Thermal acclimation of respiration in rice and chickpea

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Declaration

The entire research mentioned in this thesis was carried out by me with the following exceptions:

Chapter 2: The quantitative PCR analysis was conducted by Mr. You Zhang at the Australian National University. Transcript analysis was operated by Professor James Whelan and team at the La Trobe University and Dr. Peter Crisp at the Australian National University.

Chapter 3: Gas Chromatography-Mass Spectrometry (GC-MS) metabolite analysis was carried out by Associate Professor Nicolas Taylor and team at the University of Western Australia and Dr. Shinichi Asao at the Australian National University.

Chapter 4: The quantitative PCR analysis was conducted by Dr. Crystal Sweetman at Flinders University.

Furthermore, I certify that the contents of this thesis were not previously submitted for a degree in any university. It does not contain any materials previously published except where due reference is given.

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Abstract

The world population has grown from 3 billion in 1961 to 7.6 billion in 2018, and is expected to reach nearly 10 billion by 2050. Global crop production must at least double by 2050 in order to meet the needs of a growing world population. Achieving high yields depends on increasing biomass production in the lead up to anthesis, which is determined mainly by the balance between respiration and photosynthesis. Knowledge of how fluctuating environments, particularly temperature, affects this carbon balance in rice is of significant importance yet not well understood. The overall aim of my PhD research was to understand thermal acclimation of physiological processes in rice. I first characterised the extent of acclimation of respiration and photosynthesis to varying temperature in rice. The results showed that respiratory acclimation did occur in rice newly-developed leaves that formed subsequent to temperature transfer, although not to the extent that respiration homeostasis was maintained. Contrastingly, light-saturated photosynthesis acclimated strongly in newly-developed leaves with photosynthesis being homeostatic at their growth temperatures. The key findings of the molecular and biochemical analyses were: (1) there was a transient adjustment in transcript abundance in pre-existing leaves within the first 24 h following temperature shift, aligning with respiration and photosynthesis perturbations; and (2) with longer exposure, cytochrome *c* oxidase (COX) was declined in abundance at hotter temperature. I also explored the extent to which respiration varies throughout the day/night cycle. Independent of growth temperature, I observed acclimation capacity to significantly vary over the diurnal cycle. The strongest respiration acclimation phenotype was observed at the end of the day. Based on metabolic profiling analysis, temperatures and the time of day both drive variations in respiratory metabolite levels, independently of one another. Finally, I scaled from isolated mitochondria to whole leaf tissues of chickpea to better understand the

mechanistic basis of thermal acclimation of leaf respiration. Interestingly, there were reductions in the capacity of cytochrome pathway along with a decrease in the abundance of COX protein, and an associated decline in rates of respiration per unit mitochondrial protein. Thus, it seems that a common feature of respiratory acclimation across species is a heat-dependent reduction in respiration and associated decline in cytochrome pathway capacity, likely as a result of reduced COX protein abundance. These insights into the mechanisms of respiratory adjustments to temperature will contribute towards a better understanding of how crops respond to global warming.

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Chapter 1 – General introduction

1.1 Global demand for food is rising

Statistics from the Food & Agriculture Organization of the United Nations shows that the world population has grown from 3 billion in 1961 to 7.6 billion in 2018, and is expected to reach nearly 10 billion in 2050 (FAOSTAT, 2019). This will result in an expanding demand for food, with global crop production needing to at least double by 2050 to meet the nutritional requirements of the world's human population (FAOSTAT, 2019). The Green Revolution of the 1960s – where farming made use of high yielding varieties, improved fertilizers, irrigation and pesticides (Pingali, 2012) – resulted in an impressive increase in food production (Figure 1.1).

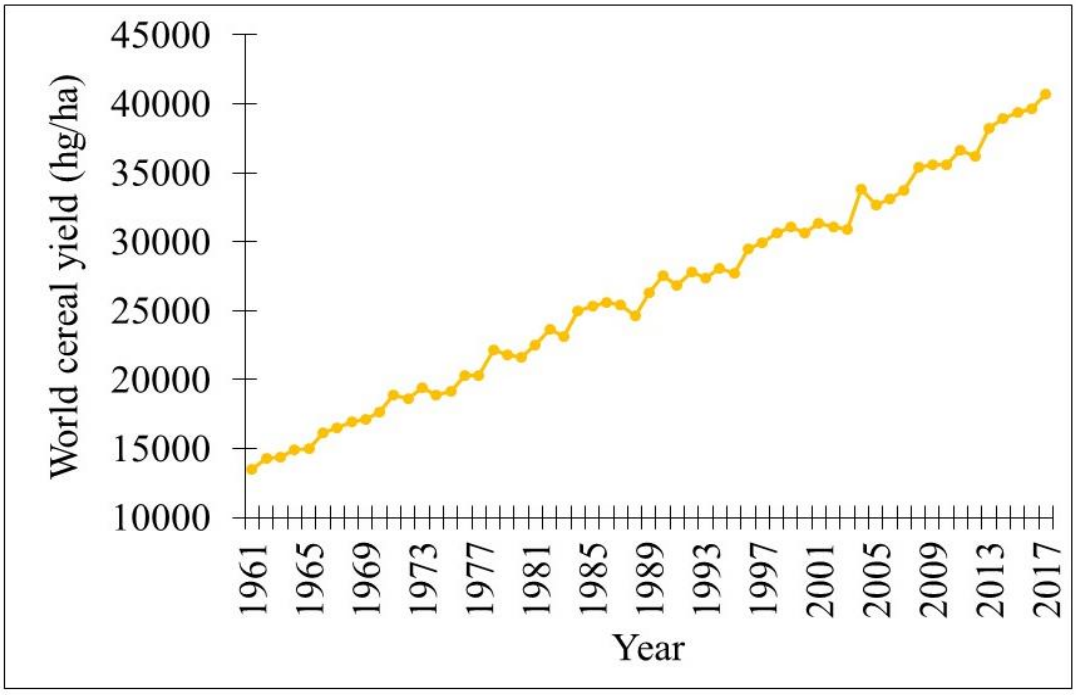

Figure 1.1. Total world yield of cereals from 1961 to 2017. Cereals include rice, wheat, barley, maize, etc. Data was obtained from FAOSTAT (2019).

However, while global crop yields are increasing, recent studies show that some regions are experiencing stagnation or declines in yield improvement (Brisson et al.,

2010; Lin & Huybers, 2012; Ray et al., 2012). For example, a survey conducted by Ray et al. (2012) indicates that yields of soybean (*Glycine max*), maize (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) – which together produce more than half of global agricultural calories – are no longer improving across their important growing regions (i.e. yields either never improved, stagnated and/or collapsed). In order to double global crop production by 2050, every year we need a ~2.4% increase in crop yield (Ray et al., 2013). However, these four staple crops are increasing only about 0.9-1.6% per year. If this trend continues, yield will only increase 38-67% by 2050, insufficient to feed the rising global population.

The good news is that a Green Revolution 2.0 may be possible, enabling step increases in crop yields, both in underperforming and high-yielding agricultural regions. For example, a study by South et al. (2019) determined that enhancing photosynthetic efficiency by altering the photorespiratory pathway could markedly increase photosynthetic efficiency and vegetative biomass, potentially improving crop productivity.

Despite the possibility that yields could be improved, there is a danger that potential increases in yield will not be realized as a result of the changing climate. For example, there is a strong evidence showing that many important cropland areas, mostly in developing countries, are vulnerable to climate change due to low capacities of adaptation [i.e. adjustment in natural systems or human activities in response to climate change (UNFCCC, 2007)], high rates of poverty and poor infrastructure (Niasse et al., 2004). With the added cost of human-induced climate change, implementation of the strategies needed to increase global crop production and achieve the goal of feeding the world's population is going to be an even greater challenge. Here, understanding the factors that limit crop yields under climate change scenarios will be vital.

1.2 Climate change and agriculture

Overall, it is understood that the growing population, along with rising wealth (which results in dietary changes) is driving up the demand for food. The first Green Revolution has successfully increased crop yields in order to meet this growing food demand. However, over time, the continuous need to increase yield is becoming ever more difficult due to rapid climate change. To mitigate the detrimental effect of climate change on yield, farmers worldwide have turned to increasing the amount of agricultural land used to grow crops, usually through deforestation. The process of removing forests, in turn generates higher atmospheric CO_2 concentration, a positive feedback loop adding to rising global temperatures.

The Fifth Assessment Report by the Intergovernmental Panel on Climate Change IPCC (2018) stated that mean surface temperature has increased by 0.87°C (likely between 0.75°C and 0.99°C) globally in the decade 2006 – 2015 and is estimated to further increase year after year. Additionally to increases in average global temperatures, heat waves are expected to become more frequent, intense, and longer-lasting (Meehl & Tebaldi, 2004). Cold winter extremes will continue to occur occasionally (IPCC, 2013). Additionally, extreme precipitation events will also occur more often and be more intense. In a report by Kukal and Irmak (2018), average temperatures across the Great Plains of America during the maize, sorghum and soybean growing season (1st May-30th September) varied from cooling (0.28°C/decade) to warming (0.45°C/decade) during the period of 1968-2013. The regions with a cooling trend had increased precipitation, whereas the regions with a warming trend showed reduced precipitation. These observed trends of temperature and/or precipitation have had significant negative implications on agriculture, particularly on crop yield (Kukal & Irmak, 2018; Rahman et al., 2017).

In contrast to a typical decline in crop yield from an increase in temperature or fluctuating precipitation, rising CO_2 concentration seems to generally increase yield by: (1) increasing rates of photosynthesis, and (2) reducing the amount of water loss through transpiration (Deryng et al., 2016). However, the effect of elevated CO_2 levels on crop yields varies regionally. For example, maize yield under high concentration of CO_2 is greater when grown in areas that rely on rain compared to arid regions where agriculture relies heavily on irrigation (Deryng et al., 2016).

