assays were performed at 25°C after the cuvette had been pre-incubated for 10 minutes. The temperature was kept constant using a circulating water bath. Optimisation experiments were run in order to determine an appropriate amount of extract to add along with substrate concentrations and length of time to run the assays.

### Fumarase activity

The maximal activity of fumarase was assayed spectrophotometrically. Following a modified procedure of Noguchi et al. (1996), enzyme activity was measured using crude and purified mitochondria. For each replicate sample 10 μL of isolated mitochondria was placed in a 1 mL cuvette containing 80 mM phosphate buffer (pH 7.5), 50 mM malate and 0.05% (v/v) Triton X-100. 10 μL of crude mitochondria was added to a 1 mL cuvette containing 80 mM phosphate buffer (pH 7.5), 50 mM malate, 4 mM DTT, and 0.05% (v/v) Triton X-100. The reaction was initiated with malate and an increase in absorbance
was monitored at 240nm for 300 seconds. For calculations of activity, the extinction coefficient for fumarate of 2.25mM⁻¹ cm⁻¹ was used (Hatch, 1978; Behal and Oliver, 1997).

**Succinate dehydrogenase activity**

Succinate dehydrogenase activity was measured following a procedure incorporating the techniques described by Kennedy *et al.* (1987); Nanos *et al.* (1994) & Singer *et al.* (2002). The reaction mixture was composed of 40mM HEPES, 2mM KCN, 20mM succinate, 0.002% (w/v) DCIP, 0.033% (w/v) PMS. 50uL of extract was dotted onto the sides of a 1mL cuvette along with DCIP and PMS. The assay was initiated by inverting the parafilm covered cuvette three times. A decrease in absorbance at 600nm was monitored in the spectrophotometer for 300 seconds. Activity was calculated using the millimolar extinction coefficient for DCIP of 19.1 (Hamilton *et al.*, 2002).

**Calculations for enzyme activity**

Once each assay has been run, the absorbance values versus time were plotted and a regression analysis performed on the data. The slope of the straight line was taken to be \( V_0 \) (initial velocity) in Δ absorbance/second. The velocity was then converted to the change in concentration per minute, and then converted again to the change in number of moles/mL/minute (velocity).

### 2.2.8.3 Total protein content

Total protein content for each ryegrass sample was determined using a modification of the Bradford procedure. The Bio-Rad assay was monitored at 595nm at room temperature and calibrated with bovine serum albumin (BSA). Each sample was incubated with Bradford dye for at least six minutes before reading the absorbance. A standard curve (Figure 3) was constructed using six BSA standards of known concentrations (2, 4, 6, 8, 10, 12 μg/ml). The ryegrass extracts collected were diluted 1/1000 and 1/2000 times before addition of the dye. Finally, the unknown concentrations
of each ryegrass extract were determined using the formula derived from the standard curve: \( x = (y - 0.0374) / 0.0362 \) (where \( x \) is protein concentration (mg/mL) and \( y \) is the absorbance).

![Graph](image)

**Figure 2.2.** Standard curve used to determine protein concentration. Bovine serum albumin (BSA) was used as the standard. Values represent the averages of 6 replicates per known concentration of BSA (± SEM).
2.2.9 Statistical analyses

Statistical analyses of data were carried out using Minitab 13 for windows and R (software package version 2.3.1). Differences were considered significant using an alpha level of less than 0.05. Analyses were performed to determine if the differences between cool- or warm-grown ryegrass were significant. Differences in nitrogen content, gas exchange measurements, oxygen electrode measurements, ultrastuctural analysis and enzyme activities for cool- and warm-grown ryegrass were tested with two-tailed t-tests. Data were checked for normal distributions with homogenous variances, to meet the assumptions. There were a few exceptions (organelle counts and dimensions) where data were identified as positively skewed (non-normal) and general linear models using a Poisson distribution and “Chi” tests were carried out.
2.3 Ryegrass Results

2.3.1 Gas exchange measurements

Figure 2.3 shows the respiratory temperature response curve for intact warm-grown (15-25 °C) & cool-grown (8-15 °C) ryegrass leaves. Respiration rate was elevated in the cool-grown plants across the temperature range measured. Full acclimation (respiratory homeostasis) was achieved, as illustrated by both treatments having identical $R_4$ at their respective growth temperatures (on the graph the two arrows indicate growth temperatures of both treatments and the dotted line between the arrows shows cool- and warm-grown leaves have the same respiration rate). In leaves, acclimation was achieved via an increase in $R_{10}$ ($P = 0.03$) while there was no significant change in $Q_{10}$ (Table 2.3).

![Graph showing respiratory response to temperature]

Figure 2.3. The response of dark respiration to temperature for intact cool- and warm-grown ryegrass. The curves represent the mean of six replicate responses fitted using the equation $R = R_{10}^{(Q_{10}^\alpha ((T_x - T_0)/10))}$. Parameters for this equation are given in Table 1. Arrows indicate the growth temperature and the dotted line indicates respiratory homeostasis over the range 8-15°C.
Table 2.3. Temperature response parameters: $Q_{10}$ (proportional change in $R_a$ with a 10°C increase in temperature) and $R_{10}$ of leaf dark respiration for ryegrass grown in either a cool or warm treatment. Mean values represent the average of six replicate plants with 15 measurements each.

<table>
<thead>
<tr>
<th>Growth treatment</th>
<th>Parameter</th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm</td>
<td>$R_{10}$</td>
<td>0.53</td>
<td>0.04</td>
<td>6</td>
<td>(P = 0.03)</td>
</tr>
<tr>
<td>Cool</td>
<td>$R_{10}$</td>
<td>0.68</td>
<td>0.06</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Warm</td>
<td>$Q_{10}$</td>
<td>1.67</td>
<td>0.09</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>Cool</td>
<td>$Q_{10}$</td>
<td>1.74</td>
<td>0.08</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

### 2.3.2 Oxygen electrode measurements

Dark respiration rates were recorded using whole leaf and root material, crude leaf and root material, and crude and pure mitochondria from both leaf and root material. Cool-grown root material had significantly greater respiratory capacity than warm-grown roots (P = 0.0026) (Figure 2.4a). However, the small difference with the cool and warm leaf material were not significant (P = 0.3835).

There was a greater respiratory capacity seen in cool-grown crude leaf material that was not quite significant (Figure 2.4b; P = 0.06). The small difference between cool- and warm-grown root materials was insignificant for crude root respiration.

The respiratory capacity of the crude mitochondrial fraction resembled that of crude leaf and root materials, although the increases in activity in cool-grown leaf and root fractions were insignificant (Figure 2.4c).

The response of isolated mitochondria mirrored that of intact tissues (Figure 2.4d) but not that of crude mitochondria. Oxygen consumption of mitochondria from cool- and warm-grown leaf material did not differ. Activity was insignificantly higher in the cool-grown roots than the warm-grown root material (P = 0.0875).
Figure 2.4. Oxygen consumption rates from warm- & cool-grown leaf and root material at 20°C in the dark. Respiration rates of (a) intact tissues, (b) crude leaf and root extract, (c) crude mitochondrial fraction, and (d) isolated mitochondria. Values represent means, ± SEM, n = six plants with the assay replicated four times. ns = nonsignificant; * P<0.05; ** P<0.01 & *** P<0.001.

2.3.3 Ultrastructural analysis

2.3.3.1 Transmission electron micrographs

Transmission electron micrographs of ultra-thin sections from cool- and warm-grown ryegrass leaves can be found in Figures 2.5 and 2.6. Mesophyll cells from the cool treatment (Fig 2.5 a) had slightly larger vacuoles and smaller cytoplasmic areas than those from the warm treatment (Fig 2.5 b). Mitochondria and chloroplasts were smaller and more plentiful in the cool leaves (Fig 2.6a) compared to those grown in the warm treatment (Fig 2.6b).
Figure 2.5. Representative transmission electron micrographs (3,000 magnification) of ultra-thin sections from ryegrass leaves. (a) mesophyll cells from cool-grown leaves; (b) mesophyll cells from the warm treatment. Scale bar = 5 μm.
Figure 2.6. Representative transmission electron micrographs (20,000 magnification) of ultra-thin sections from ryegrass leaves. (a) Mitochondria sampled from the control treatment; (b) mitochondria from the warm treatment. Solid bar = 1 μm.
2.3.3.2 Mesophyll cell areas

The only differences in cytoplasm area were found between upper and lower mesophyll cells from warm-grown ryegrass (Appendix 1a; \( P = 0.035 \)). The upper and lower layers from each replicate plant in both cool and warm treatments were combined to create a larger data set. Combined upper and lower data for mesophyll area (Figure 2.7) revealed that cell sizes did not differ. Vacuole area was larger in the cool-grown mesophyll cells although the difference was not statistically significant (\( P = 0.0858 \)). Cytoplasmic area was greater in the warm-grown than the cool-grown ryegrass (\( P = 0.0349 \)). This result was taken into consideration when calculating mitochondria and chloroplast counts per 100\( \mu \text{m}^2 \) cytoplasm area.

Figure 2.7. Average mesophyll areas from cool- and warm-grown ryegrass. Combined upper and lower epidermal mesophyll cells for each warm and cool treatment. Values represent the mean of 5 replicate plants each with 6 TEM images of mesophyll cells (+ SE). \( \text{ns} = \) nonsignificant; \( \ast P < 0.05 \)
2.3.3.3 Organelle counts

For both mitochondria & chloroplast counts from the upper and lower mesophyll cells in both the warm and cool treatments were statistically equivalent (Appendix 1b) and were therefore combined (Figure 2.8). Cool-grown ryegrass plants were found to have significantly greater numbers of mitochondria (per 100 um² cytoplasm area) (P = 0.0002) and chloroplasts (P < 0.0001) than warm-grown plants.

![Bar graph showing average mitochondria and chloroplast counts per 100 um² cytoplasmic area from ryegrass grown in either a cool or warm treatment. Values represent the mean of 5 replicate plants each with 6 TEM images of mesophyll cells (± SE). *** P<0.001](image-url)
2.3.3.4 Organelle dimensions

The differences in organelle dimensions (mitochondria major axes (lengths) and minor axes (widths)) between upper and lower sections of leaves were not statistically different for either the cool or warm grown leaves (Appendix 1c). As a result, the upper and lower data were combined (Figure 2.9). Mitochondria from warm-grown ryegrass mesophyll cells were found to be significantly larger in size compared to cool-grown (major axes 11% greater, P = 0.033, minor axes 11% greater, P = 0.009).

Figure 2.9. Average mitochondria lengths (major axis) and widths (minor axis) from mesophyll cells in either cool- or warm-grown ryegrass. Averages of major and minor axis were calculated by using 134 mitochondria from the warm treatment and 69 from the cool. * P <0.05 & ** P <0.01
Again the small differences between upper and lower section chloroplast major and minor axes were regarded to be not significantly different for either cool- or warm-grown leaves (Appendix 1d). The upper and lower section data were merged together and once combined (Figure 2.10), it was established that chloroplasts were significantly larger in the warm-grown mesophyll cells than the cool-grown (major axis 10% greater, P = 0.0015; minor axis 13% greater, P = 0.007).

![Figure 2.10](image_url)

**Figure 2.10.** Average chloroplast lengths (major axis) and widths (minor axis) from mesophyll cells in either warm- or cool-grown ryegrass. Averages were calculated by using 139 chloroplasts from the warm treatment and 171 from the cool. **P < 0.01**
2.3.4 Enzyme activities

Figure 2.11a shows maximal fumarase activity in isolated mitochondria. Activity was significantly greater in cold-grown than in warm-grown roots (P = 0.001), but not different in leaves. Succinate dehydrogenase (SDH) activity was also measured from isolated mitochondria (Figure 2.11 b). Activity was significantly higher in the warm-grown roots than the cool-grown root material (P = 0.024). SDH activity did not differ between cool- and warm-grown leaf material (P = 0.328).

![Graph](image)

Figure 2.11. Maximal activities of two TCA cycle enzymes measured from isolated mitochondria of cool- (shaded bars) and warm-grown (open bars) ryegrass leaf and root material. (a) Maximal activities of fumarase measured in the presence of saturating concentrations of malate at 25°C. (b) Succinate dehydrogenase maximal activity measured in the presence of saturating concentrations of succinate at 25°C. Each column represents an average of six plants and the assay was replicated four to six times (±SE). ns = nonsignificant; * P<0.05 & *** P<0.001.


2.4 Discussion

Previous literature has demonstrated that plants have the ability to maintain the same rates of respiration (when measured at their growth temperatures) while growing at contrasting temperatures (Larigauderie and Körner, 1995; Atkin et al., 2000b; Xiong et al., 2000; Browse and Xin, 2001; Atkin and Tjoelker, 2003; Bolstad et al., 2003; Loveys et al., 2003; Kurimoto et al., 2004b; Talts et al., 2004; Atkin et al., 2005; Armstrong et al., 2006b). This phenomenon is referred to as respiratory homeostasis or full acclimation (Atkin and Tjoelker, 2003). However, little is known on the mechanisms that underpin this phenomenon.

In this study, the first step was to investigate the response of ryegrass respiration to changes in temperature. Ryegrass was grown from seed at two contrasting growth temperatures: (1) a cool treatment of 8/15°C (night/day) and (2) a warm treatment of 15/25°C (day/night). The findings reveal that respiratory homeostasis indeed occurred in ryegrass and that type II acclimation had taken place (Fig. 2.3). The second step was to investigate the mechanisms underpinning the differences in rates of dark respiration ($R_d$) between cool- and warm-grown ryegrass. Based on previous literature (Miroslavov and Kravkina, 1991; Robertson et al., 1995; Griffin, 2001; Atkin et al., 2002; Kofidis et al., 2003; Griffin et al., 2004; Kurimoto et al., 2004b; Wang et al., 2004; Atkin et al., 2005; Armstrong et al., 2006b), several hypotheses were formed. Acclimation to warmer temperatures results in: (1) a decrease in mitochondrial respiratory capacity; (2) a decrease in mitochondria numbers and sizes; (3) a decrease in mitochondrial enzyme activity.

The findings reveal that accompanying the decrease in the rates of $R_d$ of warm-grown ryegrass (Fig. 2.3) was: (1) a decline in mitochondrial respiratory capacity in leaves and roots (Fig. 2.4); (2) a decline in mitochondrial numbers (Fig. 2.8), although, perhaps surprisingly, the overall sizes (major and minor lengths) were larger (Fig. 2.9); (3) no systematic change in the mitochondrial enzyme activity in leaves in vitro; however,
fumarase activity declined in root extracts while succinate dehydrogenase activity increased (Fig. 2.11).

2.4.1 Dark respiration of cool and warm-grown ryegrass

It was hypothesized that an increase in temperature would be associated with a decrease in dark respiration ($R_d$). Gas exchange was measured on intact ryegrass leaves during the night programmed cycle in the growth chambers. Acclimation was clearly achieved in the ryegrass leaves via an increase in $R_{10}$ (Fig. 2.3 & Table 2.3). When acclimation involves a change in net CO$_2$ flux, two types of acclimation are considered to be exhibited by plant respiration (Atkin et al., 2005). Type I acclimation involves changes in enzymatic regulation, primarily in existing tissues (and is seen as a change in $Q_{10}$). Type II mechanisms involve increases in mitochondrial protein content and or mitochondrial numbers (and primarily involve a change in $R_{10}$). Based on Atkin et al. (2005) and the results in this section and the ultrastructure section below, it is clear that type II acclimation has taken place.

These findings clearly indicate very strong thermal acclimation of respiration in ryegrass leaves, displaying respiratory homeostasis over the range of 8-15 °C night temperature (i.e. a significant decrease in rate under warm temperatures). The results also indicate that temperature is one of many important factors affecting respiratory rates of plants. Results in this study are in agreement with previous literature on other species (Collier, 1996; Bolstad et al., 2003; Kurimoto et al., 2004a; Kurimoto et al., 2004b; Talts et al., 2004; Armstrong et al., 2006b; Atkin et al., 2006; Bunce, 2007). It is important to note that there are also species that show no respiratory homeostasis (Loveys et al., 2003), and some species can have lower degrees of respiratory homeostasis than others (Tjoelker et al., 1999a).

The findings in this research are important for global carbon cycle models as they can assume respiratory homeostasis for ryegrass when predicting future CO$_2$ fluxes between the atmosphere (Gifford, 2003). This is important for future predictions of terrestrial CO$_2$ emissions as the quantities of CO$_2$ released by plants displaying homeostasis have been
estimated to be half that of plants that do not show acclimation with increasing temperatures (Atkin et al., 2000a).

### 2.4.2 Respiratory capacity in extracts and isolated mitochondria

Previous literature suggests that acclimation is associated with changes in respiratory capacity which are due to an overall increase or decrease in protein investment (Atkin & Tjoelker, 2003; Armstrong et al., 2006a, b). The second hypothesis here was that the decline in dark respiration ($R_d$) exhibited at warm temperatures would be associated with a reduction of mitochondria respiratory capacity. Respiratory capacity in isolated mitochondria generally mirrored the response of intact tissue, although this was not associated with an increase in fumarase or SDH activity (Fig. 2.11). However, the differences in respiratory capacities observed were not as strong in magnitude as the respiratory rates of intact tissues measured with the gas analyser (Fig. 2.3). The findings also reveal that root tissues display a degree of acclimation, as indicated by the lower rates of $R_d$ of warm-grown roots at 20°C. The decline in respiratory capacity could be explained by the decrease in fumarase activity from isolated mitochondria. These results are comparable to those of Kurimoto et al. (2004b) who measured rates of respiration in rice and wheat plants. Respiratory rates of intact roots of plants grown at 25°C were slower than those of plants grown at 15°C Kurimoto et al. (2004b).

Surprising, there was an overall loss in respiratory capacity between crude extracts and isolated mitochondria. A likely reason for this could be due to the homogenization process being too destructive. Kech et al. (2005) report an overall increase in respiratory activity when comparing isolated mitochondria and crude extracts of leaves. It is also highly likely that substrate supply plays a part. Substrates for mitochondrial enzymes maybe more easily accessible with isolated mitochondria than in whole tissues and crude extracts. Wiskich and Dry (1985) found that isolated mitochondria typically exceeded actual rates of intact tissues. The results (although not significant) suggest that the respiratory capacity of mitochondria (from either isolated or crude fractions or from
whole tissues) decrease in association with the decline in respiratory flux in the warm-treated plants (Fig. 2.4).

### 2.4.3 Ultrastructure of the respiratory apparatus

Based on previous studies (Miroslavov and Kravkina, 1991; Kofidis et al., 2003; Armstrong et al., 2006b) the third hypothesis was that the decrease in respiratory rates in warm grown plants (compared to cool) would be accompanied by a decrease in the size and abundance of mitochondria. Both mitochondria and chloroplasts are essential components involved in the cycling of carbon between vegetation and the atmosphere (Schimel, 1995; Wang et al., 2004). Because respiration and photosynthesis are highly sensitive to temperature, it is important to understand the responses of these processes to temperature and how they are being controlled.

Mitochondrial abundance was measured per 100 μm² of cytoplasmic area to rule out any effects of cell size. Mesophyll cell areas were unaffected by temperature, however, cytoplasmic areas of warm-grown mesophyll cells were slightly larger (Fig. 2.7). The increase in area was in disagreement with the results found by Strand et al. (1999); where mesophyll cells from 23°C had an decrease in cytoplasmic volume compared to those from 5°C. Possible reasons for the difference between Strand et al. (1999) and these results could be due to the number of electron micrograph images sampled (which was not reported). Armstrong et al. (2006b) did not report changes in mesophyll areas.

The decrease in mitochondria numbers was confirmed in the electron micrographs in Figures 2.5 & 2.6. The results demonstrate that mitochondria numbers from warm-grown ryegrass leaves were indeed less abundant than counts from cool-grown leaves (Fig. 2.8). There was an approximately 40% decrease in mitochondria numbers. However, these results are in disagreement with Armstrong et al. (2006b), who found that in contrast to epidermal cells, warm-grown mesophyll cells contained more mitochondria per cell than the cold-grown counterparts. Armstrong et al. then suggest despite the fact that density was decreased in warm-grown epidermal tissues, the apparent absence of any significant
change in mitochondria density in mesophyll tissues, indicates that changes in mitochondria density alone cannot fully account for the difference in flux observed between cold and warm leaves. The results of the present study clearly indicate that changes in mitochondrial numbers in mesophyll cells (by far the dominant cells in leaf tissue) can be associated with changes in respiratory capacity, at least in ryegrass.

This sensitivity of mitochondrial numbers to temperature is consistent with the fact that mitochondria are the organelles that supply the majority of ATP, and should thus be related to the energy demands of the cell (Taiz and Zeiger, 2002). Another likely scenario is because the respiratory enzymes in cool-grown plants are functioning at sub-optimum temperatures, so that enzyme activity is slower and will not be able to keep up with energy demands. For cool-grown plants to keep up with energy demands they need to increase the amount of enzymes present. This could be done by increasing the amounts of mitochondria that contain enzymes or up-regulation of the synthesis of certain enzymes (Atkin et al., 2005). From the results above it can be concluded that a decline in mitochondria abundance represents an important response for the decreased rates of respiration of the warm-grown leaves.

Interestingly, chloroplast numbers decreased in warm-grown mesophyll cells also. These results might provide evidence to support Strand et al. (1999), who observed decreases in photosynthetic enzymes and content in Arabidopsis leaves from 23 °C compared to those from 5°C. Using the same logic as for mitochondria, changes in protein content and enzyme activity could be correlated with changes in chloroplast numbers. An increase in chloroplast numbers and therefore enzymes would supply the increased demand for energy and carbohydrates in plants acclimating to lower temperatures. The reverse would be the case for warm-acclimated plants.

Surprisingly, both mitochondria and chloroplasts from warm-grown mesophyll cells were found to be larger than those from the cool treatment. These results are consistent with those of Armstrong et al. (2006b) who also found differences in mitochondrial volume (corresponding to size) between warm and cool grown epidermal cells from Arabidopsis
leaves. Armstrong et al. (2006b) reported that mitochondria dimensions between upper and lower layers (adaxial and abaxial) were different, whereas in this study, there was no difference between either layer (Appendix 1 c), only when the two layers were combined (Fig. 2.9). However, the results of this study were in disagreement with Griffin et al. (2001); Griffin et al. (2004) & Wang et al. (2004), where elevated CO₂ had no effect on mitochondrial dimensions. Possible reasons for this discrepancy could be due to the fact that the treatment in the Griffin and Wang studies involved CO₂ manipulation and in the present study it was temperature manipulation.

In electron micrographs, more rod shaped mitochondria than spherical could account for larger mitochondria lengths and widths in warm-grown tissues than cool (Fig. 2.9). However, there were more spherical shaped mitochondria than rods, and rod shaped mitochondria were found to be significantly fewer in the warm treatment than cool (P = 0.0028) (Appendix 1 c). Logan and Leaver (2000) were able to observe the dynamic behaviour of mitochondria in live cells with fluorescence microscopy and report that mitochondria are not all the same shape or size. Mitochondria from *Arabidopsis* cells were typically spherical, but also ranged from sausage-shaped to longer worm-like forms. They speculated that these shapes can be related to the energy status of the organelle. Mitochondria are considered to exist in one of two conformational states (Logan and Leaver, 2000), where the spherical mitochondria were suggested to be in a less energetic condensed state, while the sausage and worm-like mitochondria were in a highly energetic, orthodox state. Overall, the results suggest that mean mitochondrial and chloroplast dimensions are sensitive to temperature and also play a mechanistic role.

Future work that investigated whether the mitochondria from mesophyll cells change in number or size in real time would provide evidence on whether mitochondria numbers and sizes are altered with changes in temperature. Previous papers (Robertson et al., 1995; Gálvez et al., 1998; Logan and Leaver, 2000; Armstrong et al., 2006a; Armstrong et al., 2006b), have employed the technique of mitochondria targeted green fluorescent protein (GFP) to study the morphology and dynamics and protein contents. GFP is an excellent tool to highlight the mitochondrial number, position and shape within a living
cell in real time situation without causing any damage to tissues (Logan and Leaver, 2000).

Additional experiments using transverse sections through warm- and cool-grown leaves could be employed to investigate if mesophyll numbers or sizes changed and if gross leaf anatomy changed. Kofids et al. (2003) and Armstrong et al. (2006b) showed that leaf blades were thicker in response to raising elevations or cool temperatures, respectively. Kofids et al. (2003) and Armstrong et al. (2006b) indicate that leaf thickness was due to an increase in the size of epidermal and mesoppyl cells rather than their number. Specific leaf area (SLA), which describes the allocation of leaf biomass per unit of leaf area, was much greater (although not-significantly) in warm ryegrass leaves than cool (Tables 2.1 and 2.2). Slight differences in SLA between cool and warm-grown leaves indicate that there are differences in leaf traits, suggesting warm-grown leaves are thinner.

### 2.4.4 Enzyme activity of cool and warm-grown ryegrass

As a result of past studies (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992; González-Meler et al., 1996; González-Meler et al., 1999; Kurimoto et al., 2004b), it was hypothesised that a decline in $R_d$ due to thermal acclimation would come about due to a decrease in mitochondrial enzyme activity. Two mitochondrial enzymes (fumarase and succinate dehydrogenase) were chosen as they are considered excellent mitochondrial markers (Cooper and Beevers, 1969).

The results (Fig 2.11) demonstrate that fumarase activity in isolated mitochondria from leaf material was unaffected by temperature. However, in root extracts, fumarase activity did decline in warm-grown roots compared to cool. Fumarase activity was comparable to Noguchi & Terashima (2006), when the units are converted to μmol mg$^{-1}$ protein min$^{-1}$. Succinate dehydrogenase (SDH) activity from leaf extracts was also unaffected by temperature. Warm-grown roots on the other hand, showed an unexpected increase in activity in comparison to cold-grown roots. Again when comparing the rates of SDH
activity (µmol mg\(^{-1}\) protein min\(^{-1}\)) to those reported in Nanos et al. (1994), the values are comparable. The rates of SDH activity in the present study are lower than those reported by Kennedy et al. (1987) and Singer et al. (2002). Atkin et al. (2002) reported SDH activity increased with increasing temperatures, which is in agreement with the results of this study on SDH activity in root extracts.

Possible reasons for why there are discrepancies between the SDH values reported here to those in the literature could be due to differences in methods employed and choice of plant species. Kennedy et al. (1987) used mitochondria isolated from barnyard grass seedlings, Atkin et al. (2002) used isolated mitochondria from soybeans, and Singer et al. (2002) used mitochondria from cauliflower that was subject to two 15-second sonications. A potential reason for why there were differences between leaves and roots may have been because the homogenisation process is less destructive to root mitochondria than those from leaves. This is because mechanical disruption of plant cells for the isolation of mitochondria can lead to the rupture of the central vacuole which can release highly acidic contents, also phenols, hydrolytic enzymes, tannins, alkaloids and terpenes. These substances released into the grinding buffers can interact and harm organelles, such as mitochondrial membranes (Neuberger, 1985). Strong and prolonged grinding (especially with leaves than roots due to tough cellulosics present) can also mechanically injure mitochondria, resulting in a population of intact and damaged mitochondria (ruptured mitochondria that have resulted in the loss of matrix contents) (Neuberger, 1985). Analysing the intactness of mitochondria present in root and leaf extracts might shed light on whether extraction protocol differentially affects roots and leaves. With the use of an oxygen electrode, high rates of oxygen consumption, a high respiratory control ratio (>3) and ADP:0 ratios close to theoretical values, will indicate if mitochondria are in good shape (Hatch, 1978; Neuberger, 1985; Douce et al., 1987; Hájek et al., 2004). Despite this, any potential loss of mitochondria function should remain constant between treatments (cool and warm), and thus not affect relative changes in mitochondria respiratory capacity. The reason for why SDH activities were contrasting to those of fumarase could be due to the expression of the two enzymes being differentially regulated. Future experiments using imunoblotting techniques to
investigate whether SDH or fumarase is expressed more or less in roots than leaves could rule out this possibility.