1.3 How does temperature influence the yield of crops?

Evaluating the effects of temperature on crop yield has resulted in varying outcomes. In general, crop yields will gradually increase with temperatures up to an optimum. For example, the optimum temperature for maize, soybean and cotton is between 29 and 32°C and beyond this point yields decrease abruptly (Hatfield & Prueger, 2015; Schlenker & Roberts, 2009). Thus, if climate change resulted in a temperature above the optimum range, there is likely to be significant reductions in yield (Kucharik & Serbin, 2008; Lobell & Field, 2007). According to Zhao et al. (2017), results from multiple analyses (i.e. statistical regressions, field-warming experiments, global grid-based and local point-based models) investigating the impacts of temperature on crop yield, found that increase in global mean temperature by 1°C would reduce global yield of crops by 3.1-7.4%. Specifically, for each 1°C mean temperature increase, average yield of soybean will decline by 3.1%, rice by 3.2%, wheat by 6.0%, and maize by 7.4%.

Negative effects of temperature on plant growth (and subsequently yield production) are observed across all growth stages. For instance, elevated temperature can decrease photosynthesis (possibly caused by chloroplast damage) during the vegetative stage, induce spikelet sterility caused by reduced pollen production during the

reproductive stage, and increase respiratory energy consumption during the ripening stage (Wassmann et al., 2009). These detrimental effects are predicted to become more severe with warming, particularly in response to a warmer night, due to a faster rise of minimum night-time temperature compared to maximum day-time temperature (IPCC, 2007; Peng et al., 2004). This disparity between night and day warming and how this will influence crop function is an important consideration when investigating the impacts of temperature on plant physiological characteristics. For example, Welch et al. (2010) showed that the minimum night-time temperature and maximum day-time temperature from 1994 to 1999 had opposite impacts (negative and positive, respectively) on rice grown across tropical and subtropical Asia. However, the negative impacts of higher minimum night-time temperature (particularly during vegetative and ripening stages) exceed the positive impacts of higher maximum day-time temperature during all growth stages.

Apart from the fact that global temperature is rising as a whole, low temperature events, although less common, still occur (Powell & Reinhard, 2016), and hamper growth and yield (Buti et al., 2018). Zhang et al. (2014) stated that approximately 15 million hectares of rice throughout the world experience cold damage at one or more growth developmental phases. Low temperature can decrease the seed germination percentage (Yoshida, 1981), slow down vegetative growth (Ali et al., 2006) and reduce tillering (Shimono et al., 2002). At the reproductive stage, low temperature can increase spikelet sterility and subsequently reduce grain yield (Shimono et al., 2002). Thus, while it is important to understand how high temperatures influence processes controlling growth and yield, attention also needs to be given to the role of lower temperatures on crop physiology.

1.4 Plant biomass and the carbon economy

The maximum yield is defined as the yield obtainable from a cultivar grown in the environment to which it is adapted, under optimal conditions, i.e. without any biotic or abiotic stress and without resource limitations (Evans & Fischer, 1999; Tollenaar & Lee, 2002). One aspect of improving maximum yield is increasing biomass production (Evans, 2013), which is determined mainly by the balance between photosynthesis and respiration (Sakai, Yagi, Kobayashi, & Kawashima, 2001). Both photosynthesis and respiration are very sensitive to temperature (Berry & Bjorkman, 1980; Way & Yamori, 2014). Photosynthesis creates biomass by capturing light to oxidise water, releasing O_2 and to reduce CO_2, forming carbohydrates, primarily sugars (Simkin et al., 2019; Taiz et al., 2014).

Between 30 and 60% of the carbon fixed by photosynthesis (which incorporates carbon lost as photorespiration) is released back to the atmosphere through respiration (Amthor, 1989; Beer et al., 2010; Poorter et al., 1990). Respiration breaks down sugars to provide energy and carbon skeletons needed for growth and survival. Respiration can be categorised into two main functions. Firstly, there is growth respiration. Growth respiration is responsible for the generation of energy and carbon skeletons to accumulate biomass and produce new cellular structural components (Amthor, 2000; De Vries et al., 1974; Lambers et al., 2008; Weraduwage et al., 2015). It is also responsible for the accumulation of nutrients from the soil (Thornley & Cannell, 2000). Unsurprisingly, growth respiration is proportional to the plants growth rate (Lambers et al., 2008; Poorter et al., 1991). In theory, higher growth rates require higher ATP and carbon skeleton production (Amthor, 2000; De Vries et al., 1974). The second category is maintenance respiration. Maintenance respiration does not directly contribute to new biomass accumulation. Instead, maintenance respiration (as the name suggests), is required for

metabolic processes needed to maintain the existing plant biomass (Amthor, 1984; Thornley, 2011). These metabolic processes include macromolecule turnover, and maintenance of ion concentration/gradient across membranes (Thornley, 1970, 2011). One can expect that the elimination of wasteful energy use – from both growth and maintenance respiration – will reduce CO_2 loss, which in turn will enhance biomass accumulation and agricultural production (De Block & Van Lijsebettens, 2011). Synthesis of more stable proteins which require less energy expensive turnover, or building membranes which are less leaky, are two such examples of where respiratory energy costs might be spared.

As its name suggests ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) can fix both CO_2 and O_2, with the latter considered a wasteful process that consumes valuable energy while re-releasing fixed carbon (Dusenge et al., 2019; Lopez & Barclay, 2017). Photorespiration increases with increasing temperature mainly because the Rubisco specificity for CO_2 relative to O_2 decreases with temperature (Peterhansel et al., 2010; South et al., 2018). In C3 plants, photosynthesis and photorespiration are competitive. At high temperature, as much as 50% of the ATP synthesised by photosynthesis may be consumed for photorespiration (Peterhansel et al., 2012; Walker et al., 2016). This energy-consuming process certainly reduces the efficiency of photosynthesis, biomass production (South et al., 2019; South et al., 2018), and lowers the yield potential of C3 crops (Walker et al., 2016).f

1.5 Temperature responses of photosynthesis and respiration

1.5.1 Response of photosynthesis to temperature

Similar to photorespiration, it is well established that both photosynthesis and respiration are also temperature sensitive. Photosynthesis increases with temperature until it reaches

an optimum (A_{opt}) at a given temperature (T_{opt}) before decreasing at temperature above the T_{opt} (Evans, 2013; Hüve et al., 2011; Mathur et al., 2014; Song et al., 2014; Warren & Dreyer, 2006). In general, plants have an ability to acclimate to growth temperature (Demmig-Adams et al., 2008; Hughes & Dunn, 1996; van Zanten et al., 2013). Acclimation is defined as the adjustment in metabolism to compensate for the decline in plant performance following changes in the environment (Demmig-Adams et al., 2008; Lambers et al., 2008). In photosynthesis, plants that are grown under cold temperature regime exhibit lower T_{opt}, and an increase in growth temperature results in an increase in T_{opt} (Way & Yamori, 2014; Yamasaki et al., 2002). This phenotypic plasticity in photosynthetic characteristics enable plants to perform the maximum rate of photosynthesis at their growth temperature (i.e. photosynthetic acclimation; Figure 1.2). The degree of the linear relationship between T_{opt} and growth temperature shows considerable variation between species although the shift in T_{opt} is generally less than one-half that in the growth temperature. For example, Yamori et al. (2014) found the regression slopes of T_{opt} on growth temperature across 282 C_3 species, 35 C_4 species and 27 CAM species were 0.5°C C^{-1} (i.e. T_{opt} increased by 1°C with an increase in growth temperature by 2°C), 0.2°C C^{-1} and 0.3°C C^{-1}, respectively. These adjustment in T_{opt} to growth temperature are usually controlled by several limitations of photosynthesis (Slot & Winter, 2016; Way & Yamori, 2014; Yamori et al., 2014), which are discussed in Section 1.8.1. In some species, full photosynthetic acclimation (i.e. photosynthetic homeostasis; Figure 1.2) could be achieved with plants grown at contrasting temperatures show similar A_{opt} at the T_{opt}. (Berry & Bjorkman, 1980; Hikosaka et al., 2006; Yamori et al., 2005).

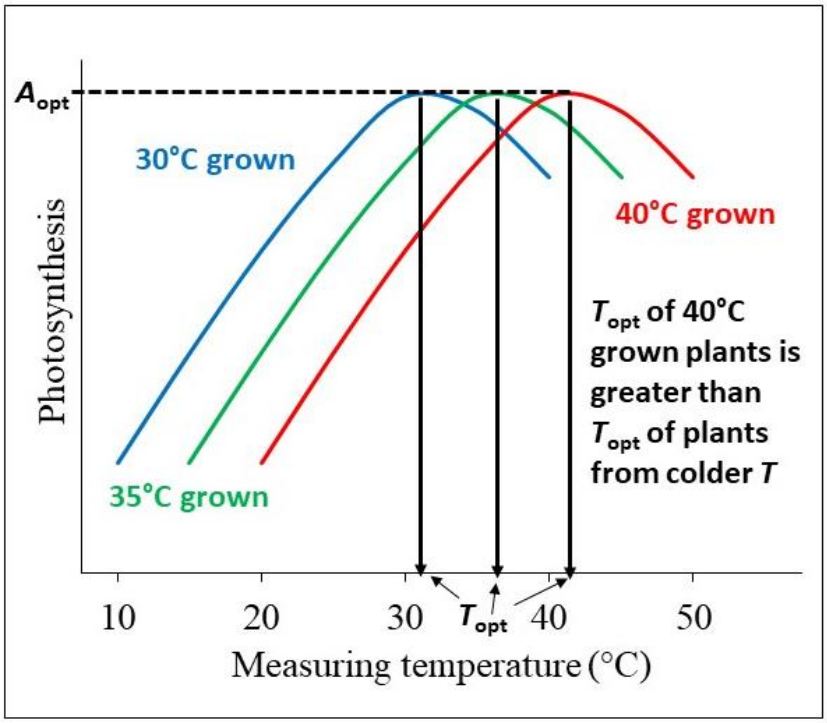

Figure 1.2. Diagrammatic representations of the temperature response of photosynthesis in plants acclimated to colder (blue) and hotter environment (red). Green represents the warm control plants. Acclimation of photosynthesis would be seen if the plants grown in cold environment exhibit lower T_{opt} [temperature at maximum photosynthesis (A_{opt})] compared to plants grown in hot environment. For example, in a situation where a set of plants is transferred from 35°C growth temperature to 30°C growth temperature, the T_{opt} would be decreased, but if the plants are transferred to 40°C growth temperature, the T_{opt} would be increased. Full photosynthetic acclimation (i.e. photosynthetic homeostasis) is achieved when the A_{opt} at T_{opt} are similar, regardless of their growth temperatures.

1.5.2 Response of respiration to temperature

The rates of respiration generally increase with temperature until it reaches the temperature optimum where respiration occurs at maximal rate (Atkin et al., 2005c; Prasad et al., 2016). The proportional change in respiration rates which is known as Q_{10} is often assumed to be 2.0 (i.e. respiration rate doubles per 10°C rise). However, this value is only found over a narrow range of temperatures, and usually decreases with increasing temperature (Atkin & Tjoelker, 2003). The Q_{10} value also varies across biomes. For example, plants growing in the tundra (colder biomes) have a greater Q_{10} value compared

to those growing in the tropics (warmer biomes) (Atkin & Tjoelker, 2003; Heskel et al., 2016).

In response to sustained temperature changes, acclimation of respiration occurs with plants grown in the cold exhibiting higher respiration rates than plants grown at warmer temperature when measured at a common temperature (Figure 1.3). Respiratory acclimation can be categorized as Type I or Type II acclimation. Type I acclimation commonly occurs in mature tissues that have developed prior to a change in temperature. Type I acclimation is characterised by a change in the Q_{10} value (i.e. an altered respiratory response to an immediate change in temperature). In contrast to Type I, Type II acclimation does not result in changes in the slope of the temperature response curve (i.e. no change in Q_{10}). Instead, Type II acclimation affects the intercept of a respiratory temperature-response curve by decreasing or increasing the entire elevation of the curve. Type II acclimation usually occurs in tissues that emerged and developed under the prevailing temperature regime. If Type I, Type II, or a combination of both occurs to a great enough extent, plants grown at different temperatures will exhibit identical rates of respiration when measured at their respective growth conditions (Figure 1.3), referred to as respiratory homeostasis (Atkin et al., 2000a). The extent to which respiration acclimates, whether it is Type I or II acclimation, or if homeostasis is achieved, is still unclear for many species including rice.

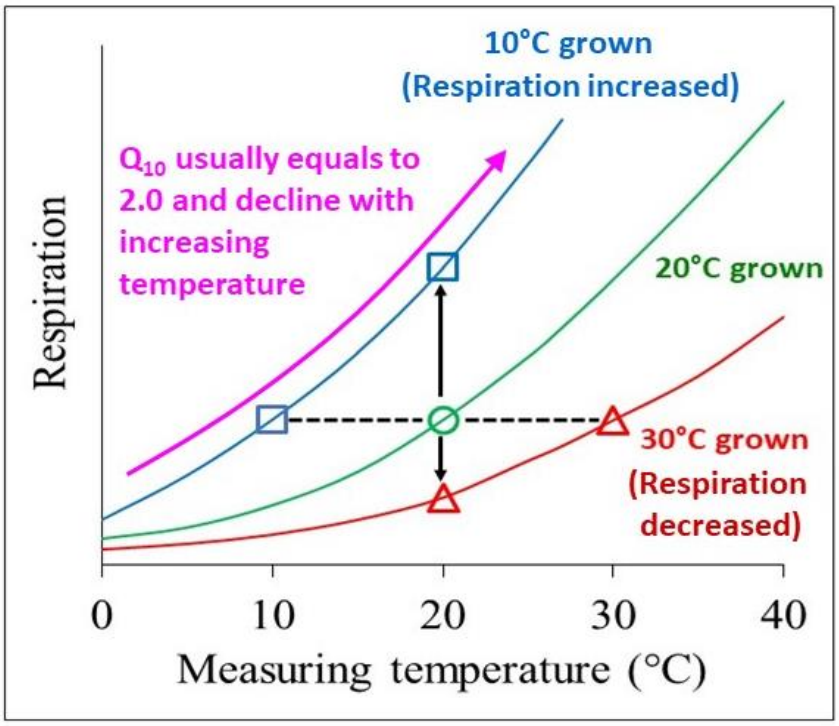

Figure 1.3. Diagrammatic representations of the temperature response of respiration in plants acclimated to colder (blue) and hotter environment (red). Green represents the warm control plants. In respiration, the temperature sensitivity can be seen in Q_{10} values, with the respiration rate usually doubles per 10°C rise in temperature (i.e. Q_{10} = 2.0) (purple arrow). However, this value will steadily decline with increasing temperature. The Q_{10} values are often higher in cold grown plants when compared with warmer grown plants. Temperature acclimation of respiration would be seen if the plants grown in cold environment exhibit higher rates of respiration than plants grown at warmer temperature when measured at a common temperature (solid black arrow). For instance, if a set of plants is transferred from 20°C growth temperature to 10°C growth temperature, the rates of respiration would be increased. By contrast, if a set of plants is transferred from 20°C growth temperature to 30°C growth temperature, the rates of respiration would be decreased. Full respiratory acclimation (i.e. respiratory homeostasis) could be observed if the rates are identical when measured at the contrasting growth temperatures (dashed black line).

1.6 Response of respiration to diurnal temperature fluctuations

Plants are continually facing changes in temperature, not only due to seasonal changes, but also due to the day/night cycle. Often, measurements of respiration are conducted on dark-adapted leaves (generally 30 minutes of exposure to darkness) at only one time-point during day (Aspinwall et al., 2017; Benomar et al., 2017; Talts et al., 2004). Not much is known about the extent to which respiration varies throughout the diel cycle despite the

importance of respiration to the carbon budget. To date, only a few studies have measured the variation of respiration on a diel basis. For example, Yang et al. (2014) found that stem respiration of *Larix principis-rupprechtii* trees in Northern China showed clear diurnal cycles with the highest peak in respiration at 15:00 before a decline. In root respiration of *Quercus crispula* and *Fagus creanata*, the rates peaked at around 10:00-12:00 and then decreased (Makita et al., 2014). These examples demonstrate that respiration can vary throughout the day but a greater unknown is whether respiratory acclimation also varies throughout the day. This is particularly relevant when considering plants experience contrasting growth temperatures between day and night and the greater increase in night minimums versus day maximums in global warming trends.

1.7 Respiration-photosynthesis coupling

Respiration and photosynthesis are inter-reliant. Respiration breaks down photosynthates to produce ATP and carbon skeleton. On the other hand, photosynthesis relies on the product of respiration for the synthesis and maintenance of photosynthetic machinery and products, such as for sucrose synthesis, and the transport and repair of photosynthetic proteins (Atkin et al., 2007; Benomar et al., 2017; Chi et al., 2013; Gandin et al., 2014; Gifford, 1995, 2003). Given the contrasting temperature dependence of the two processes, the ratio of respiration to photosynthesis typically increases with temperature (Atkin et al., 2006a; Drake et al., 2016; Loveys et al., 2003). However, over sustained periods of contrasting temperatures, physiological acclimation dampens this increase in the ratio of respiration to photosynthesis (Dewar et al., 1999; Lombardozzi et al., 2015; Loveys et al., 2003; Ow et al., 2010; Smith & Dukes, 2013). As a result, the ratio is often constant (Campbell et al., 2007; Dewar et al., 1999; Drake et al., 2016). Though, the ratio can be different above a certain temperature due to several factors, especially if the

measurements are conducted on whole plant level (Atkin et al., 2006b). The balance between respiration and photosynthesis has been studied in many species; however, relatively little information is available for major crop species such as rice. As previously mentioned, understand the carbon balance, more specifically, how we can maximise carbon gain and minimise carbon loss, are very crucial in order to achieve enhanced crop yield. For this to be achieved we need to understand the factors that might underpin the responses of respiration and photosynthesis to temperature, in rice and other important crops.

1.8 Factors that might explain the physiological responses to temperature changes

1.8.1 Factors that might explain the response of photosynthesis to temperature

Photosynthetic metabolism consists of two stages. Firstly, the light dependent reaction which takes place across the thylakoid membranes, producing ATP and NADPH. These high energy compounds are used in the second stage known as the Calvin cycle to recycle the 5-carbon sugar substrate ribulose-1,5-bisphosphate (RuBP). In the stroma of the chloroplast, the Calvin cycle is the second stage of photosynthesis. The Calvin cycle is responsible for the synthesis of carbohydrates by fixing inert atmospheric CO_2 to RuBP (Figure 1.3) (Berg et al., 2002b; Johnson, 2016; Lopez & Barclay, 2017). Limitation in the generation of ATP and NADPH by the light reaction is commonly referred to as RuBP regeneration limited photosynthesis, while limitation in CO_2 fixation by Rubisco is commonly referred to as RuBP carboxylation limited photosynthesis (von Caemmerer, 2000).

In response to temperature changes, associated with a shift in T_{opt}, photosynthetic rate is potentially limited by RuBP carboxylation, RuBP regeneration or a balance of these two processes (Farquhar & Von Caemmerer, 1982; Hikosaka et al., 2006; Hikosaka

et al., 1999; Yamori et al., 2010). A third limitation can occur when the triphosphate sugar product of photosynthesis is not utilised but this limitation is often associated with maximal photosynthesis rates when CO_2 is above physiological relevant concentration (Sharkey et al., 2007). RuBP carboxylation seems to be the main limitation on photosynthesis above the T_{opt} (Busch & Sage, 2017; Hermida-Carrera et al., 2016). This heat limitation is attributed to the heat-labile nature of the protein Rubisco activase, an enzyme required for Rubisco function by removing inhibitors from the active site (Portis et al., 2008; Salvucci & Crafts-Brandner, 2004; Salvucci et al., 2001). Further, the affinity of Rubisco for carbon relative to oxygen declines with temperature (i.e. photorespiration increases), another limitation on RuBP carboxylation in warmer environments (Peterhansel et al., 2010; Sage & Kubien, 2007; Zhang et al., 2013). In some studies, RuBP regeneration rather than carboxylation has been postulated to limit photosynthesis above T_{opt} (Cen & Sage, 2005; Makino & Sage, 2007), associated with membrane stability (Sharkey, 2005). However, a revisiting of this work more recently points to RuBP carboxylation being the more likely limitation, again linked to the heat susceptibility of Rubisco activase (Busch & Sage, 2017).