Fumarase activity was much greater than SDH activity overall in isolated mitochondria. The difference was very large; fumarase activity was approximately 30 times greater than SDH. A potential reason for the discrepancy between fumarase and SDH could be due to the differences in methods employed. Triton x-100 was used to disrupt the mitochondria when testing fumarase activity and a freeze/thaw method was adopted for SDH. Because fumarase had higher rates, it seems triton x-100 is an excellent disruptor of mitochondrial membranes to ensure the enzyme in question can react with its substrate. However, several papers have also reported differing levels of enzyme activity. Nanos et al. (1994) examined several mitochondrial enzymes (including SDH) and also had large differences in enzyme activities. Cooper and Beever (1969) analysed 5 enzymes (including fumarase and SDH) from caster bean endosperm, in which the enzymes had differing rates. Fumarase was five times more active than succinate dehydrogenase and 40 times greater than the other three enzymes. Noguchi & Terashima (2006) and Noguchi et al. (1996) also compared the responses of several mitochondrial enzymes from spinach leaves of differing nitrogen treatments and growth irradiances. There were differences among the respiratory enzyme activities (by up to a factor of 10).

It is somewhat surprising that fumarase and SDH had different activities, considering both enzymes belong to the citric acid cycle and the product from SDH is the substrate for fumarase. SDH is a membrane bound protein, unlike fumarase, and plays a part in the electron transport chain. There may have been a need for the energy producing pathway to be upregulated, but in this case fumarase activity would be expected to increase if SDH activity did. Davis & Merrett, (1974) studied the effect of light on fumarase and SDH activity in Euglena (algae) cultures. Both enzymes showed similar patterns of activity - in comparison to the light there was a rapid doubling of fumarase and SDH activity when subjected to the dark. Fumarase activity was generally twice that of succinate dehydrogenase when testing the effect of light on enzyme synthesis. Interestingly, Kennedy et al. (1987) studied the activities of SDH and cytochrome c oxidase from
aerobically and anaerobically germinated barnyard grass seedlings. Each enzyme showed differences in activity, cytochrome c oxidase increased over time, whereas SDH activity declined. These two enzymes are linked via the energy producing pathway (electron transport chain) and SDH activity would be expected to increase if cytochrome c oxidase did. This is because SDH also supplies electrons (as do several other enzymes) that cytochrome c oxidase uses to pump protons for ATP to be produced by the last complex in the chain (ATP synthase).

Further investigations assaying fumarase and SDH activity from crude mitochondria and crude extracts may be a good way of identifying if the results seen in isolated mitochondria are reproducible and follow the same trend. Numerous attempts were made in this study, but it appeared that there were interfering contaminants in the crude extracts that made it difficult for the spectrophotometer to read changes in absorbance. Bruhn et al. (2007) reports that the degree of inhibition observed due to elevated CO₂ was different between intact tissues, isolated mitochondria and electron transport chain enzymes investigated, so it is perhaps not surprising that differences were observed in this study.

Future work analysing other respiratory enzymes (such as alternative oxidase and cytochrome c oxidase from the TCA cycle) might create a more complete picture on patterns of enzyme activity that underpin thermal respiratory acclimation. Cooper and Beevers (1969) also indicate isocitrate dehydrogenase and α–ketoglutarate oxidase (2-oxoglutarate dehydrogenase) are associated with mitochondria and can be located in the TCA cycle (along with SDH and fumarase). It would be interesting to test the response of these two enzymes to see whether their activities are increased or decreased with increasing temperatures.

Additional experiments employing immunolabelling techniques could investigate whether protein levels increased or decreased in roots and leaves. Immunoblotting enzymes from isolated mitochondria are an effective way to estimate the amount of protein present. It is also an additional tool to see whether activities of these enzymes agreed with protein levels. Several reports have analysed respiratory chain proteins from
various plants and show that the proteins (such as alternative oxidase and cytochrome c oxidase) increase with decreasing temperatures (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992; González-Meler et al., 1999; Kurimoto et al., 2004b).

Collectively, the results suggest fumarase and SDH in leaves are insensitive to temperature. Enzymes other than fumarase and SDH could be involved in thermal respiratory acclimation of leaves. Noguchi et al. (1996) has compelling evidence that concentrations of carbohydrates might also determine the respiratory rate, as the maximal activities of respiratory enzymes did not explain the trends seen with respiratory rates. Future investigations involving the concentrations of carbohydrates (respiratory substrates) could be undertaken to see if these are associated with changes in respiration in response to temperature.

Interestingly, in this present study, the results indicate that the two mitochondrial enzymes isolated from roots are sensitive to temperature. This is an important finding given that root respiration is a major component of CO₂ exchange between the soil and atmosphere. Soil respiration is the second largest flux of carbon in terrestrial ecosystems, and up to two thirds of CO₂ released by the soil is from roots (Ekblad and Högb, 2001; Xu et al., 2001; Bhupinderpal-Singh et al., 2003). Belowground respiration exerts a large control on terrestrial carbon cycling (Ryan and Law, 2005). Loveys et al. (2003) results show that acclimation was greater in both leaves and roots that developed at the growth temperature than in pre-existing leaves and roots shifted from one temperature to another. The intact root respiratory capacities from the warm treatments were greater than those in the cool treatment. These rates were even greater in magnitude compared to the whole leaves. Respiratory rates of intact roots of plants grown at 15°C were faster than those of plants grown at 25°C Kurimoto et al. (2004b). Therefore it is important to understand the responses of root respiration to changes in climate for future predictions of carbon exchanges within the ecosystem.
2.5 Summary

The hypothesis was that an increase in temperature would be associated with a decrease in dark respiration ($R_d$), and this decrease would be associated with: (1) a decline in mitochondrial respiratory capacity; (2) a decline in mitochondria numbers and sizes; (3) a decline in enzyme activity and mitochondria numbers. The results indicate that: (1) ryegrass leaves showed evidence of acclimation in response to changes in temperature; (2) a decline in mitochondrial numbers and respiratory capacity is associated with thermal acclimation. (3) However, there were no consistent differences in enzyme activities in leaves of cool- and warm-grown ryegrass, indicating that fumarase and SDH may not be sensitive to temperature.

Microscopic evidence (Robertson et al., 1995; Strand et al., 1999; Griffin, 2001; Griffin et al., 2004; Wang et al., 2004; Armstrong et al., 2006b) indicates that leaf structure is modified in response to environmental changes. In particular, the decrease in mitochondrial numbers was accompanied by a reduction in respiration rates when acclimation to warm temperatures occurred. There is persuasive evidence that elevated temperatures affects plant mitochondria numbers (Armstrong et al., 2006a; Armstrong et al., 2006c). This suggests that mitochondria numbers play an important role in acclimation. Biochemical evidence (Strand et al., 1999; Ribas-Carbo et al., 2000; Kurimoto et al., 2004b; Noguchi et al., 2005; Noguchi and Terashima, 2006; Bruhn et al., 2007) indicates that changes in mitochondria capacity and enzymes play important roles in response to environmental changes also, although the results of the present study are not conclusive.

Because there is little information existing on the effects of elevated temperature on the mechanisms of the respiratory apparatus, the findings of this research are important for understanding the responses of plants to changes in the environment and estimating the respiratory $CO_2$ released into the atmosphere. Ongoing research to investigate the responses of mitochondria and chloroplast structures and the activity of other key
enzymes (e.g. cytochrome oxidase & alternative oxidase) will be valuable in increasing the knowledge of the mechanisms involved in thermal respiratory acclimation.
CHAPTER THREE
MECHANISMS REGULATING THE THERMAL ACCLIMATION OF DARK RESPIRATION IN NATIVE SNOW TUSSOCK
CHAPTER THREE
MECHANISMS REGULATING THE THERMAL ACCLIMATION OF DARK RESPIRATION IN NATIVE SNOW TUSSOCK

3.1 Introduction

Many studies have examined the influence temperature has on plants in laboratory conditions. Under field conditions the mechanisms of acclimation and the extent to which this happens is not well understood. This chapter examines how plants from the field regulate respiratory capacity in response to long-term changes in temperature. The same hypotheses as described in chapter two were tested. Dark respiration rates ($R_d$), enzyme activities and leaf ultrastructure was measured from two species of native grass, *Chionochloa rubra* & *C. pallens* growing over different altitudinal ranges on Mount Hutt, New Zealand. The aim of the field-based experiments described in this chapter was to identify any differences in respiratory acclimation attributable to temperature by comparing plant species of different altitudes. These results will be important for understanding alpine plant responses to current and predicted climate changes (IPCC, 2007) and for calculating/modelling carbon exchanges in New Zealand.

Snow tussock grasses were chosen because they dominate many mountainous terrains of New Zealand, predominating in many alpine and subalpine grassland areas (Wardle, 1991). These plants are slow growing perennial tussock grasses. There are about 22 endemic species belonging to the genus *Chionochloa* in New Zealand (Edgar and Connor, 2000). *C. rubra* are a distinctive red coloured tussock. These plants are widespread and found abundantly in the North, South and Stewart Islands (Mark, 1975). Some tussock grasses can reach up to 2m high and 1m wide in optimum growing
conditions (Dawson, 1988). They are lowland to low alpine species, usually found in poorly drained soils. On the slopes of Mount Hutt, *C. rubra* can be found occupying an altitudinal range from 450m (at the base of the mountain) to 1060m (Sullivan and Kelly, 2002). Low alpine species, *C. pallens* can be found widespread in the North and South Islands (Mark, 1975). The grasses have a pale leaf colouration and can be distinguished from other tussocks by their midribbed leaf. They can be located growing at an altitudinal range of approximately 1070m to 1620m on the slopes of Mount Hutt (McKone, 1990). These grasses are usually present on well drained soils, compared to *C. rubra*.

### 3.2 Materials and Methods

#### 3.2.1 Location of field sites

Sites at four different altitudes were chosen for the fieldwork component of this thesis. The study site is located at Mount Hutt, Canterbury, New Zealand (43°32’S, 171°33’E), on the eastern edge of the central Southern Alps, approximately 110 Km west of Christchurch (Map 3.1). Sampling was undertaken at four elevations on Mt Hutt, where two dominant species of *Chionochloa* (snow-tussock) densely cover the slopes. *Chionochloa rubra* and *C. pallens* were selected to measure response of respiratory acclimation to temperature. *C. rubra* was sampled at 450m and 1060m while *C. pallens* was sampled at 1070m to 1600m (Map 3.2).

Long-term weather stations were located at the chosen sites to provide accurate climatic readings during the study season. For ease of assessment, the data from each of the field sites has been displayed in the order of increasing altitude (and hence decreasing ambient air temperature). Therefore, *C. rubra* sampled at 450m was regarded as a warm site, and at 1060m a cool site. *C. pallens* growing at 1070m was regarded as a cool site and 1600m was considered the coldest site.
Map 3.1: Arrow indicates location of Mt. Hutt, Canterbury. MapToaster TopoNZ. Scale 1:1,000,000.
Map 3.2. Arrows indicate the location of the four field sites at Mount Hutt.

MapToaster Topo/NZ. Scale 1:50,000.
3.2.2 Experimental plants

Six replicate plants were randomly selected at each elevation for experimental purposes. Whole plants and soil surrounding the roots were removed from the field for subsequent respiratory and enzyme measurements in the laboratory. The plants were potted up and placed in four growth chambers set to the original temperatures and day & night periods which they naturally experienced during the growing season. *C. rubra* plants that were removed from 450m were kept at a minimum and maximum temperature of 8.5 and 19°C. *C. rubra* and *C. pallens* from the middle sites were kept at 6.2 and 14.2°C (minimum and maximum). *C. pallens* taken from 1600m was kept at 1.0 and 9.1°C. Sections of leaf material used for electron micrographs, SLA, nitrogen analysis and gas exchange work were taken from the middle third of each leaf. Leaves from whole plants were harvested for mitochondria isolation; dead or unhealthy material was discarded.

3.2.3 Ambient air temperatures

The weather stations located at 450m, 1000m and 1600m recorded the minimum and maximum air temperatures during the years of 2005 and 2006. A summary is shown in Figure 3.1 below. Mean monthly air temperatures were generally similar between the two years. Generally, 2006 was slightly cooler than 2005 for all sites (Appendix 2h).

The average ambient air temperature (maximum and minimum) at each site in the month preceding the sample collection was used to program the growth chambers in 2006. The average air temperatures for the growth chamber containing plants from 1000m were calculated on the 2005 meteorological data. The average minimum and maximum air temperature during November that *C. rubra* plants from 450m experience were 8.5 and 19°C (minimum and maximum). *C. rubra* and *C. pallens* from the middle site had average ambient air temperatures of 6.2 and 14.2°C. *C. pallens* plants from 1600m had an average ambient air temperature of 1.0 and 9.1°C.
Figure 3.1. Mean monthly maximum and minimum air temperatures at three altitudinal sites (450m, 1000m & 1600m) for 2005 and 2006. Values are the average of two recordings each day for each month.
3.2.4 Specific leaf area

Fully expanded leaves were collected from well established healthy plants. Senescent or damaged leaves were avoided. From each plant four leaves of 10 cm in length were cut from the middle third of each leaf blade. The width of each segment was measured using digital vernier callipers. Leaf area was then calculated. Leaves were weighed to determine their fresh weight. The samples were then oven-dried at 60°C for three days and their dry weights determined. Specific leaf area (SLA) was expressed as the ratio of leaf area to leaf dry mass (m²kg⁻¹). SLA did not differ significantly between sites for either species (Table 3.1).