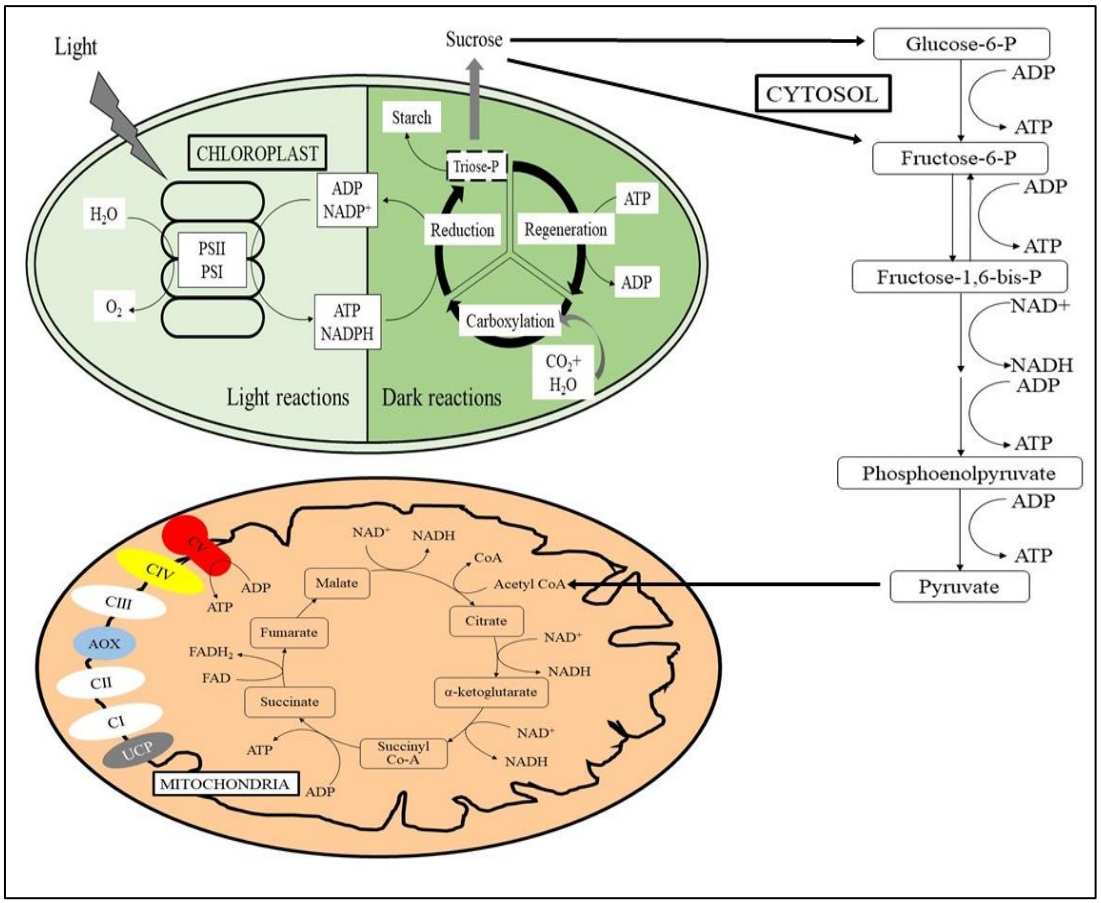

Figure 1.4. Summary of interactions between photosynthesis in chloroplasts and respiration in mitochondria. Substrates for respiration generated by photosynthesis enter glycolysis in the cytosol and converted into organic acid such as pyruvate, then oxidized in the matrix of mitochondria to produce NADH and $FADH_2$ which would be used in electron transport chain to synthesize ATP.

At lower growth temperatures, RuBP regeneration is often the rate-limiting step of photosynthesis rather than RuBP carboxylation. For example, in a study by Onoda et al. (2004) on the leaves of *Polygonum cuspidatum*, a perennial herb widely distributed in the temperate zone of Asia, the ratio of RuBP regeneration (expressed as J_{max}; maximal photosynthetic electron transport rate) to RuBP carboxylation capacity (expressed as V_{cmax}; the maximal velocity of Rubisco carboxylation) was higher in autumn compared to summer. Similarly, in a more recent study by Zaka et al. (2016) on alfalfa (*Medicago sativa*) cultivars, the degree of J_{max} was greater than V_{cmax} in plants grown in the cold, suggesting that more nitrogen was allocated in the RuBP regeneration process at lower

growth temperatures (Hikosaka, 2005; Hikosaka et al., 2006). The response of the balance between J_{max} and V_{cmax} to temperature is suggested to be species specific. Yamori et al. (2010) studied the photosynthetic temperature acclimation among cold-tolerant and cold-sensitive crop species and found that the J_{max}:V_{cmax} ratio was strongly correlated with Rubisco content, with cold-tolerant species [e.g. spinach (*Spinacia oleracea*), winter wheat (*Triticum aestivum*) and broad bean (*Vicia faba*)] exhibiting greater ratio compared to cold-sensitive species [e.g. tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*)]. Plants acclimated to cold environments usually need greater abundance of photosynthetic proteins, especially Rubisco (Campbell et al., 2007; Goulas et al., 2006), in order to compensate for a decrease in enzyme activity at low temperatures (Yamori et al., 2014). Similarly, sucrose phosphate synthase (SPS), an enzyme involved in sucrose biosynthesis increases in abundance during cold acclimation (Stitt & Hurry, 2002). Moreover, a greater ratio of unsaturated to saturated fatty acids also underlies photosynthetic acclimation to low temperature (Niu & Xiang, 2018).

1.8.2 Factors that might explain the responses of respiration to temperature

Respiratory metabolism involves reducing equivalents from glycolysis (in the cytosol) and from the tricarboxylic acid (TCA) cycle (in the matrix of mitochondria) being oxidized by the electron transport chain (ETC) localized in the inner membrane of mitochondria (Affourtit et al., 2001; Li et al., 2013). Variation in Q_{10} is strongly controlled by several limitations in response to short-term temperature changes. This includes the temperature dependent enzyme activity that determines the speed at which substrates can be metabolised. The temperature dependent substrate supply (i.e availability of sugars) and product demand (i.e. the ratio of ATP:ADP) will also have a major impact on respiratory response to temperature and are control points at different steps of the

respiratory pathway (Atkin & Tjoelker, 2003; Lambers et al., 2008; O'Leary & Plaxton, 2016).

Following a shift of pre-existing tissues to a new temperature, respiratory acclimation is usually associated with changes in Q_{10} values (Type I acclimation) as mentioned in Section 1.5.2 (Atkin et al., 2005b; Atkin & Tjoelker, 2003; Reich et al., 2016). The changes are probably influenced by changes in the physical and chemical components within existing organelles in the cell, such as substrate supply (Hüve et al., 2012). Sugars, for example, are the main respiratory substrate, and commonly accumulate in the cold (Lukaszuk et al., 2016; Partelli et al., 2010; Rosa et al., 2009; Stitt & Hurry, 2002). Sugar accumulation in the cold can be associated with an increase in respiration (Lee et al., 2005). Feedback regulation of respiration through adenylate control exerted at Complex I (NADH ubiquinone oxidoreductase), complex III (cytochrome b/c_1 complex) and complex IV (COX; cytochrome *c* oxidase) in the mitochondrial ETC (Figure 1.4) also plays a role in altering respiration rates under changing environmental conditions (O'Leary et al., 2018). Electrons that flow through these complexes are coupled to proton transport via ATP synthase, resulting in the production of ATP. In addition to the ATP synthase, another means of dissipating the proton gradient is by using uncoupling proteins (UCP). However, engagement of UCP is not coupled to ATP synthesis (Fernie et al., 2004; Jastroch et al., 2010).

A further means of respiratory O_2 consumption is via the non-phosphorylating alternative oxidase (AOX) pathway which is not coupled to proton transport; thus, no production of ATP is involved (Plaxton & Podestá, 2006; Vanlerberghe, 2013). High engagement of AOX pathway can reduce the degree of adenylate restriction under high ATP/ADP ratios (Plaxton & Podestá, 2006). Other enzymes that do not contribute to the proton-motive force, and reduce the efficiency of ATP synthesis, including rotenone-

insensitive external and internal NAD(P)H dehydrogenases (Rasmusson et al., 2004; Svensson et al., 2002; Sweetman et al., 2019). These dehydrogenases provide electrons directly to the UQ pool by oxidizing cytoplasmic and matrix NADH and NAD(P)H, respectively (Møller, 2001; Taiz et al., 2014). It seems likely that the function of NAD(P)H dehydrogenases, in part, is to maintain the redox balance in the cytosol and matrix of mitochondria (Svensson et al., 2002). In the case of tissues that emerged and developed at the new temperature regimes, a higher degree of respiratory acclimation is involved (Type II acclimation), as described in Section 1.5.2. Underpinning this, often, are changes in the capacity of respiratory enzymes. Either capacity per unit mitochondrion, or even changes in the number of mitochondria per unit volume of tissue (Crous et al., 2017; Slot & Kitajima, 2015). Armstrong et al. (2006), showed that associated with an increase in respiration rates of cold-developed *Arabidopsis thaliana* leaves were an increase in mitochondrial density per unit volume of tissue of epidermal cells, an increase in the density of cristae within mitochondria of mesophyll cells, as well as an increase in the cytochrome pathway capacity at the whole leaf level.

In rice, not much is known about how respiration is altered following sustained changes in temperature, both in pre-existing and newly-developed leaves. Characterising respiratory gene expression, protein abundance and metabolite pools will be used in this thesis to answer some of these unknown aspects of rice respiratory acclimation.