3.2.5 Leaf nitrogen analysis

Nitrogen (N) concentrations of healthy leaf material of C. rubra and C. pallens were measured. Total N content was determined from dried and ground leaf material from each replicate plant. Physiologically similar leaf material was collected by cutting the leaves from the base of the plant and then dividing into thirds. The middle section of the leaf was selected for sampling. Six to ten leaves of 10cm in length from each plant were dried overnight in a 60°C oven then ground to powder with a ball mill. The samples were placed in small sealable vials, labelled and sent for analysis. The percentage N content (%N), i.e. g of N per 100 g tissue measured from each sample, was then determined. Nitrogen content calculated on a mass- and area-basis (Table 3.1) was significantly greater in leaves of C. rubra at 450m compared to leaves at 1060m (P_{Nm} = 0.003 & P_{Na} = 0.04). There was no significant difference in nitrogen content between leaves of C. pallens at 1070m and 1600m.
Table 3.1. Nitrogen content sampled from *Chionochloa* leaf material from four different elevations. Values are based on specific leaf area (SLA), N on a mass-basis \( (N_m) \) and N on an area basis \( (N_a) \). Values represent averages, ± SE, n = 8 samples per altitude.

<table>
<thead>
<tr>
<th>Altitude</th>
<th>Species</th>
<th>SLA</th>
<th>( N_m )</th>
<th>( N_a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>450m</td>
<td><em>C. rubra</em></td>
<td>6.66 ± 1.17</td>
<td>0.88 ± 0.04</td>
<td>132.42 ± 6.15</td>
</tr>
<tr>
<td>1060m</td>
<td><em>C. rubra</em></td>
<td>6.19 ± 0.75</td>
<td>0.72 ± 0.02</td>
<td>116.89 ± 3.64</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>SIG (0.0029)</td>
<td>SIG (0.0357)</td>
<td></td>
</tr>
<tr>
<td>1070m</td>
<td><em>C. pallens</em></td>
<td>12.11 ± 2.07</td>
<td>0.79 ± 0.04</td>
<td>65.40 ± 3.44</td>
</tr>
<tr>
<td>1600m</td>
<td><em>C. pallens</em></td>
<td>12.59 ± 0.96</td>
<td>0.72 ± 0.07</td>
<td>57.51 ± 5.23</td>
</tr>
</tbody>
</table>

3.2.6 *Ultrastructure analysis*

Ultrastructure methods are described in section 2.2.5.

3.2.7 *Gas exchange measurements*

Please refer to chapter 2 methods (section 2.2.6) for the protocol on gas exchange measurements. The method differed slightly for the plants collected from the field. A healthy tiller with roots was removed from each experimental plant and placed into a sealable labelled plastic bag containing moist paper. These then were placed into a separate growth chamber where the temperature was altered to monitor the response of dark respiration \( (R_{dark}) \). Respiration rates were recorded in the dark over a range of temperatures of 5, 10, 15, 20, 25°C set in both the growth chamber and cuvette. Each respiration measurement was the average of 3-5 values logged at 30 second intervals after the respiratory gas exchange had equilibrated (usually after 2-3 minutes after closing the cuvette).
3.2.8 Oxygen electrode analysis

Please refer to chapter 2 methods (section 2.2.7) for the protocol on oxygen electrode measurements. The methods were slightly different to those in the controlled experiments with ryegrass. Five replicate plants from each field site were used to measure respiratory capacity from crude and pure mitochondria extracts. Optimisation experiments were run prior to this experiment to determine if fresh or frozen samples showed any difference in respiratory capacity. There was no significant difference between fresh and frozen samples. Respiratory capacity was therefore measured using frozen samples once all replicate plants had been harvested and mitochondria isolated.

Four wells containing 2 mLs of reaction buffer were simultaneously used for each replicate plant. The air-saturated reaction buffer (pH 7.5) contained 0.3 M sucrose, 10 mM TES-KOH, 5 mM KH₂PO₄, 10 mM NaCl, 2 mM MgSO₄, and 1% (w/v) BSA. 5.5 mM succinate and 0.27 mM ATP were also included to activate succinate dehydrogenase (SDH).

3.2.9 Biochemical analysis

There was no deviation from the ryegrass methods (section 2.2.8) for the extraction protocol, enzyme assays and measurements of total protein content for C. rubra and C. pallens.

3.2.10 Statistical Analyses

Analyses were performed to determine if any differences between C. rubra plants (growing at 450m and 1060m) or C. pallens (growing at 1070m and 1600m) or between 450m and 1600m were significant. Statistical analyses were carried out using Minitab 13 for windows and R (software package version 2.3.1). Differences were considered significant using an alpha level of less than 0.05. Differences in nitrogen content, gas exchange measurements, oxygen electrode measurements, ultrastructural analysis and enzyme activities were tested with one-way ANOVAs and Tukey tests.
3.3 Tussock Results

3.3.1 Gas exchange measurements

Figure 3.2a shows the response of dark respiration ($R_d$) to temperature of *C. rubra* leaves sampled at 450m and 1060m. The cooler grown plants of *C. rubra* (1060m) showed higher rates of $R_d$ than the warmer plants (450m) over the range of measurement temperatures. Full acclimation (adjustment in the rates of respiration to compensate for a change in temperature) can be identified on the graph where the two growth temperatures have identical $R_d$ values (arrows indicate average ambient air temperatures of 4 and 6°C for the month preceding sample collection and the dotted line between the arrows shows plants at 450m and 1060m have the same respiration rate). The parameters from the fitted response describing the relationship can be found in Table 3.2 - neither the $R_{10}$ nor $Q_{10}$ values were found to be significantly different.

![Graph showing the response of dark respiration to temperature for *C. rubra* and *C. pallens*](image)

Figure 3.2. The response of dark respiration to temperature for intact *Chionochloa* leaf material. (a) *C. rubra* growing at 450m (solid line) and 1060m (broken line); (b) *C. pallens* growing at 1070m (solid line) & 1600m (broken line). The curves represent the mean of six replicate responses fitted using the equation $R = R_{10}^* (Q_{10}^* (T_{v}/T_0)/10)$. Parameters for this equation are given in Tables 3.2 and 3.3.
Figure 3.2b shows the dark respiration temperature response curve for intact \textit{C. pallens} leaves sampled at 1070m and 1600m. Following the same trend as \textit{C. rubra}, plants at the cooler site had a greater respiratory capacity than plants at the warmer 1070m site, but this tended to be more pronounced at higher temperatures. Full acclimation at the respective ambient air temperatures was not evident (see dotted line between the two temperatures of 2 and 4°C that is represented by arrows). The parameters from the fitted response describing the relationship can be found in Table 3.3 - only the $Q_{10}$ values were found to be significantly different between altitudes ($P = 0.039$).

Altitude had a significant affect on respiration, as one-way ANOVA and Tukey analysis reveals that the $R_{10}$ values between 450m and 1600m are significantly different ($P<0.001$) whereas $Q_{10}$ was not ($P= 0.394$).

\textbf{Table 3.2. Comparison of the temperature response parameters: $Q_{10}$ (proportional change in $R$ with a 10°C increase in temperature) and $R_{10}$ of leaf dark respiration for \textit{C. rubra} growing at a cool (1060m) or warm (450m) altitude.}

<table>
<thead>
<tr>
<th>Altitude</th>
<th>Parameter</th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>450m</td>
<td>$R_{10}$</td>
<td>0.07</td>
<td>0.02</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>1060m</td>
<td>$R_{10}$</td>
<td>0.09</td>
<td>0.02</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>450m</td>
<td>$Q_{10}$</td>
<td>2.37</td>
<td>0.44</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>1060m</td>
<td>$Q_{10}$</td>
<td>2.22</td>
<td>0.22</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

\textbf{Table 3.3. Comparison of the temperature response parameters: $Q_{10}$ (proportional change in $R$ with a 10°C increase in temperature) and $R_{10}$ of leaf dark respiration for \textit{C. pallens} growing at a cold (1600m) or cool (1070m) altitude.}

<table>
<thead>
<tr>
<th>Altitude</th>
<th>Parameter</th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1070m</td>
<td>$R_{10}$</td>
<td>0.28</td>
<td>0.02</td>
<td>5</td>
<td>ns</td>
</tr>
<tr>
<td>1600m</td>
<td>$R_{10}$</td>
<td>0.32</td>
<td>0.06</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>1070m</td>
<td>$Q_{10}$</td>
<td>1.94</td>
<td>0.17</td>
<td>5</td>
<td>$P=0.039$</td>
</tr>
<tr>
<td>1600m</td>
<td>$Q_{10}$</td>
<td>2.71</td>
<td>0.36</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
3.3.2 Oxygen electrode measurements

Mean rates of respiration from crude leaf extract were significantly lower in *C. rubra* growing at 450m compared to 1060m (P = 0.0024) (Figure 3.3 a). Respiration rates for *C. pallens* however, were significantly higher in the lower altitude plants (1070m) compared to plants from the higher altitude (1600m) (P < 0.0001) (Figure 3.3 b).

Respiratory capacity of crude mitochondria isolated from *C. rubra* leaf material was slightly (but not significantly) greater in high altitude grown plants (Figure 3.3 c). Respiratory capacity of crude mitochondria from *C. pallens* did not differ significantly between low and high altitude plants (Figure 3.3 d).

Oxygen consumption measured from pure mitochondria of *C. rubra* leaves mirrored that of crude leaf and crude mitochondria respiratory capacity, except there was a gain in the overall rate (Figure 3.3 e). The rates were greater in mitochondria from 1060m compared to 450m; however this difference was not statically significant (P = 0.1756). Respiratory capacity of pure mitochondria from *C. pallens* leaves (Figure 3.3 f) was significantly greater from 1600m compared to 1070m (P = 0.0186). One-way ANOVA and Tukey tests reveal that the difference in respiratory activity of isolated mitochondria between 450m and 1600m was not significant.
Figure 3.3. Rates of respiration in crude leaf material (a) & (b), crude mitochondria extract (c) & (d), and isolated mitochondria (e) & (f) from *C. rubra* and *C. pallens* leaves growing at a warm (shaded bar) or cool altitude (open bar). Values represent means, ± SEM, n = 20 (5 plants and 4 wells per altitude. ns = nonsignificant; * P<0.05, ** P<0.01 & *** P<0.001.
3.3.3 Ultrastructural analysis

3.3.3.1 Transmission electron micrographs

Transmission electron micrographs of ultra-thin sections from leaves of *C. rubra* at 450m and 1060m and *C. pallens* at 1070m & 1600m are shown in Figures 3.4, 3.5, 3.6 & 3.7. *C. rubra* mesophyll cells from 450m (warm site) had slightly smaller vacuoles and smaller cytoplasmic areas than those from the higher altitude plants at 1060m (cool site) (Figure 3.4). This was also seen in *C. pallens* mesophyll cells when comparing 1060m to 1600m (cool and cold site) (Figure 3.5). Mitochondria were smaller and less abundant at 450m in *C. rubra* compared to those at 1060m (Figure 3.6). However, for *C. pallens*, mitochondria numbers were equivalent between 1060m and 1600m (Figure 3.7). Their sizes were slightly larger at 1070m when compared to 1600m.
Figure 3.4. Representative transmission electron micrographs (50,000 magnification) of ultra-thin sections from *C. m/bi* leaves. (a) mesophyll cell from 450m and (b) mesophyll cell sampled from 160m. Arrows indicate mitochondria, solid bar = 5μm.
Figure 3.5. Representative transmission electron micrographs (5,000 magnification) of ultra-thin sections from *C. pallens* leaves. (a) mesophyll cell from 1070m and (b) mesophyll cell sampled from 1600m. Arrows indicate mitochondria, solid bar = 5μm.
Figure 3.6. Representative transmission electron micrographs (20,000 magnification) of ultra-thin sections from *C. rubra* leaf material at 450m (a) and 1000m (b). Mitochondria are represented by letter m. Solid bar = 1μm.
Figure 3.2. Representative transmission electron micrographs (20,000× magnification) of ultra-thin sections from C. pulchra leaf material at 1,070m (a), and 1,600m (b). Mitochondria are represented by the letter m. Scale bar = 1μm.
3.3.3.2 Mesophyll cell areas

Generally, there were no differences between mesophyll cell, vacuole or cytoplasm areas between upper and lower layers for _C. pallens_ and _C. rubra_ at each site (refer to Appendices 2 a & b). Based on these observations, data for the two layers were combined into one so that there was a greater number representing each replicate plant at each altitude. _C. rubra_ mesophyll cell sizes were slightly smaller in the 450m plants than the 1060m plants (P = 0.0223) (Figure 3.8 a). There was also a small (non-significant, P=0.075) difference observed in cytoplasm area and vacuole areas. For the higher altitude plants (_C. pallens_), the cytoplasmic area was significantly smaller at the cool site (1070m) than at the cold site at 1600m (P = 0.0188) (Figure 3.8 b). The differences in cell and vacuole areas between the two sites were not statistically significant. Comparisons between the lowest and highest sites reveal that mesophyll cell and cytoplasm area were significantly smaller at 450m than 1600m (P_{mesophyll} = 0.0161 & P_{cytoplasm} = 0.0085), vacuole sizes did not differ significantly (P = 0.9490).

![Figure 3.8. Average areas of mesophyll cells from _C. rubra_ (a) and _C. pallens_ (b) leaf material growing in a warm (shaded bars) or cool altitude (open bars). Values represent means, ± SE, n = 40 TEM images per altitude. ns = nonsignificant & * P<0.05.](image-url)
3.3.3.3 Organelle counts

Measurement of organelle number revealed that the upper and lower epidermal layers were found to be equivalent (with the exception of mitochondria from *C. pallens* at 1070m and 1600m) (Appendices 2 c & d). Based on these observations, both layers were merged to form a larger data set.

Mitochondria counts from *C. rubra* were lower at the warmer 450m site than at 1060m (Figure 3.9 a). This increase in mitochondria numbers with increasing altitude was highly significant (*P* = 0.0086). Chloroplasts counts were not significantly different between the two altitudes (*P* = 0.2228). There was no difference between the 1070m and the 1600m sites in mitochondrial count for *C. pallens* leaf tissues (Figure 3.9 b).

Chloroplast counts were found to be significantly greater at the warmer site (1070m) compared to the 1600m site (*P* = 0.0262). Comparisons of mitochondria and chloroplast counts reveal that there were no significant difference in abundances between 450m and 1600m (*P*<sub>mitochondria</sub> = 0.4270 & *P*<sub>chloroplast</sub> = 0.5680).