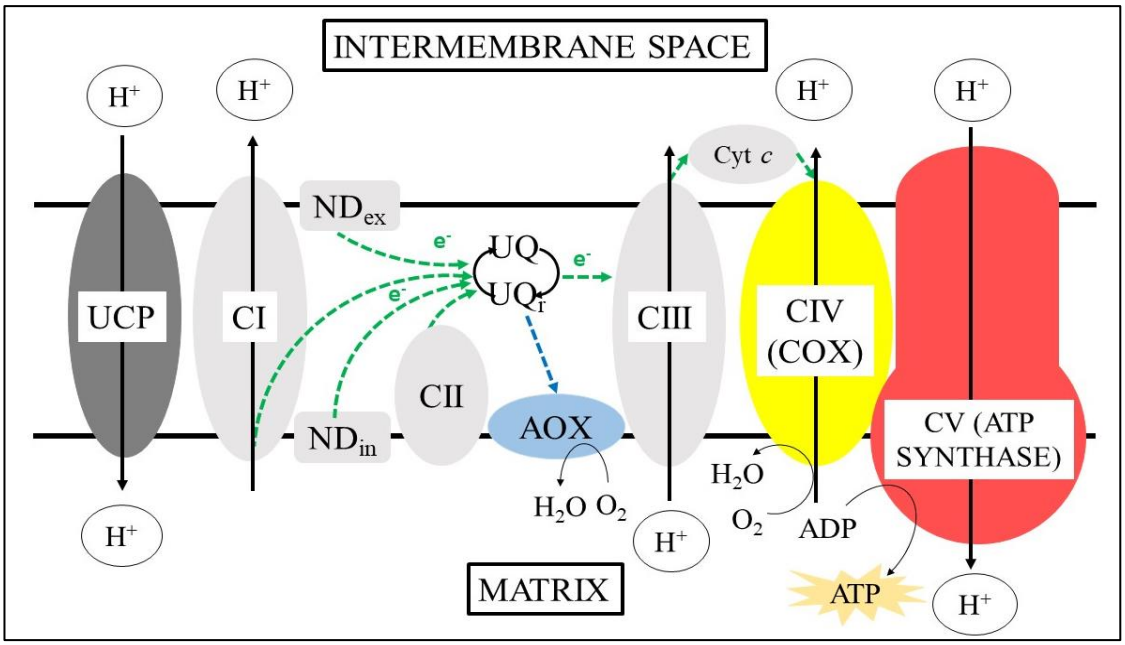

Figure 1.5. Plant mitochondrial electron transport chain (ETC) oxidizes NADH and $FADH_2$ generated by TCA cycle and reduce oxygen to water. Oxidation by CI in which electrons are transferred to CIV (COX) indirectly is coupled to proton transport from the matrix to the intermembrane space of mitochondria whereas oxidation by AOX is not coupled to the proton transport. The former generates a proton motive force that is subsequently dissipated by CV (ATP synthase) to produce ATP. In contrast, electrons flow via AOX does not result in ATP production. Abbreviations: CI: NADH ubiquinone oxidoreductase; CII: succinate dehydrogenase; CIII: cytochrome *b/c*$_1$ complex; CIV: cytochrome *c* oxidase (COX); CV: ATP synthase; UCP: uncoupling protein; AOX: alternative oxidase (AOX); e^-: electrons; ND_{ex} and ND_{in}: external and internal-oriented alternate NAD(P)H dehydrogenases, respectively; UQ and UQ_r; oxidized and reduced ubiquinone, respectively.

1.9 Rice and chickpea as important crops

Rice is arguably the most important crop in the world, with more than half of world's population relying on rice every day (Khush, 2005). Data provided by FAOSTAT (2017) highlights the extent of rice importance. Rice is a staple food for over four billion people in Asia, around one billion in Africa and hundreds of millions in Latin America. The world's largest producer of rice is China, followed by India and Indonesia. In China, more than 208 million tonnes (6.8 tonnes per hectare) of rice was produced in 2014, whereas in India and Indonesia, 157 (3.5 tonnes per hectare) and 70 million tonnes (5.1 tonnes per hectare) of rice were produced, respectively. These countries together produce nearly

60% of the global production of rice. The world distribution of rice-growing regions is presented in Figure 1.5 (Laborte et al., 2017). Many of these regions have current temperatures approaching critical limits for optimal grain production during their susceptible growth stages (Peng et al., 2004; Prasad et al., 2006). Exceeding these critical upper limits for optimal rice production is predicted to significantly reduce rice yield (Redfern et al., 2012). Reduced rice yield caused by climate change will have major implications for global food security (Adhikari et al., 2015; Gautam et al., 2013; Pachauri et al., 2014; Tripathi & Mishra, 2017).

There are two main subspecies of Rice: Japonica (*Oryza sativa subsp. japonica*) and Indica (*O. sativa subsp. indica*) (Lu et al., 2009; Xu et al., 2009). Japonica rice plants are usually grown in high altitudes, and in temperate regions such as Japan, Spain and Portugal. Indica rice plants are mainly grown in tropical and subtropical regions with either low latitudes or altitudes, such as in the lowlands of the Philippines, India and Pakistan (Agrama et al., 2010; Lu et al., 2009). Japonica are relatively short plants that have narrow and dark green leaves, with medium-height tillers. The grains are short and round, do not easily shatter and have lower amylose content compared to Indica. By contrast, Indica plants are taller and have broad to narrow light green leaves. The grains are long to short, slender, and shatter more easily compared to Japonica (Awan et al., 2017).

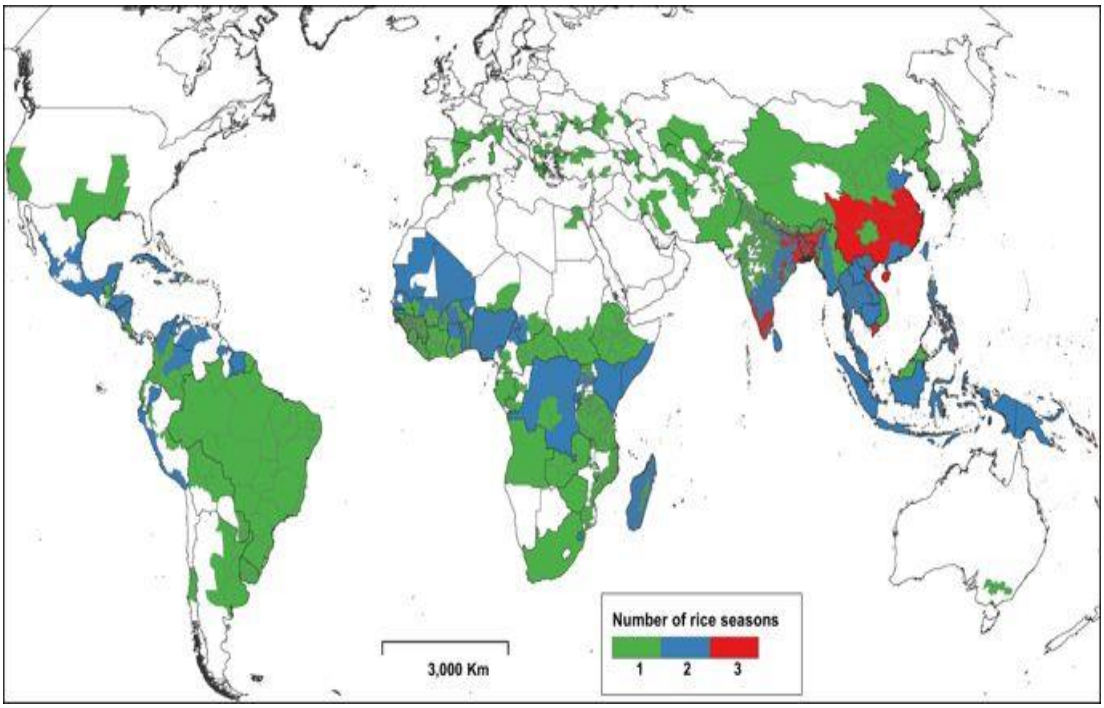

Figure 1.6. Rice growing regions and the number of rice growing seasons provided by RiceAtlas (Laborte et al., 2017).

Some studies have examined photosynthesis and respiration temperature acclimation in rice but not in detail or in combination with one another. In regards to photosynthesis, Nagai and Makino (2009), grew rice plants at colder temperature (i.e. 19°C day, 16°C night) and noted maximum rates of photosynthesis at this lower temperature. An increase in growth temperature (i.e. to 25°C day, 19°C night) resulted in an increase in the T_{opt}, showing acclimation potential of photosynthesis can occur in rice. Associated with the observed shift in T_{opt} was temperature dependent changes in the allocation of nitrogen to Rubisco. In regards to respiratifon, not many studies have been conducted in rice, particularly during the vegetative stage. There is some data available during the reproductive and ripening stages. For example, Peraudeau et al. (2015) grew rice plants at a constant day temperature of 29°C with varying night temperature (i.e. 21°C and 26°C) and light (i.e. shaded and normal light condition) preceding measurements of respiration rates at night. Respiration rates increased with warmer night

temperatures. Acclimated leaves from the plants grown under normal light conditions, with a colder temperature of 21°C at night exhibited higher rates of respiration compared to warmer night grown plants that were shaded. The results suggested that respiration did acclimate to temperature changes in rice, but was dependent on light driven carbohydrate status (Peraudeau et al., 2015).

Increased respiration stimulates the production of reactive oxygen species (ROS), which can overwhelm antioxidant levels and decrease membrane thermal stability, leading to cell damage. Mohammed and Tarpley (2009) investigated the effect of high night temperature of 32°C on respiration, membrane stability, antioxidant capacity (all measured during ripening stage) and yield of rice plants. The ambient night temperature of 27°C was used as a control. They found that high night temperature increased respiration rates, decreased membrane thermal stability and negatively affected the yield by 95%. In contrast to plants applied with salicylic acid, the reduction in yield due to high night temperature was lowered to 76% by decreasing respiration rates, increasing membrane thermal stability and antioxidant capacity. Although it is known that enhanced respiration due to high temperature reduces yield, the extent to which respiration in rice acclimates to sustained changes in growth temperature during the vegetative stage is still not extensively studied and unclear. Furthermore, investigations of how respiration and photosynthesis simultaneously respond to temperature changes in rice is lacking.

Besides rice, chickpea (*Cicer arietinum*) is also important to the growing population due to the richness in nutrients. Chickpea is a cool-season, leguminous crop, cultivated widely in the Asia Pacific region (Sharma et al., 2006), predominantly in India (Verma et al., 2014). In 2014, India produced approximately 9.5M tonnes of chickpea, accounting for more than 70% of global production. Besides India, chickpea is also produced by Australia and Pakistan with 629,400 and 399,030 tonnes in 2014,

respectively (FAOSTAT, 2017). There are two varieties of chickpea: desi and kabuli, mainly distinguished by the size, shape and colour of the seeds (Knights et al., 2011; Purushothaman et al., 2014). Desi typically have small angular seeds that are wrinkled at the beak. Colour of desi seeds usually darker than kabuli with range in colour including brown, light brown, fawn, yellow, orange, black or green. By contrast, kabuli typically have larger and rounder seeds, with white to cream in colour. Colour of the flowers is also different with desi typically have pink flowers, whereas kabuli have white flowers (Bantilan et al., 2015; Knights et al., 2011; Sharma et al., 2013).

Chickpea is a cool season crop, however, a low temperature stress typically encourages a prolonged vegetative phase and reduces pollen viability and germination (on the stigma) during reproductive phase (Devasirvatham et al., 2015). Chickpea is also a heat sensitive crop where the exposure of chickpea to increasing temperature due to climate change is expected to markedly reduce yield (Kaushal et al., 2011; Lake et al., 2016). Previous reports have indicated adverse effects of high temperature on chickpea. For example, in Devasirvatham et al. (2012), gradual increase in temperature (29/16°C to 40/25°C) affected the reproductive development of chickpea, which eventually caused major loss of yield, by decreasing pollen production per flower, pollen germination, pod set and seed number, relative to the control treatment at 27/16°C. Similarly in Wang et al. (2006), seed yield was decreased by 53-59% when the high temperature stress (35/16°C) was imposed for 10 days during the pod development stage of the 20/16°C grown plants. With increasing demand, the yield reduction of this new alternative cash crop, besides maize and soybeans, is really worrying. Knowledge on the basic mechanisms that control the growth process in chickpea, particularly with regard to carbon balance in current fluctuating environments is still unclear.

1.10 Thesis objectives and outline

The thesis I present here is in response to the importance of carbon balance (i.e. respiration and photosynthesis) to crop biomass and yield, the importance of crops, particularly rice, to world food security, and the impact climate warming will have on these processes. This thesis consists of five chapters:

- Chapter 1 – This chapter describes some background of the research presented in the thesis including how climate change influencing plant physiological processes and eventually affecting the yield of crops. Chapter 2 – I used rice plants in this chapter. I first quantify photosynthesis and respiration in fully expanded: (1) pre-existing leaves (PE leaves) which were the leaves that emerged in warm control condition and being transferred to either a cold and hot environment, and; (2) newly developed leaves (ND leaves) which were the leaves that developed in their new temperature regimes. This is to determine the extent of thermal acclimation of both photosynthesis and respiration in rice to varying temperature. Thereafter, I investigated mechanisms associated with variability in rice leaf respiration rates following sustained changes in temperature – at the levels of transcript, protein and carbohydrates. Generally, I hypothesised that thermal acclimation of photosynthesis and respiration is greater in ND leaves compared to PE leaves. Associated with the acclimation to sustained temperature changes would be the alteration in protein and transcript abundance in key pathways.
- Chapter 3 – I used rice plants in this chapter. I determined if the rate of dark respiration, particularly in cold and hot acclimated plants, is affected by fluctuating diurnal temperature regimes. Further, I profiled the metabolites of carbon metabolism across the day/night cycle to assess how growth temperature and time of day affect the pool sizes of respiratory metabolites. I hypothesised

that rice grown in hot would exhibit greater diurnal variation in respiration compared to cold grown rice, when measured at a common temperature, reflecting greater diurnal variation of substrate availability over the day/night cycle.

- Chapter 4 – I used chickpea plants in this chapter. This chapter focusses on the measurements of O_2 consumption in isolated mitochondria *in vitro* of plants grown at different temperature treatments. The initial plan for this chapter was to isolate mitochondria from rice leaves. However, due to the leaf toughness, it was difficult to liberate intact mitochondria without also disrupting them. Thus, I switched to another important crop – chickpea, to study the mechanistic basis of thermal acclimation of leaf dark respiration, adding the organelle level work needed for this thesis. I hypothesised that thermal acclimation of respiration is linked to concomitant changes in the rates of O_2 uptake per unit mitochondrial protein due to changes in the COX and AOX capacity in isolated mitochondria.
- Chapter 5 – This final chapter of the thesis discusses the findings in general. The significance of the results and how the results fit the broad research field is discussed. Future directions of the research is also mentioned here.

Chapter 2 – Molecular-physiological responses during thermal acclimation of photosynthesis and leaf respiration in rice

2.1 Abstract

To further my understanding of how sustained changes in temperature affect the carbon economy of rice (*Oryza sativa*), hydroponically-grown plants of the IR64 cultivar were developed at 30/25°C (day/night) before being shifted to 25/20°C or 40/35°C. Leaf mRNA and protein abundance, sugar and starch concentration, gas-exchange and elongation rates were measured on pre-existing leaves (PE) already developed at 30/25°C, or leaves newly-developed (ND) subsequent to temperature transfer. There was a transient adjustment in metabolic gene transcript abundance of PE leaves before homeostasis was reached within 24 h, aligning with R_{dark} (leaf dark respiratory CO_2 release) and A_n (net CO_2 assimilation) perturbations. With longer exposure, the central respiratory protein cytochrome *c* oxidase (COX) declined in abundance at 40/35°C. In contrast to R_{dark}, A_n was maintained across the three growth temperatures in ND leaves. Soluble sugars did not differ significantly with growth temperature, and growth was fastest with extended exposure at 40/35°C. The results highlight that acclimation of photosynthesis and respiration is asynchronous in rice, with hot acclimated plants exhibiting a striking ability to maintain net carbon gain and growth when exposed to heat-wave temperatures.

2.2 Introduction

The response of net CO_2 assimilation (A_n) and leaf respiration in the dark (R_{dark}) to changes in temperature (T) is often dynamic, with adjustments in metabolic rates taking place following short- and long-term changes in T. Acclimation – physiological changes

that enable adjustments in the rate of A_n and R_{dark} at a given measuring T in response to sustained changes in growth T – is observed over a wide range of species, in natural and under controlled experimental conditions (Atkin et al., 2005a; Berry & Bjorkman, 1980; Campbell et al., 2007; Reich et al., 2016; Smith & Dukes, 2017; Tjoelker et al., 2001). Acclimation, which can occur within 1-2 day period following a T transfer can also lead to metabolic homeostasis (i.e. similar rates of A_n and R_{dark} being exhibited by hot and cold acclimated plants, when compared at their respective growth temperatures). Determining the extent to which R_{dark} and A_n acclimate to sustained changes in growth T is of growing interest, as climate warming is resulting in plants of natural and managed ecosystems experiencing higher average growth Ts, often in conjunction with more frequent and severe heat waves (CSIRO & BOM, 2018; Hartmann et al., 2013). The impact of heat on A_n and R_{dark} of cereal crops – including that of rice (*Oryza sativa*) – is of particular interest, given the need to increase food production to meet the requirements of a growing and more affluent world population (Godfray et al., 2010). Rice contributes substantially to global food demand, particularly in Asia where it makes up more than 30% of all dietary energy intake (Seck et al., 2012). However, in recent years rice yields have declined in regions such as South-East Asia, with the declines being more strongly correlated with nightly minimum T than daytime maximum T, (Peng et al., 2004; Welch et al., 2010). Reduced yields and grain quality were also observed for rice in North America when exposed to warmer night T (Lanning et al., 2011). Given this, and the importance of A_n and R_{dark} for grain production, it is crucial that we develop a better understanding of how changes in T affect these key metabolic processes in rice.

In a range of crops (including rice), RNA sequencing analysis has shown large scale perturbations to the gene profile when plants are exposed to colder and warmer T, with the changes occurring over a period of hours to days and across multiple functional

categories, but especially in primary metabolic pathways (Bhardwaj et al., 2015; Hu et al., 2014; Shen et al., 2014). The vast gene regulatory response to *T*-perturbations is likely mediated through heat shock transcription factors, which regulate changes in transcriptional networks (Ohama et al., 2017). These are induced by abiotic stimuli including heat stress (Morimoto, 1998). Importantly, *T*-mediated changes in RNA abundance may not necessarily be reflected in changes in protein abundance, as transcription and RNA turnover rates are influenced by *T* independent of active regulation (Sidaway-Lee et al., 2014). In cases where heat stress results in changes in protein concentration, the greatest changes are seen in proteins associated with primary metabolism, with ≈ 50% of all leaf protein abundance changes seeming to be in A_n and R_{dark} metabolic pathways (Scafaro & Atkin, 2016). In rice, cold-stress similarly perturbs a large proportion of energy metabolism pathways (Neilson et al., 2011), emphasising the importance of changes in protein abundance in chloroplasts and mitochondria as part of the thermal acclimation process. There is growing evidence that plants can acclimate by stimulating energy metabolism at colder *T* - and suppressing energy metabolism at warmer *T* - either through regulation of associated enzymatic velocities or changes in enzyme abundance (Armstrong et al., 2008; Badger et al., 1982; Campbell et al., 2007; Hikosaka et al., 2006; Scafaro et al., 2017b; Strand et al., 1999; Yamori et al., 2005). Whether the same is true for rice remains unclear, both for pre-existing (PE) leaves that experience a sustained change in growth *T*, and in newly-developed (ND) leaves that form under a new thermal regime. In plants other than rice, the extent of changes underpinning thermal acclimation (including changes in leaf structure, nitrogen partitioning and organelle abundance), is typically greater in ND than PE leaves (Armstrong et al., 2008; Gorsuch et al., 2010; O'Leary et al., 2018; Tjoelker et al., 1999; Yamori et al., 2005).