![Figure 3.9](image_url)

*Figure 3.9. Number of mitochondria and chloroplasts sampled from mesophyll cells of *C. rubra* (a) and *C. pallens* (b) leaf material growing at a warm (shaded bars) or cool altitude (open bar). Values represent means, ± SE, *n* = 40 TEM images per altitude. ns = nonsignificant; * P<0.05 & ** P<0.01.*
3.3.3.4 Organelle dimensions

Because mitochondria data were generally not significantly different between the upper and lower layers at each altitude (Appendix 2 e & f), the data were combined.

![Graph showing mitochondria dimensions](image)

**Figure 3.10.** Major (length) and minor (width) axes of mitochondria sampled from mesophyll cells of *C. rubra* (a) and *C. pallens* (b) leaf material growing at a warm (shaded bars) or cool altitude (open bars). Values represent means, ± SE, n = 40 TEM images per altitude. ns = nonsignificant & *** P<0.001.

Mitochondria from *C. rubra* at 450m were found to have smaller major and minor axes (lengths and widths) compared to the cooler altitude at 1060m (Figure 3.10 a). These differences were statistically significant (major axes 12% smaller, P = 0.0007 & minor axes 11% smaller, P = 0.0001). Mitochondria dimensions were found to be different in *C. pallens* leaf material from 1070m and 1600m (Figure 3.10 b). The major axes from the lower altitude (1070m) were found to be significantly larger in comparison to the higher altitude (major axes 12% larger, P = 0.001). There was no difference identified in the minor axes between the 1070m and 1600m. Comparisons of mitochondria major and minor axes between 450m and 1600m reveal that mitochondria were significantly smaller at 450m than at 1600m (P<0.0001 & P<0.0028).
Interestingly, *C. pallens* mitochondrial sizes were generally much larger than those of *C. rubra*. When comparing major axes at the lower sites of both species, *C. pallens* major axes were 34% larger and minor axes were 5% larger than mitochondria from *C. rubra*. Examining mitochondria sizes at the higher sites of both species reveals that the major axis was 5% larger and the minor axis was 2% smaller in *C. pallens*.

![Figure 3.11](image)

**Figure 3.11.** Major (length) and minor (width) axes of chloroplasts sampled from mesophyll cells of *C. rubra* (a) and *C. pallens* (b) leaf material growing at a warm (shaded bars) or cool altitude (open bars). Values represent means, ± SE, n = 40 TEM images per altitude. ns = nonsignificant & *** P<0.001.

Because chloroplast data were generally not different between the upper and lower layers at each altitude (Appendix 2 g & h), the dimension data were combined. Analysing chloroplast dimensions from *C. rubra* leaf material (Figure 3.11 a) revealed differences in major axes between warm- and cool-grown plants. Chloroplast major axes were significantly smaller from the 450m site compared to 1060m site (major axes 12% smaller, P = 0.0005). The minor axes of chloroplasts at 450m and 1060m were not significantly different. There was no difference in chloroplast major axis between 1070m and 1600m from *C. pallens* leaf material (Figure 3.11 b). However, the chloroplast minor axes from 1070m were significantly smaller than those from 1600m (minor axes 23%
smaller, P < 0.0001). Comparisons of chloroplast dimensions between 450m and 1600m reveal that were smaller than those at 1600m (P\textsubscript{major} = 0.0072 & P\textsubscript{minor} = 0.0001).

Chloroplast sizes were generally the same for both species at each site. The exceptions were *C. rubra* had 25% smaller major axes at the lower site and *C. pallens* had 22% larger minor axes at the higher site.

### 3.3.4 Enzyme activities

Fumarase activity was not significant difference between the sites of 450m and 1060m (P = 0.3691) and 1070m and 1600m (P = 0.2577) (Figure 3.12 a). However, when comparing 450m and 1600m, fumarase activity significantly increased (P = 0.0004).

Succinate dehydrogenase (SDH) activity measured from *C. rubra* leaf extracts was significantly greater at 1060m than 450m (P = 0.0001) (Figure 3.12 b). SDH activity sampled from *C. pallens* extracts were not different at either site (P = 0.2238). When comparing the 450m site to 1600m, SDH activity was not significantly different (P = 0.0789).

![Figure 3.12. Average maximal activities of (a) fumarase and (b) succinate dehydrogenase, from isolated mitochondria from leaves sampled at 450m and 1060m (*C. rubra*) and 1070m and 1600m (*C. pallens*). Values represent the means, ± SEM, n = 15. ns = non-significant, P>0.001 = ***.](image-url)
3.4 Discussion

Using the results from the present work with ryegrass as a foundation, it was hypothesised that growth in warmer temperatures will reduce $R_d$, as a consequence of: (1) a reduction in mitochondrial respiratory capacity; (2) a reduction in mitochondrial size and numbers; (3) and the reduction in the activities of two key mitochondrial enzymes. There are numerous literatures studying the effects of elevated CO$_2$ and/or temperature on respiration from plants grown in controlled environment chambers (Strand et al., 1999; Kurimoto et al., 2004a; Kurimoto et al., 2004b; Talts et al., 2004; Bunce, 2005; Armstrong et al., 2006a; Armstrong et al., 2006b). The relevance of these observations to field situations is uncertain, as light, humidity and temperature is not kept constant in the field. Recently, a number of studies concerning thermal acclimation of respiration in the field (Larigauderie and Körner, 1995; Atkin et al., 2000b; Xiong et al., 2000; Bolstad et al., 2003; Loveys et al., 2003; Bunce, 2005) provide reliable/convincing observations on how plants function in their natural environment. Predicting how rising temperatures may affect carbon balance in plants in the field is of importance for global carbon budgets and understanding plant growth in response to changing environments.

The results of this study support the hypothesis that growth at low altitudes (and relatively warm temperature) results in the reduction of $R_d$ as a consequence of: (1) temperature sensitivity of the respiratory apparatus, resulting in the reduction of the respiratory capacities of mitochondria; (2) a reduction in mitochondrial size and numbers; and as a consequence of this (3) a reduction in the activities of mitochondrial enzymes (because of a reduced total enzyme contents).

3.4.1 Dark respiration in the field

In this study dark respiration ($R_d$) was measured on field-grown plants transported to growth chambers. *Chionochloa* plants (snow tussocks) were sampled from four field sites, where *C. rubra* was found growing over the range of 450m to 1060m and *C. pallens* growing over the range of 1070m to 1600m. It was hypothesised that growth in
low altitudes (corresponding to warmer temperatures) would result in the reduction of rates of $R_d$. The hypothesis was tested by measuring gas exchange on dark adapted leaves under controlled conditions in growth chambers with the Li-Cor 6400 portable gas-exchange system.

The results in this study reveal several important points. Firstly, increasing ambient air temperature resulted in increased instantaneous rates of $R_d$, as evident from the shape of the temperature response curves illustrated in Figure 3.2. These results are consistent with Atkin et al. (2000b), Xiong et al. (2000) and Larigauderie & Körner (1995), who also report short-term increases in temperature resulting in the exponential increase in leaf respiration. The temperature sensitivity of respiration (i.e. the $Q_{10}$; the proportional change in respiration rates per 10 °C) varies among plants species (Larigauderie and Körner, 1995) and tissues (Loveys et al., 2003). The results of this present study clearly indicate that respiration is sensitive to changes in temperature.

The next interesting point was that there were very different respiration ranges for the two species (Fig. 3.2). There were larger differences between the two species, with *C. rubra* displaying much lower rates of $R_d$ than *C. pallens*, than there was at different altitudes within each species. At the species level, $R_d$ was greater (but not significantly) at cooler sites than at warmer sites). These results are consistent with Larigauderie and Krömer (1995), who also found varying degrees of respiration between species. This lack of significant difference between sites is probably due to the fact that average ambient air temperature at each site between *C. rubra* plants (at 450m and 1060m) and/or *C. pallens* plants (at 1070m and 1600m) were not contrasting enough (Section 3.3.3 Fig. 3.1). Collectively, the combined results of $R_d$ from *C. rubra* at the lower altitudes and *C. pallens* from the higher altitudes support the general prediction that an increase in temperature (at low altitudes) results in the reduction of respiration in comparison to plants from high altitudes.

The third point in this experiment was evidence for the occurrence of acclimation of dark respiration to temperature from plants of contrasting altitudes. This is an important
finding, as the extent of respiratory CO₂ release by leaves under natural conditions is also dependent on the degree of respiratory acclimation (adjustment in the rates of respiration to compensate for a change in temperature) (Atkin et al., 2000b). Respiration in leaves has been shown to acclimate to changes in temperatures, such that cold-grown plants exhibit higher rates than plants in warmer conditions (Billings and Mooney, 1968; Klikoff, 1968; Larigauderie and Körner, 1995; Arnone and Körner, 1997; Atkin et al., 2000b). Perfect acclimation, or respiratory homeostasis, results in identical rates of respiration in plants grown at contrasting temperatures when measured at their original growth temperature (Ziska and Bunce, 1994; Larigauderie and Körner, 1995; Atkin et al., 2000b; Xiong et al., 2000). Thermal acclimation results in warm-grown plants exhibiting lower rates of R at a set measuring temperature than plants grown at cooler temperatures (Atkin et al., 2000b). Larigauderie and Körner (1995) speculate that acclimation to higher temperatures would prevent exhaustion of carbohydrate reserves if respiration rates in cold-adapted plants become very high when exposed to high temperatures. Previous literature has also shown that acclimation can vary between species, some with little or no acclimation (Larigauderie and Körner, 1995; Tjoelker et al., 1999a; Loveys et al., 2003).

In this study, a tendency was observed toward acclimation (non-significantly) of $R_d$ to temperature for *C. rubra* plants between 450m and 1060m (Fig. 3.2 a). However, there was little evidence of acclimation for *C. pallens* between the two sites of 1070m and 1600m (Fig. 3.2 b). When comparisons were made at the respective ambient air temperatures, plants from the warmer site (1070m) displayed greater respiration rates than the coolest site (1600m), indicating only partial acclimation occurred. These results are consistent with previously publications where partial acclimation has also been observed (Ziska and Bunce, 1994; Larigauderie and Körner, 1995). Full acclimation did not occur possibly because the ambient air temperatures were not contrasting enough between the high and low sites of both species (differences of approximately 2 and 5°C) (Section 3.3.3 Fig. 3.1). Another possibility is suggested by Xiong et al. (2000), in which species native to habitats with large temperature variations during their growing seasons
generally display a strong ability to acclimate photosynthetically, whereas species from relatively stable regimes tend to possess a poor ability for acclimation. Interestingly, the findings of Larigaurderie and Körner (1995) indicate that alpine plants, on average, did not acclimate to temperature differently from lowland plants. They found that origin had no effect on the absolute rates of respiration of plants when grown and measured at 10°C. However, origin did have an effect on respiration of plants grown and measured at 20°C, where alpine plants displayed greater respiration rates than the lowland species. Larigaurderie and Körner, (1995) suggest that acclimation depends upon the genus more than the origin, so that predictions at the community level cannot be made based on a single species because of the variety observed in the respiration responses. However, the results in this present study indicate that there are differences between species from the same genus (Chionochloa), where the alpine plants (C. pallens) had only partial acclimation, and lowland plants (C. rubra) showed stronger acclimation. Conducting future experiments using plants from different genera from the same sites as Chionochloa may also reveal whether there are differences among genera more so than due to origin.

Larigaurderie and Körner, (1995) also suggest that plants native to cool and warm habitats display similar dark respiration rates in their respective environments, which is due to a combination of adaptation and acclimation. They indicate that adaptation can be studied by growing species originating from thermally contrasting areas under similar thermal regimes. Under these circumstances the short-term temperature response curve of the cold-adapted plant should be above that of the warm-adapted plant. The difference is due to genetic differences and reflects adaptation to prevailing habitat temperature. The acclimation component can be studied within a plant species by collecting plants from the same site and growing them at various temperatures, typically 20°C and 10°C. Acclimation is illustrated by a downward shift of their short-term temperature response curve (Larigaurderie and Körner, 1995; Loveys et al., 2003). Usually warm-grown plants have greater respiratory rates than cold-grown (partial acclimation) or equal respiration rates to cold-grown plants (full acclimation). The results of the present study reflect the extent of adaptation (which might also be considered as analogous to long-
term acclimation) of high altitude plants to differences in growth temperature. Future experiments may reveal the extent of true acclimation by analysing C. rubra and C. pallens plants that have been taken from similar altitudes and exposed to variable thermal regimes.

In the investigation by Loveys et al. (2003), $Q_{10}$ values and the degree of respiratory acclimation among leaves and roots of several contrasting plant species (herbs, grasses, shrubs and trees) was assessed. They conducted two experiments: (1) The short-term (ST acclimation) experiment assessed the extent to which acclimation occurs in warm-grown plants shifted to low-growth temperature; (2) In contrast, the long-term (LT acclimation) experiment assessed the degree of acclimation in leaves developed under contrasting temperature. They concluded acclimation was greater in both leaves and roots that developed at the growth temperature (LT acclimation) than in pre-existing leaves and roots shifted from one temperature to another (ST acclimation). Studies with Arabidopsis have also shown that new leaves are required for photosynthetic acclimation to occur (Strand et al., 1997). However, Loveys et al. (2003) compared 16 species that were fast and slow growing, and found no difference in the degree of respiratory homeostasis. A study on photosynthetic and respiratory acclimation has also shown that acclimation to a new growth temperature can occur in pre-existing leaves, formed at the previous growth temperature (Atkin et al., 2000b). The results of this study are consistent with previous work (Ziska and Bunce, 1994; Larigauderie and Körner, 1995; Atkin et al., 2000b; Loveys et al., 2003) - in this case partial long–term acclimation occurred in the long-lived tussock species.

Collectively, the results in this study support the findings of Larigauderie and Korner, (1995), Tjoelker et al. (1999a) and Loveys et al. (2003), who found varying degrees of long-term respiratory acclimation between alpine and lowland plants. In the present study, only the broad differences in respiration rates between the low altitude (450m) and the high altitude plants (1600m) were significant, exhibiting greater temperature sensitivity and higher rates than within-species comparisons. Atkin et al. (2000b) concluded that cold-acclimation of dark respiration in snow gum leaves is associated with
changes in both the temperature sensitivity and the capacity of the respiratory apparatus. The results from this study reveal that acclimation to changing temperature results in changes in apparent respiratory capacity, and the sensitivity of respiration to short-term changes in temperature (i.e. a decrease in $Q_{10}$). The different $Q_{10}$ values between the two C. rubra sites and between 450m and 1600m (although not significant) seem to provide some support for this notion.

### 3.4.2 Respiratory capacity

Based on previous studies, long-term acclimation likely involves an increase in respiratory capacity (Klikoff, 1968; Atkin et al., 2000b; Atkin and Tjoelker, 2003; Armstrong et al., 2006c). The next step in this study was to investigate the hypothesis that an increase in temperature (at low altitude sites) results in a decrease in respiratory capacity of mitochondria.

The differences in respiratory capacities observed for crude and pure mitochondria extracts using the oxygen electrode (Fig. 3.3) were not as great in magnitude as the respiratory rates of intact tissues measured with the gas analyser (Fig. 3.2). However, the respiratory capacities did generally show the same trend as $R_d$ values, were warm-altitude plants of C. rubra (growing at 450m) had lower respiratory capacities than the cool-altitude plants (at 1060m). Likewise for C. pallens plants, where cool-altitude plants (at 1070m) had lower respiratory capacities compared to the coldest-altitude plants (excluding results from crude leaf extracts). Surprisingly, the respiratory capacities from C. pallens extracts were not greater than C. rubra respiratory capacities, although $R_d$ values were. In any case, overall these results suggest that an increase in temperature results in the decrease in $R_d$, and associated with this decrease is a reduction in mitochondrial respiratory capacity.

In Figure 3.3b the oxygen consumption result for C. pallens (at 1600m) is unusually much lower than the values for extracts from 1070m. This result was the opposite trend to leaf gas exchange by a considerable margin and was not expected. However, the pure mitochondria response better reflects the overall response and these results shall be concentrated on in the following discussion. A possible reason for such a large
discrepancy between the crude extract and isolated mitochondria could be due to some
component in the crude extract interfering with the assay. It is worthy to note that the
overall gain in respiratory rate for pure mitochondria over crude extract is comparable to
Keech et al. (2005). They report an overall increase in respiratory activity when
comparing isolated mitochondria to crude extracts of leaves.

The respiratory capacity results from isolated mitochondria for both species (C. rubra at
450m and 1060m & C. pallens at 1070m and 1600m) are consistent with the hypothesis
that plants from warm sites (low altitudes) have lower respiratory capacity in comparison
to plants from cool sites. It is interesting to note that an overall comparison of the two
species shows that respiratory capacity of isolated mitochondria in greater in C. rubra
than in C. pallens, although the reverse was true in $R_d$. This is more likely to reflect
differences in the effectiveness of extraction or the carryover effects of interfering
cellular components than systematic differences in actual capacity. The results are
comparable to Loveys et al. (2003) and Kurimoto et al. (2004b), who found that the
intact root respiratory capacities from the warm treatments were smaller than those in the
cool treatment. These rates were even greater in magnitude compared to the whole
leaves.

### 3.4.3 Ultrastructure

Based on previous literature, it was hypothesised that growth at lower elevations
(corresponding to warming) would result in a decrease in mitochondria numbers
(Miroslavov and Kravkina, 1991; Larigauderie and Körner, 1995). This was
investigated by use of transmission electron microscopy to measure mitochondrial
abundance and dimensions from mesophyll cells of leaf material (Figures 3.4 – 3.7).

Firstly, mesophyll cell areas were measured to see if there were any differences in cell
area with altitude (Fig. 3.8). When comparing the lower and higher sites of both species
(C. rubra and C. pallens), mesophyll cell areas were smaller at the lower sites (although
not significantly for C. pallens). Vacuole areas were not significantly different between
the higher and lower sites. However, cytoplasmic area was smaller in the lower sites of each species (although not significantly for *C. rubra*). When comparing cell areas between species, *C. pallens* plants had slightly larger mesophyll, vacuole and cytoplasmic areas than *C. rubra* mesophyll cells (Because of this, mitochondrial abundance was measured per 100 μm² of cytoplasmic area to rule out any effects of cell size). These cell size results were in disagreement with Kofidis *et al.* (2003), who cut leaf cross sections of *Origanum vulgare* (oregano) plants and observed thinner leaf blades due to a decrease in the sizes of mesophyll cells (not the number) in lowland plants. In the present study, specific leaf area (SLA) was also measured, which describes the allocation of leaf biomass per unit of leaf area (Table 3.1). SLA was not-significantly different between the two altitudes of each species. However, when SLA is compared between both species, *C. rubra* clearly has smaller SLA than *C. pallens*, indicating there are differences in leaf traits between species and that *C. rubra* leaves are thicker. These results are consistent with those reported by Mark (1975) who reports *Chionochloa rigida* (from low altitudes) had thicker leaf blades than the mid and high altitude plants (*C. macra* and *C. oreophila*).

The study by Mark (1975) also reports that differences in leaf respiration rates between the three species reflects the different proportions of metabolically active tissue present. Mesophyll cells in leaves of *C. rigida* (low altitudes) occupied about 50% of the cross section. *C. rigida* from two altitudes (910m, 1220m) had the lowest dark respiration rates compared to the two higher altitude plants (*C. macra* at 1590m and *C. oreophila* at 1650m). Mesophyll cells occupied 58% of the leaf cross section in *C. macra* and 62% in *C. oreophila* (which had the highest rates of respiration and *Q*₁₀ values among the three species at all the temperatures). Future observations of leaf cross sections sampled from the field in the present study may reveal whether mesophyll composition of leaf blades also varies with altitude in *C. rubra* and *C. pallens*.

Interestingly, each species (*C. rubra* and *C. pallens*) had different trends with respect to mitochondria and chloroplast counts and their dimensions, indicating that there is variability in responses among related species. When comparing mitochondria counts
(Fig. 3.9), *C. rubra* had more abundant mitochondria at the high site in comparison to the low site (44% increase). However, there was no difference in numbers for *C. pallens* between the two altitudes. Overall, when comparing mitochondria numbers from the lowest site (450m) to the highest site (1600m) there was a small (non significant) increase in mitochondrial numbers. The results indicate that mitochondria may be less abundant in mesophyll cells from warmer sites (low altitudes), although the response is species specific. These results are consistent with Miroslavov and Kravkina (1991), who found that mitochondria per unit leaf area decreased with decreasing altitude. For example, *Poa pratensis* possessed fewer mitochondria than *Poa alpina*.

The next hypothesis was that growth at lower altitudes would result in a decrease in mitochondrial size (Fig. 3.10). This holds true for *C. rubra*; however, *C. pallens* at the highest elevation (1600m) did not follow this trend and displayed a 10% decline in length. Further investigations on the shape of mitochondria revealed that there were more rod shaped mitochondria at 1070m than 1600m, which could explain why mitochondria were larger at 1070m than at 1600m. Logan and Leaver (2000) observed the dynamic behaviour of mitochondria in live cells with fluorescence microscopy and report that mitochondria are not all the same shape or size. They suggest that mitochondria are considered to exist in one of two conformational states, where spherical mitochondria are in a less energetic condensed state, while the sausage and worm-like mitochondria are in a highly energetic, orthodox state. Interestingly, *C. pallens* mitochondria sizes were generally much larger than that of *C. rubra*. When comparing dimensions at the lower sites of both species, *C. pallens* major axes were 34% larger and minor axes were 5% larger than mitochondria from *C. rubra*. Overall, when comparing mitochondrial sizes between the highest site (1600m) and the lowest site (450m) there is a general decrease in the mitochondrial length and width (20% and 10% respectively). These results are in agreement with Miroslavov and Kravkina (1991), in which mitochondria were less abundant and slightly smaller in the lower altitudes.

Chloroplast counts and dimensions were also analysed to see if these followed a similar trend as mitochondria. Chloroplast numbers measured from both species showed a
similar pattern (Fig. 3.9). Chloroplast counts from *C. rubra* mesophyll cells at both sites were very similar; there was a slight (non significant) decrease in counts from the lower site. However, chloroplasts were more abundant from *C. pallens* at the higher site (20% increase). Comparison of chloroplast counts between 1600m and 450m indicates that chloroplast abundance does not differ with decreasing altitude. These results are in disagreement with Miroslavov and Kravkina (1991) who found that chloroplasts were less abundant at low altitudes.

Chloroplast dimensions were also investigated (Fig. 3.11). The average chloroplast major axis (length) in *C. rubra* was smaller (11%) at 450m but the minor axis (width) did not differ. Chloroplast lengths were similar between the two elevations of *C. pallens* plants but there was a 33% decrease in widths at the lower (1070m) site. The overall comparison of chloroplast dimensions between the lowest (450m) and highest (1600m) sites revealed that the lengths and widths decreased with decreasing altitude (32% and 22% respectively). These results are consistent with Kofidis *et al.* (2003), who observed a decrease in the size of chloroplasts with decreasing elevations. They report that this was a result of starch grains being absent in the lower sites. Seasonal differences in chloroplast dimensions were also observed. In spring (growing season) the dimensions increased compared to summer and autumn. They suggested that from previous studies (Miroslavov and Kravkina, 1991) the low starch contents and small chloroplasts at high altitudes were a result of the high respiration rates as judged by the numerous mitochondria occurring in the cells.

Collectively, the results of this study indicate that in field plants, a decrease in mitochondrial abundance and size is likely responsible for the lower respiration rates and mitochondria respiratory capacity observed in response to growth at higher temperatures. Miroslavov and Kravkina (1991) suggest that tissues of lower altitude plants have fewer mitochondria and this will correspond to a reduced total content of respiratory enzymes. This is because higher altitude plants experience harsher environments and therefore will have a higher energy demand for maintenance and repair.
3.4.4 Enzyme activity

This experiment investigated whether respiratory capacity and dark respiration \( R_d \) was associated with an increase in fumarase and/or succinate dehydrogenase activity. Based on past experiments (Vanlerbergh and McIntosh, 1992; González-Meler et al., 1999; Ribas-Carbo et al., 2000; Atkin et al., 2002; Kurimoto et al., 2004b), it was hypothesized that a decline in \( R_d \) and respiratory capacity would come about due to a decrease in mitochondrial enzyme activity.

The activities of two key mitochondrial enzymes were measured with saturating substrates at 25°C (Fig. 3.12). Interestingly, there was a significant species difference for fumarase activity, with greater activities in high-altitude *C. pallasii* than in low-altitude *C. rubra*. However, when comparing fumarase activity between the sites of each species, there were no significant differences. Previous work on maximal activities of fumarase by Noguchi *et al.* (1996) reveal that different growth rates did not affect fumarase activity between a sun (*Spinacia oleracea*) and shade (*Alocasia macrorrhiza*) species. However, activities of other TCA cycle enzymes did differ between the two species with decreasing growth irradiance. In *S. oleracea*, enolase and pyruvate kinase increased. However, with the shade species *A. macrorrhiza*, enolase increased and pyruvate kinase decreased. They indicate is that respiratory rates of sun plants might be determined by concentration of substrates and those of shade species determined by the demand for ATP.

Kurimoto *et al.* (2004b) found that respiratory rates of intact roots of rice and wheat cultivars (with different degrees of respiratory homeostasis) grown at 15°C were greater than those plants grown at 25°C. The maximal COX activity (cytochrome c-dependent oxygen consumption in detergent solubilised mitochondria) was consistently higher in mitochondria isolated from roots grown at the lower temperature than those at the higher temperature. Noguchi *et al.* (2005b) measured fumarase activity from leaves of *Spinacia oleracea* and *Alocasia odora*. Fumarase activity was greater in *S. oleracea* plants than in *A. odora*. Fumarase activity was also greater in plants grown in the high light conditions
than those from low lights. Noguchi also was able to measure respiratory capacity from isolated mitochondria from leaves of these plants. For *S. oleracea*, the capacities measured from high light grown plants were greater than those from low light conditions, (which was related to increased enzyme activities in high light conditions). The increase in capacity in *A. odora* was associated with an increase in succinate oxygen uptake and not fumarase activity. Noguchi suggests that an increase in respiratory capacity is achieved by a general increase in mitochondrial content rather than by an increase in specific activity of electron transport components.

Succinate dehydrogenase (SDH) activity measured from isolated mitochondria from *C. rubra* leaf extracts was greatly reduced at 450m compared to 1060m. There was a similar trend for *C. pallens* at 1070m and 1600m, although this difference was not significant. Surprisingly, SDH activity was not different between the 450m and 1600m site. The between-species comparisons of SDH are comparable to Atkin *et al.* (2002), who reported that SDH activity in soybean mitochondria was high at 25°C and decreased with decreasing temperature. It was expected that plants would display an overall decrease in SDH activity much like that of fumerase between the two *Chionochloa* species. Noguchi *et al.* (2005) reported a decrease in oxygen uptake rates from isolated mitochondria between the high and low light treatments of *S. oleracea*, however, for *A. odora* oxygen consumptions increased. These results indicate species differences in enzyme rates, which were associated with differences seen in capacities of the cytochrome pathway, cytochrome c oxidase and alternative oxidase from isolated mitochondria. Additional experiments with the crude leaf and crude mitochondrial extracts could provide clues to whether SDH activity follows the same trend as the isolated mitochondria or has similar rates to those of fumarase. Further investigations assaying other respiratory enzymes (such as alternative oxidase and cytochrome c oxidase also belonging to the TCA cycle) might create a more complete picture on patterns of enzyme activity that underpin long-term thermal respiratory acclimation in the field.