Past studies on rice conducted during the vegetative (Glaubitz et al., 2014; Kurimoto et al., 2004a) and reproductive (Bahuguna et al., 2017; Mohammed et al., 2013) phases of development have reported limited and variable levels of acclimation of R_{dark}. By contrast, A_n of rice shows stronger thermal acclimation, with rates of net CO_2 uptake measured at the prevailing growth *T* being homeostatic or increasing in rice as growth *T* is increased from 15 to 37°C (Nagai & Makino, 2009; Yamori et al., 2010). While such studies point to a possible asynchrony of A_n and R_{dark} thermal acclimation in rice, a finding supported by work on a range of other crops and non-crop species (Campbell et al., 2007; Drake et al., 2016; Way & Oren, 2010), further work simultaneously comparing thermal acclimation of A_n and R_{dark} in rice is needed. Such studies need to integrate simultaneous measurements of A_n and R_{dark} with analyses of changes in chemical composition, protein abundance and gene expression.

It is with the above issues in mind that I conducted a study using the IR64 cultivar of *Oryza sativa* to address the following aims: (1) characterise the extent of thermal acclimation of R_{dark} and A_n; and (2) link physiological acclimation to the underlying processes through analysis of molecular (transcript abundance), biochemical (protein abundance), and metabolic (sugar and starch concentration) changes at different time points after changes in growth *T*. I hypothesised that: (1) initial exposure to very high growth *T* increases rates of respiratory CO_2 release and decreases net photosynthetic CO_2 uptake; (2) subsequent acclimation is associated with recovery of A_n, and reduced rates of R_{dark}; and (3) thermal acclimation of R_{dark} and A_n is associated with dynamic changes in transcript and protein abundance, starting within the first 24 h following *T* transfer. For example, increases in AOX and uncoupling protein that potentially reduce ATP production at high growth temperatures.

2.3 Materials and methods

2.3.1 Plant materials and temperature treatments

Rice (*Oryza sativa*) cultivar IR64 plants were grown hydroponically in a glasshouse facility at the Research School of Biology, Australian National University in Canberra between October and December 2015. Seeds were initially incubated at 40-42°C for two days before soaking in water for eight hours, placed on wet Whatman filter papers in petri dishes and kept in the dark at 30°C for five days. The germinated seedlings were then transferred to trays of vermiculite and placed in temperature-controlled glasshouses (30°C day and 25°C night) under natural sunlight and photoperiod with photosynthetically active radiation (PAR) between 400 and 1200 µmol m^{-2} s^{-1}. When the third leaf had emerged, seedlings were transplanted from vermiculite to a hydroponic system. Individual plants were placed within PVC tubes with a 3.7 cm diameter and 13 cm height. Tubes were then suspended at the top of 20 L capacity hydroponic tanks (12 tanks in total), with each tank holding a maximum of 20 plants. Each tank across all three temperature treatments was filled with 16 mL hydroponic solution containing NH_4NO_3 (1.4 mM), $NaH_2PO_4.2H_2O$ (0.6 mM), K_2SO_4 (0.5 mM), $CaCl_2.2H_2O$ (0.2 mM), $MgSO_4.7H_2O$ (0.8 mM) and micronutrients: Fe-EDTA (0.07 mM), H_3BO_3 (0.037 mM), $MnCl_2.4H_2O$ (0.009 mM), $ZnSO_4.7H_2O$ (0.00075 mM), $CuSO_4.5H_2O$ (0.0003 mM), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.0001 mM), NH_4VO_3 (0.000138 mM) and Na_2SiO_3 (0.0012963 mM) (Supplementary Table S2.1) (Hubbart et al., 2007). The nutrient solution was replaced weekly. Sulphuric acid or sodium hydroxide were used to adjust the pH to between 5-6, with pH monitored daily using a portable pH meter (Rowe Scientific Pty. Ltd., NSW, AU). The hydroponic solution was aerated continuously using Infinity AP-950 air pumps (Kong's Pty. Ltd., Ingleburn, AU).

After two weeks of hydroponic growth at 30/25°C ('warm' treatment), the most recently fully-expanded leaves were labelled as pre-existing (PE) leaves. Following labelling, four tanks were randomly chosen and shifted to an adjacent glasshouse room set to 25°C day and 20°C night (25/20°C – 'cold' treatment), and another four tanks moved to a room set at 40°C day and 35°C night (40/35°C – 'hot' treatment); four tanks were retained at 30/25°C as controls. Newly-developed (ND) leaves that emerged in each thermal regime were labelled, with all measurements reported on ND leaves being made 21-days after *T* transfer. For all experiments, four separate leaves from separate plants, one from each hydroponic tank (pot replicates) per *T* treatment were sampled.

2.3.2 Determination of transcript abundance

To quantify transcript abundance following changes in thermal regimes, the labelled pre-existing (PE) leaves were harvested two, six and 24 h after the shifting of the plants three hours into the day period so that all sample points were collected during daylight hours (i.e. under light). For each time point and temperature treatment, approximately 8 cm of middle leaf section (less than 100 mg) were sampled by immediately freezing leaf material in liquid N_2 and storing at -80°C until RNA extraction. Total RNA was extracted using the RNeasy plant mini protocol (Qiagen, Doncaster, VIC, AU) and treated with DNase I (Qiagen, Doncaster, VIC, AU) to remove any contaminating DNA.

For qPCR analysis, 1 µg of total RNA in a 10 µL volume was reverse transcribed into cDNA using SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The reverse-transcribed cDNA samples were diluted 10-fold. Transcript levels of six selected genes and one reference gene (refer to Supplementary Table S2.2 for gene accession numbers and primer sequences – each primer was designed specifically for a single gene target) were analysed

using a Light-Cycler® 480 System (Roche Holding AG, Basel, Switzerland) with SYBR Green I Dye (QIAGEN, Doncaster, Victoria, AU). cDNA samples from each biological replicate were assayed in two technical duplicates. The reaction-mix in each qPCR contained 0.4 μM of each pair of primers, 5 μL of SYBR Master Mix, and 4.6 μL of the diluted cDNA sample in a 10 μL total reaction volume. The raw fluorescence data was analysed using LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009). Data acquired from each sample was then normalized to the reference gene eukaryotic initiation factor 5c (*EIF5C*; LOC_Os11g21990.1) and were expressed as fold changes against control conditions.

RNAseq libraries were prepared using the Illumina Stranded Total RNAseq kit with RiboZero rRNA depletion as per the manufacturer's guidelines (Illumina). Libraries were pooled and sequenced on a HiSeq1500 for 61 cycles in single end mode at the Centre for AgriBioscience, University of LaTrobe. Analysis pipelines for pre-processing and mapping of sequence data are available online on GitHub (https://github.com/pedrocrisp/NGS-pipelines). Quality control was performed with *FastQC* v.0.11.2. Adapters were removed using *scythe* v.0.991 with flags -p 0.01 for the prior and reads were quality trimmed with *sickle* v.1.33 with flags -q 20 (quality threshold) -l 20 (minimum read length after trimming). The trimmed and quality filtered reads were aligned to the rice reference genome Os-Nipponbare-Reference-IRGSP-1.0 from the MSU Rice Genome Annotation Project Database v7 (http://rice.plantbiology.msu.edu/) using the *subjunc* aligner v1.5.0-p1 with -u and -H flags to report only reads with a single unambiguous best mapping location, -P 3 for phred+33 encoding (Liao et al., 2013). Reads were then sorted, indexed and compressed using *samtools* v1.1-26-g29b0367 (Li et al., 2009) and strand-specific bigwig files were generated using *bedtools genomecov* v2.16.1 (Quinlan & Hall, 2010) and the UCSC

utility *bedGraphToBigWig* for viewing in IGV (Robinson et al., 2011). Summary statistics for each sample are provided in Supplementary Dataset S1: Summary of transcriptomic datasets.

For standard differential gene expression testing, the number of reads mapping per IRGSP-1.0 gene loci was summarised using *featureCounts* v1.5.0-p1 (Liao et al., 2014) with flags -P and -c to discard read pairs mapping to different chromosomes and the -s flag set to 2 for strand specificity for a strand specific library, multimapping reads and multioverlapping reads were not counted. Reads were summarised to parent IRGSP-1.0 gene loci rather than individual splice variants by summarising to the genomic coordinates defined by the feature "gene" in the IRGSP-1.0.gff reference (last modified 7/2/2012 ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/all.gff3). Only loci with an abundance of at least 1 CPM (approximately 5 reads) in at least 4 samples were retained. Statistical testing for relative gene expression was performed in R following the 'edgeR-limma-voom' approach (https://www.bioconductor.org/help/workflows/RNAseq123/); using, 'edgeR' v.3.4.2 (McCarthy et al., 2012; Robinson et al., 2011; Robinson & Oshlack, 2010; Robinson & Smyth, 2007, 2008), and 'voom' in the 'limma' package 3.20.1 (Law et al., 2014; Smyth, 2004; Smyth et al., 2005).

To investigate the extent to which the present genes have a photosynthetic or respiratory function, MapMan analysis was performed using MapMan v3.5.1R2 (Thimm et al., 2004) using the annotated rice glycolysis and TCA; photosynthesis and mitochondrial electron transport chain pathways. MapMan annotates each pathway using the rice genes (MSU v7 annotation), based on similarity matches to Arabidopsis genes. In the glycolysis and TCA pathway 209 genes were annotated, in the photosynthesis

pathway 202 rice genes were annotated and in the mitochondrial electron transport chain pathways 176 genes were annotated. MapMan performs a statistic test (Wilcoxon Rank Sum Test with Benjamin Hoochberg correction for multiple testing) to check if any 'bins' (subsets of the pathways) exhibit a different behaviour in terms of expression profile compared to all the other remaining bins.

Gene Ontology enrichment analysis was also conducted based on a dataset from http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/all.GOSlim_assignment. Thereafter, a statistical analysis was performed in R using 'topGO' tool (Alexa & Rahnenfuhrer, 2019).