Low nutrients could be a likely explanation for low enzyme activities. Table 3.1 reveals that plants at the 450m site had significantly greater amounts of nitrogen on a mass and
area basis compared to 1060m. There was no difference between the two sites were *C. pallens* is found. It was not surprising that the 450m site has higher N content in the leaves as this is a farm site. Surprisingly, enzyme activities were lower at 450m even though the plants had higher nutrient levels available. Therefore, nitrogen content is not a likely factor affecting differences in the enzyme activities seen between each species and along the altitudinal gradient. A slight reduction in the nitrogen levels of leaves at 1600m compared to 1070m (not significant) is unlikely to account for why both fumarase and SDH at 1600m did not have the expected greater rate compared to 1070m. These results are supported by Atkinson *et al.* (2007) who found that the temperature response of root respiration is insensitive to tissue nitrogen concentrations in several herbaceous species grown in solution culture.

Respiration is often stimulated by the addition of substrates such as glucose and sucrose. Atkin *et al.* (2000b) were able to show under controlled-environmental conditions, acclimation of snow gum (*Eucalyptus pauciflora*) leaves to low temperature was associated with the increase in the concentration of soluble carbohydrates. However, under controlled conditions, cold-grown and cold-treated plants exhibited greater rates of respiration and associated with these plants were higher concentrations of sugars than their warm-grown counterparts. Atkin’s research shows that there was no correlation between respiratory acclimation in the field to changes in sugars, as these plants had very high levels of sugar compared to those in the controlled experiments. They indicate that factors other than sugar must be responsible for acclimation of leaf respiration at the field site. A study by Bannister and Ward (1981) measured changes in carbohydrate levels of tussocks over winter and reveal that the high altitude plants (*C. macra*) had greater carbohydrate levels in the leaves compared to those of mid and low altitudes (*C. rigida*). They suggest that the high levels of carbohydrates in the leaves of *C. macra* are necessary for protection of its tissues to winter damage and are likely to be associated with the high rates of respiration reported by Mark (1975). Atkin *et al.* (2000a) also suggest that respiratory flux for any given temperature is not determined simply by potential enzyme activities, but rather a combination of the availability of substrate supply, the demand for respiratory products (i.e. ATP) and/or the maximal capacity of respiratory enzymes.
Covey-Crump et al. (2002) investigated the effects of short- and long-term changes in temperature on the regulation of root respiration of two Plantago species. The results suggest that adenylates and/or substrate supply exert the greatest control over respiration at moderate temperatures (15-30°C) and at low temperatures (5-15°C) glucose had no effect on respiration, suggesting that respiration was limited by enzyme capacity alone. Future investigations of sugar concentrations in the leaves of the plants involved in this research could be undertaken to determine if sugar levels are associated with respiratory acclimation.

Interestingly, Strand et al. (1999) observed changes in cytoplasmic and vacuolar volume underpinned the 2- to 3- fold increases in protein and enzyme activity of cold-acclimated leaves. They measured protein content from Arabidopsis leaves in 5°C and 23°C and found that the protein content was lesser in the warmer leaves, which was responsible for the decrease in enzyme activity. To understand the mechanisms underlying acclimation to a warm temperature, they also investigated how the decrease in protein was accommodated in the leaf cells. The results reveal that a decrease in the volume of cytoplasm may provide an important mechanism for the decrease in enzymes and metabolites of warm-acclimated leaves. The results from Strand et al. (1999) are in agreement with the results of this present study, where there was a decrease in the cytoplasmic volume of both species from the lower sites. However, the mechanism is more likely that the abundance or dimensions of organelles containing the respiratory enzymes is responsible for a reduction in enzyme activity (Atkin et al., 2000a).

3.5 Summary

The altitudinal gradient is associated with changes in many environmental factors, such as air temperature, water precipitation, wind exposure, light intensity, UV-B radiation, soil fertility, ozone density, oxidizing air pollutants and partial CO₂ pressures (Kofidis et al., 2003). Plants are also subjected to environmental changes due to seasonal changes
during the growing period, such as photoperiod, air temperature and water availability. At increased altitudes, plants are exposed to lower mean temperatures and higher light intensities, so they must develop mechanisms to prevent damage caused by chilling, freezing and photodestruction (Kofidis et al., 2003). In this study, plants were selected along an altitudinal gradient that corresponds to a gradient of mean ambient air temperatures (average minimum air temperature over the period of November 2006 ranged from 1°C at 1600m, 6°C at 1000m and 8.5°C at 450m - see section 3.2.3 Figure 3.1).

In this research, enzymes activities are interpreted in the context of the responses of mitochondrial numbers and sizes, mitochondrial O₂ consumption and leaf gas exchange along the altitudinal gradient. At the species level, it was concluded for C. rubra growing at low altitudes, the decrease in enzyme activities (in particular succinate dehydrogenase) is associated with a decrease in respiratory capacity and \( R_d \) in response to warmer growing temperatures. The decrease in enzyme activities was associated with a reduction in mitochondrial abundance and sizes. Mitochondrial number, size and enzyme activities in C. pallens on the other hand did not respond over the range of altitude sampled (1070-1600 m). These results suggest that for these higher altitude plants (or at least in this species), enzyme activities may not play a major role as a mechanism controlling the differences in respiratory capacity and leaf gas exchange. The large differences in the respiratory flux between 1070m and 1600m is more likely to be determined by a combination of the availability of substrate supply, the demand for respiratory products (i.e. ATP) more so than the enzyme activities.

Comparing enzyme activities between the species (i.e. from the 450m site to the 1600m site reveals that fumarase activity may be involved in the differences in respiratory capacity and \( R_d \) between the two sites. There was a large decrease in fumarase activity (and a slight but not quite significant (\( P = 0.0789 \)) decrease in SDH activity) between the two sites (1600m and 450m). The large difference between the two sites for fumarase activity is comparable to the large difference in \( R_d \) (Figure 3.2) and reduction in mitochondrial abundance and dimensions seen between the two sites (Figs 3.9 & 3.10).
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This supports the overall hypothesis that for cool-grown plants to keep up with energy demands they need to increase the amount of enzymes present. This may be achieved by increasing the abundance or size of mitochondria which will possess a greater content of enzymes (Taiz and Zeiger, 2002). The reverse would be the case for warm-acclimated plants.

The results of this study support the hypothesis that growth in low altitudes will result in the reduction of $R_d$ as a consequence of: (1) temperature sensitivity of the respiratory apparatus, resulting in the reduction of the respiratory capacities of mitochondria; (2) a reduction in mitochondria size and numbers; and as a consequence of this (3) a reduction in the activities of mitochondrial enzymes (because of a reduced total enzyme contents). However, these responses are species specific and vary according to the range of temperatures experienced by plants in the field. Responses are likely a consequence of the reduced demand for respiratory energy needed for maintenance and repair of enzymes at low altitudes. As a result of this long-term acclimation to higher temperatures, carbohydrate exhaustion is prevented (Larigaurderie and Körner, 1995).
CHAPTER FOUR
CONCLUDING DISCUSSION
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4.1 Concluding Discussion

4.1.1 Mechanisms underpinning the response of respiration to changes in temperature

This study investigated whether there is a relationship between changes in the rates of dark respiration with mitochondrial capacity, mitochondrial numbers and sizes, and key mitochondrial enzyme activities. Controlled and field experiments were conducted using leaves of Lolium perenne (ryegrass) and Chionochloa (snow tussocks) to identify the underlying mechanisms underpinning respiratory acclimation to changes in temperature.

The first step was to investigate the response of dark respiration in leaves to changes in temperature. The second step was to investigate the mechanisms underpinning the differences in rates of dark respiration ($R_d$). Based on previous literature, several hypotheses were formed (Miroslavov and Kravkina, 1991; Robertson et al., 1995; Griffin, 2001; Atkin et al., 2002; Kofidis et al., 2003; Griffin et al., 2004; Kurimoto et al., 2004b; Wang et al., 2004; Atkin et al., 2005; Armstrong et al., 2006b). It was hypothesized that acclimation to warmer temperatures results in: (1) a decrease in mitochondrial respiratory capacity; (2) a decrease in mitochondria numbers and sizes; (3) a decrease in mitochondrial enzyme activity.

In the laboratory experiment, ryegrass plants were grown from seed in contrasting temperatures (either a cool regime of 8/15°C night/day, or a warm regime of 15/25°C night/day). Gas exchange measurements were carried out at night to measure rates of dark respiration in intact warm- and cool-grown ryegrass leaves. Dark respiration rates
for warm-grown ryegrass were greatly reduced compared to cool-grown grasses (Fig. 2.3). In the field experiment, *Chionochloa* plants were brought back from the field and kept at their original ambient air temperatures in growth chambers (minimum air temperatures of 1°C, 6°C and 8.5°C). Gas exchange measurements of intact leaves in the dark (Fig. 3.2) reveal that $R_d$ were slightly (non significantly) lower for *C. rubra* growing at the base of the mountain (450m) compared to plants at a higher altitude (1060m). The dark respiration rates were also slightly lower for *C. pallens* growing at 1070m than at 1600m. However, there was a significant reduction in $R_d$ when comparing the lower site species (*C. rubra* at 450m & 1060m) to the higher site species (*C. pallens* at 1070m and 1600m). Collectively, the combined results of $R_d$ from *C. rubra* of low altitudes and *C. pallens* from the higher altitudes support the general prediction that an increase in temperature (at low altitudes) results in the reduction of respiration in comparison to plants from high altitudes. The findings for ryegrass in the controlled experiment support this prediction also, displaying respiratory homeostasis over the range of 8-15 °C night temperature (i.e. a significant decrease in rate under warm temperatures).

Oxygen consumption rates were measured in the controlled experiment with ryegrass (Fig. 2.4) and indicate that accompanying the decrease in the rates of $R_d$ of warm-grown ryegrass was a trend toward a decline in mitochondrial respiratory capacity in leaves and roots from whole tissues, crude extracts and isolated mitochondria. Oxygen consumption rates between both species of *Chionochloa* also show temperature sensitivity of the respiratory apparatus, in which there was a reduction of the respiratory capacities of mitochondria in low altitude plants compared to high altitude plants. However, there was no significant difference in mitochondrial respiratory capacity between 450m and 1600m as was observed in the gas exchange measurements. It is concluded for ryegrass in the controlled experiment, and for tussocks growing in the field, that the response of dark respiration to changes in temperature is a likely consequence of other factors such as substrate availability (sugars) and/or the reduced demand for respiratory energy (ATP) needed for maintenance and repair of enzymes at low altitudes more so than a change in the capacity of the respiratory apparatus.
Transmission electron micrographs of mesophyll cells were utilized to investigate whether changes in mitochondrial abundance or size is a mechanism involved in the small changes in respiratory capacity and large differences in $R_d$ between warm and cool grown plants. For ryegrass in the controlled experiment, a decline in mitochondrial numbers (Fig. 2.8) was observed, although, perhaps surprisingly, the overall sizes (major and minor lengths) were larger in warm-grown plants than cool (Fig. 2.9). Analysis of electron micrographs of mesophyll cells from tussocks revealed that at the lower altitudes, where *C. rubra* grows, there was a significant decrease in mitochondrial numbers and dimensions with decreasing elevation (Figs. 3.9 & 3.10). Surprisingly, at higher altitudes, where *C. pallens* grows, there was no change in mitochondrial numbers and the dimensions increased with decreasing elevations. A likely reason for the lack of significant change would have been because of a small difference in ambient air temperatures, but actually there was a large difference for *C. pallens* at each site (1 - 6°C) compared to *C. rubra* at each site (6 – 8.5°C). Comparisons of mitochondrial abundance and dimensions between the 450m and 1600m sites revealed a slight decrease in abundance (non significant) and size with decreasing elevation. It is concluded for ryegrass in the controlled experiment, that mitochondria abundance (not size), is a likely mechanism associated with the change in $R_d$. However, for tussocks growing in the field, the response of dark respiration to changes in temperature was a likely consequence of a reduction in size of mitochondria from 1600m to 450m more so than mitochondria abundance (as this was only evident between *C. rubra* at 450m and 1060m).

Lastly, differences in the activities of two key mitochondrial enzymes, succinate dehydrogenase and fumarase were investigated. In the controlled experiment with ryegrass there was no systematic change in mitochondrial enzyme activity in leaves *in vitro*, however fumarase activity declined in root extracts while succinate dehydrogenase activity increased at higher growth temperatures (Fig. 2.11). The results from mitochondrial enzyme activities isolated from leaves of tussocks at each field site were difficult to interpret as there were species specific reductions in activities that varied accordingly to the range of temperatures experienced by plants in the field (Fig. 3.12). There was a significant reduction in fumarase activity between 450m and 1600m, but not
between the altitudinal ranges of both species. The results from the field and controlled experiment support the hypothesis of a reduction in enzyme activity (fumarase). The response of succinate dehydrogenase (SDH) activity, on the other hand, was not consistent in ryegrass, but was consistent with the notion of a reduction in enzyme activity with decreasing altitude. A small (non significant) reduction in activity between 450m and 1600m was detected and a significant large reduction in SDH activity between altitudes of 1060m and 450m was observed (between *C. rubra* plants).

A likely reason for discrepancies observed in respiratory capacities and enzyme activities between both tussock species and between ryegrass tissues could be due to the homogenisation process. Homogenisation may have been less destructive to root mitochondria than for mitochondria from leaves. The disruption of tissues can cause vacuole contents to be released into the solution, and depending on how much is released, could have significant effects on organelles (such as mitochondria). The extraction buffers are supposed to buffer changes in pH and also prevent damage to organelle membranes; however these methods were designed for potatoes, cauliflower and *Arabidopsis* plants and not tussocks containing a considerable amount of celluloses. Strong and prolonged grinding (especially with leaves than roots due to tough celluloses present) can also mechanically injure mitochondria, resulting in a population of intact and damaged mitochondria (ruptured mitochondria that have resulted in the loss of matrix contents) (Neuburger, 1985). Another possibility is that there are differences in damaging compounds between leaves and root tissues, some compounds are leaf-or root-specific. It is also likely that the two *Chionochloa* species in the field will contain differing compounds and amounts in their leaves. Any discrepancies arising from the homogenisation process is unlikely to have a large impact on the treatment effects, as these differences should have remained constant between ryegrass and tussocks, but could explain the differences observed between the two species of tussocks, and between leaf and root material in the ryegrass. Further investigations to optimise the isolation procedure could be employed for future measurements in oxygen consumption and enzyme activities from isolated mitochondria.
Conclusions

The clearest factors responsible for the dynamic responses of respiration to changes in temperature in this project are clearly mitochondria abundances and dimensions. Future investigations (as highlighted in the previous discussions) should be targeted toward a determination of whether concentrations of carbohydrates and the demand for energy (instead of mitochondrial capacities), are the major drivers involved in the response of respiration to changes in temperature.

The results of this study are supportive of the hypothesis that growth in low altitudes and warm conditions will result in the reduction of $R_d$ as a consequence of: (1) temperature sensitivity of the respiratory apparatus, resulting in the reduction of the respiratory capacities of mitochondria; (2) a reduction in mitochondria size and numbers; and as a consequence of this (3) a reduction in the activities of mitochondrial enzymes. However, these responses are species specific and vary according to the range of temperatures experienced by plants in the field and controlled environments.

Further investigations assaying other respiratory enzymes (such as alternative oxidase and cytochrome c oxidase also belonging to the TCA cycle), carbohydrates concentrations and the demand for respiratory products (i.e. ATP) might create a more complete picture on patterns of enzyme activity and sugars that underpin thermal respiratory acclimation in controlled environments and in the field. Investigating the mechanisms behind the responses of respiration to changing temperature is important for two reasons: (1) Understanding of likely plant responses to climate change; (2) calculating/modelling of carbon exchange between terrestrial ecosystems and the atmosphere.
4.1.2 Global significance of respiration to changes in temperature

This research analysed possible underlying mechanisms responsible for the changes in plant respiration in response to changes in temperature in controlled environments and in the field. It is important to know whether current and future environmental changes are likely to affect plant processes (and how) before investigating the relevance at a global level. Temperature is one of the most important factors affecting both photosynthesis and respiration of plants, and their responses will play significant roles in future cycling of carbon. Because both photosynthesis and respiration are sensitive to temperature, changes in the climate are likely to affect carbon storage in plants and ultimately an ecosystem’s carbon balance. Increases in temperature and CO$_2$ have been shown to stimulate photosynthesis, resulting in the increase of carbon being stored both below-ground and above-ground (Reddy et al., 1998; Centritto et al., 2002). It is predicted that stimulation of photosynthesis by CO$_2$ and temperature would increase ecosystem carbon uptake (Drake et al., 1997; Norby et al., 1999). Higher rates of respiration in a warmer climate could lead to the release of more CO$_2$ to the atmosphere from terrestrial ecosystems. However, it has been shown that over time, respiration can acclimate to warmer temperatures (Larigaurderie and Körner, 1995; Atkin et al., 2000b; Xiong et al., 2000; Browse and Xin, 2001; Atkin and Tjoelker, 2003; Bolstad et al., 2003; Loveys et al., 2003; Kurimoto et al., 2004b; Talts et al., 2004; Atkin et al., 2005; Armstrong et al., 2006b). Plants grown in controlled conditions at higher temperatures often show a reduction in the rates of respiration with further increases in temperature compared to plants grown at a cooler temperature. A better understanding of the acclimation phenomenon is becoming increasingly important in the context of global climate change; it is a vital component for models predicting the effects of possible climate change on plants (Luo et al., 2001). It is suggested that respiratory acclimation to continued warming would improve plant carbon balance by reducing carbon losses (Xiong et al., 2000). Acclimation of respiration could reduce the annual respiratory CO$_2$ flux into the atmosphere (Atkin et al., 2000a), and failure to take acclimation into account will result in an over-estimation of the effects of global warming on CO$_2$ release (Luo et al., 2001).
Measuring whole plant respiration \((R)\) rates would have been desirable in this project, especially for modelling perspectives. Harvesting of roots from the plants in the field would have been destructive, and would not have been realistically completed in the time frame of this project. Transmission micrographs of root tissues to investigate changes in mitochondria counts and sizes in the ryegrass experiment were also planned but could not be completed in time. Understanding the effects of temperature in both leaves and roots will be very important for predicting responses of plant functioning and future CO\(_2\) concentrations in the atmosphere. It has been shown that air and soil temperatures can fluctuate during the day and seasonally (as evident in Fig. 2.1 from section 2.2 and Fig. 3.1 from section 3.3). The role of temperature in mediating a positive feedback on respiratory CO\(_2\) flux to the atmosphere is of concern for its effects on global atmospheric CO\(_2\) concentrations and its contribution to further greenhouse warming (Atkin and Tjoelker, 2003). Up to two thirds of photosynthetic carbon gain is released back into the atmosphere by plants, and at a global scale plant \(R\) represent a greater flux (~ 60 gigatonnes of carbon per year) compared to the release of CO\(_2\) by combustion of fossil fuels, cement production and land use changes (~ 7.1 Gt C year\(^{-1}\) in total). The balance between photosynthesis and respiration will determine whether an ecosystem is a net source or sink of CO\(_2\) from the atmosphere. Therefore, it is essential to investigate not just leaves but root respiration to understand the impact that global warming could have on plants. Root respiration (not just leaves) is also a major component of CO\(_2\) exchange between the soil and atmosphere. Soil respiration is the second largest flux of carbon in terrestrial ecosystems, and up to two thirds of CO\(_2\) released by the soil is from roots (Ekblad and Högberg, 2001; Xu et al., 2001; Bhupinderpal-Singh et al., 2003). Belowground respiration exerts a large control on terrestrial carbon cycling (Ryan and Law, 2005), therefore it is important to understand the responses of root respiration to changes in climate for future predictions of carbon exchanges within the ecosystem. Soil respiration and aboveground processes are strongly linked. Photosynthesis in leaves supplies the carbon substrates for root metabolism and growth, and a decrease in substrate supply can decrease soil respiration within days (Ryan and Law, 2005). Therefore, even if soil temperatures may not change as dramatically as air temperatures may, changes in temperature will have an effect on root respiration and ultimately CO\(_2\).
efflux. Future experiments conducted on roots to investigate the effects temperature has on respiration would be an important addition to the results of this present study.

Clearly, climate change has gained and continues to have considerable scientific and political attention. This is because increases in atmospheric CO₂ and temperature and altered precipitation directly affects biological and chemical processes, causing a cascade of effects in individual organisms up to the ecosystem scale (Beier, 2004), which in turn has an effect on the climate. Ultimately full-scale studies in natural environments will provide the necessary information to predict ecosystem responses to changes in climate. However, to do this, a general understanding of how individual factors (and their complex interactions) affecting individual processes is needed. Integrating these single factors into multifactor experiments at the ecosystem scale will be crucial to generate information for models in the future (Beier, 2004).
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generated by a mitochondrial localized acetyl-coenzyme A carboxylase. Plant Physiology 133, 875-884.


Literature Cited


APPENDIX

Appendix 1

Ryegrass results

Work by Armstrong et al., (2006c) have indicated that in Arabidopsis thaliana leaves there are differences in leaf structure and cell ultrastructure in upper (adaxial) and lower (abaxial) sections of leaves. Contrary to Armstrong’s study, the detailed observations on ryegrass mesophyll cell areas, organelle counts and their dimensions revealed that the upper and lower epidermal layers were found to be equivalent (Appendices 1 a-d). Based on these observations, both layers were combined to form a larger data set.

![Diagram of mesophyll areas](image.png)

Appendix 1 a. Average mesophyll areas of upper and lower layers from cool- (a) and warm-grown (b) Ryegrass leaves. Values represent the mean of 5 replicate plants with 6 EM (electron micrographs) images each, where n = 35 per treatment (± SEM). ns = nonsignificant.
Appendix 1 b. Average number of mitochondria & chloroplasts from upper and lower mesophyll cells of Ryegrass leaf material sampled from a cool (a) or warm (b) treatment. Values represent the mean of 5 replicate plants with 6 EM (electron micrographs) images each (n = 35 per treatment), (± SEM). ns = nonsignificant.

Appendix 1 c. Mean mitochondria major axis (length) and minor axis (width) from mesophyll cells of upper or lower layers from cool- (a) and warm-grown (b) Ryegrass leaves. Values represent the mean ± SEM (n_{cool} = 70 & n_{warm} = 130). ns = nonsignificant.
Appendix 1 d. Average chloroplast major (length) and minor axes (width) sampled from upper and lower mesophyll cells of cool- (a) or warm-grown (b) Ryegrass leaves. Values represent the mean ± SEM (n_{cool} = 170 & n_{warm} = 140). ns = nonsignificant.
Appendix 1 e. Average number of mitochondria shapes in cool-grown (hatched bars) and warm-grown (open bars) mesophyll cells sampled from ryegrass. Values represent averages, ± SEM, n = 50. ns = nonsignificant; * P<0.05, ** P<0.01 & *** P<0.001.

Cool-grown mesophyll cells had a significantly greater number of spherical mitochondria (P = 0.0206) and rod (P = 0.0028) shaped mitochondria than warm-grown cells.

Importantly, there were more spherical shaped than rod shaped mitochondria in the cool- (P = 0.0099) and warm-grown (P = 0.0007) mesophyll ryegrass cells.
Appendix 2

Tussock results

The observations on mesophyll cell areas, organelle counts and their dimensions sampled from leaves of *C. rubra* and *C. pallens* were in agreement with the results obtained from the ryegrass data. Because both the upper and lower epidermal layers were generally found to be equivalent (Appendices 2 a-d), both layers were combined to form a larger data set.

There were a few exceptions: (1) There was a significant difference between the upper and lower mitochondria counts for *C. pallens* at 1070m ($P_{1070m} = 0.0018$) and 1600m ($P_{1600} = 0.05$) (Appendix 2d). (2) A significant difference was seen between the upper and lower mitochondria minor axes from *C. rubra* at 450m ($P = 0.0288$) (Appendix 2e). (3) Analysis of chloroplast dimensions from 450m revealed a significant ($P = 0.0004$) difference between upper and lower chloroplast major axes (Appendix 2 g (a)). The small difference between chloroplast minor axes from upper and lower layers was insignificant ($P = 0.0853$). (4) At 1060m (Appendix 2 g (b)) the difference between chloroplast minor axes from upper and lower mesophyll cells was statistically significant ($P = 0.0085$). (5) Examining chloroplast dimension of *C. pallens* leaf material showed there was a difference ($P = 0.0222$) between upper and lower major axes (Appendix 2 h (a)).
Appendix 2 a. Average areas of upper (shaded bars) and lower (open bars) mesophyll cells from *C. rubra* growing at 450m (a) & 1060m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM). ns = nonsignificant.

Appendix 2 b. Average areas of upper (shaded bars) and lower (open bars) mesophyll cells from *C. pallens* growing at 1070m (a) & 1600m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM). ns = nonsignificant.
Appendix 2 c. Average counts per 100 μm² cytoplasmic area of mitochondria & chloroplasts from upper and lower mesophyll cells of *C. rubra* leaf material from 450 m (a) and 1060 m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM). ns = nonsignificant.

Appendix 2 d. Average counts per 100 μm² cytoplasmic area of mitochondria & chloroplasts from upper and lower mesophyll cells of *C. pallens* leaf material from 1070 m (a) and 1600 m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM). ns = nonsignificant; * P<0.05 & ** P<0.01.
Appendix 2 c. Average mitochondria lengths (major axis) and widths (minor axis) from mesophyll cells of the upper and lower layers of *C. rubra* leaf material at 450m (a) & 1070m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM), \( n_{1060m} = 300 \) & \( n_{450m} = 150 \). \( ns = \) nonsignificant & * \( P < 0.05 \).

Appendix 2 f. Average mitochondria lengths (major axis) and widths (minor axis) from mesophyll cells of the upper and lower layers of *C. pallens* leaf material at 1070m (a) & 1600m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM), \( n_{1600m} = 250 \) & \( n_{1070m} = 200 \). \( ns = \) nonsignificant.
Appendix 2 g. Average chloroplast lengths (major axis) and widths (minor axis) from mesophyll cells of the upper and lower layers of *C. rubra* leaf material at 450m (a) & 1070m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM), \( n_{1070m} = 200 \) & \( n_{450m} = 130 \). ns = nonsignificant; ** P<0.01 & *** P<0.001.

Appendix 2 h. Average chloroplast lengths (major axis) and widths (minor axis) from mesophyll cells of the upper and lower layers of *C. pallens* leaf material at 1070m (a) & 1600m (b). Values represent the averages from 4 plants per altitude with 5 EM per layer (±SEM), \( n_{1600m} = 210 \) & \( n_{1070m} = 190 \). ns = nonsignificant & * P<0.05.
Appendix 2 i. Average number of mitochondria shapes from mesophyll cells sampled from (a) C. rubra at 450m (hatched bars) and 1060m (open); (b) C. pallens at 1070m (hatched) and 1600 (open). Values represent averages, ± SEM, n_{450m} = 108, n_{1060m} = 268, n_{1070m} = 118, n_{1600m} = 243. ns = nonsignificant; * P<0.05, **P<0.01 & *** P<0.001.

There were significantly fewer spherical shaped mitochondria at 450m than 1060m (P = 0.0141) (Appendix 2 a (a)). There was no difference in the number of spherical shaped mitochondria at 1070m and 1600m (Appendix 2 a (b)).

There was no difference in the number of rod shaped mitochondria between 450m and 1060m. However, there was significantly more rod shaped mitochondria at 1070m than 1600m (P = 0.0153).

Importantly, the number of spherical shaped mitochondria was significantly greater than the number of rods at 450m (P = 0.0119), 1060m (P = 0.0001), and 1600m (P = 0.0163).
Appendix 2 j. Average maximum (A) and minimum (B) ambient air temperatures for 2005 and 2006. Values represent the means, ± SEM, n = 365.

At 450m the maximum and minimum ambient air temperatures for 2006 were significantly lower than 2005 (P = 0.0001).

For the 1000m field site the maximum and minimum air temperatures were significantly lower in 2006 than in 2005. (P_{min} = 0.0496 & P_{max} = 0.0176).

At 1600m the maximum air temperature was significantly higher in 2006 than in 2005 (P = 0.05). However, the minimum air temperature was significantly lower in 2006 than in 2005 (P = 0.0001).