2.3.3 Determination of protein abundance

Samples of frozen PE leaf material six and 24 h after *T* transfer, as well as frozen ND leaf material, were ground to a fine powder using a chilled mortar and pestle, and protein was extracted in extraction buffer containing 100 mM tricine pH 8.0, 1 mM EDTA, 1 mM PMSF, 1x protease inhibitor cocktail, 2% (w/v) PVPP, 10 mM (w/v) ascorbate, 5 mM DTT and distilled water. The sample was then solubilized in a NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) with 10% (v/v) DTT, then heated for 10 minutes at 95°C, and centrifuged for 30 sec at 12,000 RPM. Thereafter, supernatant was collected and 8 uL were loaded and separated on 4-12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) using the MOPS-based buffer system. To blot, proteins from the gel were transferred to Immobilon-P Polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Kilsyth, VIC, AU) using an XCell II Blot module (Invitrogen, Carlsbad, CA, USA). Membranes were then blocked for 2 h with 5% (w/v) skim-milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST). To probe for cytochrome *c* oxidase (COX) subunit II, alternative oxidase (AOX), uncoupling protein (UCP) and

voltage-dependent anion-selective channel protein (*VDAC1*-porin), the membranes were incubated for 2 h in primary antibody solution (5% w/v skim milk powder in TBST) containing commercially available polyclonal antibodies (Agrisera, Vännäs, Västerbotten, Sweden). All antibodies were diluted 1:5000. An antibody for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was received from Assoc. Prof. Spencer Whitney (Research School of Biology, Australian National University, Canberra, ACT, AU) and used at a dilution of 1:10 000. After washing with TBST, the membranes were incubated for 1 h in goat anti-rabbit antibody solution (5% w/v skim milk powder in TBST) at a dilution of 1:8000. Proteins were then visualized using the AttoPhos AP fluorescent substrate system (Promega, Madison, WI, USA), imaged using a Versa-Doc (Bio-Rad, Hercules, CA, USA) imaging system and quantified using Image Lab software (Bio-Rad, Hercules, CA, USA). Protein concentration were determined by Bradford method using Bovine Serum Albumin (BSA) as a standard.

2.3.4 Determination of soluble sugar and starch concentration

Fully-expanded PE and ND leaves were collected from 9:30 to 10:00 am, corresponding to 3 h into the day period, frozen and stored at -80°C before freeze-drying at -105°C for two days (Virtis Benchtop™ "K", SP, Scientific, Gardiner, NY, USA), then ground to a fine powder and 5-10 mg separated. Five-hundred μL of 80% (v/v) ethanol was added and vortexed for 20 sec. Thereafter, leaf tissues were incubated at 80°C while being centrifuged at 500 RPM for 20 min. Following further centrifugation at 12,000 RPM for 5 min, the resulting pellet and supernatant was separated. This procedure was repeated a further two-times pooling pellet and supernatant. The supernatant and pellet were used for determination of the concentration of soluble sugars and starch using a Fructose Assay Kit (Sigma-Aldrich, St Louis, MO, USA) and a Total Starch Assay Kit (Megazyme,

Chicago, IL, USA), respectively. Measurements were made following manufacturer's instructions in triplicate using a microtitre plate reader (Infinite® M1000 Pro; Tecan US, Morrisville, NC) and a standard curve generated for soluble sugars using sucrose, glucose and fructose (Sigma-Aldrich, St Louis, MO, USA) at known concentrations.

2.3.5 Gas exchange measurements using Licor 6400XT 6 cm^2 cuvettes

Gas-exchange was measured on fully-expanded pre-existing (PE) leaves just prior to and one, two, three, five and seven days after *T* transfer, as well as on fully-expanded newly-developed (ND) leaves 21 days after transfer using two matched LI-6400 instruments with 6 cm^2 cuvettes and a 6400-02B red-blue light source (Li-Cor, Lincoln, NE, USA). At each time point, light-saturated net CO_2 assimilation (A_n) and then dark respiration (R_{dark}) were measured during the day period (between 10:00 and 14:00 hours) in the glasshouses at the prevailing day-time *T* of each treatment as well as a common temperature of 30°C for ND leaves 21-days after transfer to the cold and hot treatments. In all cases, A_n was measured first, with the following settings: 1000 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), relative humidity of 60–70%, 400 ppm reference CO_2, and a flow rate of 500 μmol s^{-1}. Photosynthesis was measured when CO_2 concentration in the sample Infrared Gas Analyzer (IRGA) had stabilized, typically within 10 minutes of exposure to 1000 μmol m^{-2} s^{-1} PPFD. Thereafter, R_{dark} was measured as above but with the flow rate slowed to 300 μmol s^{-1} and turning off the light source for at least 30 minutes of darkness before taking measurements.

2.3.6 Gas exchange measurements using Walz chambers

High resolution temperature response curves of R_{dark} and light-saturated A_n were made on intact ND leaves using two matched LI-6400XT portable gas exchange systems (Li-Cor,

Lincoln, NE, USA) each connected to a 14 x 10 cm well-mixed, temperature-controlled Walz Gas-Exchange Chamber 3010-GWK1 (Heinz Walz GmbH, Effeltrich, Germany). For each temperature-response curve, leaf T was measured with a small-gauge wire copper constantan thermocouple pressed against the lower surface of the leaf and attached to a LI-6400 external thermocouple adaptor (LI6400-13, Li-Cor Inc., Lincoln, NE, USA) that enabled leaf temperature to be recorded by the LI-6400XT. As leaves were heated, net CO_2 exchange was recorded at 30 s intervals using the LI-6400XT portable gas exchange systems fitted with an empty and closed 6 cm^2 chamber that was plumbed into the airstream exiting the Walz leaf chambers. A_n was measured as described for the 6 cm^2 cuvette but using a Walz LED-Panel RGBW-L084 light source (Heinz Walz GmbH, Effeltrich, Germany). Rates of A_n were monitored as leaves were heated at 1°C min^{-1} from 20 to 45°C. For R_{dark}, on separate leaves to those measured for A_n, the flow rate was reduced to 300 μmol s^{-1} and black cloth used to cover the chamber with no light source applied, and leaves heated at 1°C min^{-1} from 20 to 60°C. When quantifying the temperature-response curve of R_{dark}, I also measured minimal chlorophyll *a* fluorescence (F_o) in the presence of a low-intensity far-red light pulse (necessary to maintain PSII in the oxidized state) every 30 sec using a Mini-PAM portable chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany) fitted above the glass surface of the leaf chamber. The temperature at which F_o increased was used as an indication of heat-induced damage to photosystem II, which I refer to as T_{crit}, calculated using the template of O'sullivan et al. (2013). At the cessation of measurements, the leaf materials used were imaged for leaf area calculation (ImageJ) and stored in paper bags, oven-dried at 70°C for two days and weighed to obtain the dry mass.

2.3.7 Leaf elongation rates

The leaf elongation rates (LER) of four leaves from separate plants from each temperature regime were measured at five separate time-points over a 24 h period. Measurements were made using a ruler, starting from the ligule of the second youngest leaf to its tip.

2.3.8 Statistical analyses

For all T treatments and collection times, four separate leaves from four separate previously unsampled plants, one plant from each of the four hydroponic tanks (pot replicates), were sampled. One-way analysis of variance was performed on Rdark and An gas-exchange experiments comparing temperature treatments. Two-way analysis of variance was performed on leaf mass to area ratio (LMA) and protein, starch, and sugar concentrations comparing time of sampling and temperature treatments. Gas-exchange and leaf biochemical statistical analysis was performed using GraphPad Prism (v 7) software. Statistical analysis of transcript abundance was performed using R statistical software (v 3.6.1) and packages as mentioned above.

2.3.9 Data availability

RNA-seq data are available under the GEO identifier GSE136045.

2.4 Results

2.4.1 Molecular and biochemical responses of leaves to temperature

Quantitative PCR was performed on specific genes of interest to elucidate the genetic response of PE leaves exposed to a change in *T* (Figure 2.1). Transcripts from the gene encoding the central electron transport chain cytochrome *c* complex (*cox*) exhibited a slight increase in PE leaves transferred from 30/25°C to 40/35°C, but a general decline over the 7 days post-transfer period for both 25/20°C and 40/35°C transferred leaves.

Genes encoding respiratory proteins that potentially reduce the production of ATP – alternative oxidase (*aox*) and uncoupling protein (*ucp*) – both showed an initial increase in expression within the first 24 h of transfer to the hotter 40/35°C, with the latter returning to maintained 30/25°C levels by 48 h. The photosynthetic electron transport gene ferredoxin NADP reductase (*fnr*), and a gene encoding the Calvin/Benson cycle enzyme phosphoribulokinase (*prk*) generally showed an increase in expression in the first 48 h at 40/35°C before being supressed for up to 5 days post transfer, suggesting a longer-term downward shift in photosynthetic machinery after an initial increase. A gene encoding sucrose phosphate synthase (*sps*) which is involved in the synthesis of sucrose from its precursors also spiked initially for the 40/35°C heat transfer, before being suppressed after 48 h of transfer. The sucrose phosphate synthase gene followed a similar expression profile to the respiratory and photosynthetic genes for both the cold and heat transferred leaves suggesting assimilate production/consumption and sucrose synthesis were coordinated in response to *T* perturbations. In general, the greatest perturbation to gene expression occurred within the first 24 h after transfer.

Based on the qPCR results showing that gene perturbations predominantly occurred within 24 h of shifting *T*, RNA-seq at two, six and 24 h after transfer of pre-existing (PE) leaves to new *T* were conducted. Following data quality control and filtering, transcript abundance for 19,308 rice genes were retained for differential expression testing. Around 20 M reads were obtained per sample, which were aligned to the Os-Nipponbare-Reference-IRGSP-1.0 rice reference genome [see Rashid et al. (2020) for Dataset S1]. Principal component analysis showed a substantial treatment effect in gene expression for the hot 40/35°C exposed leaves during the first six hours after *T* transfer compared to the other two 30/25°C and 25/20°C growth regimes (Figure 2.2). There was little variation between the cold 25/20°C and the warm control conditions.

After 24 h of growth at new *T*-regimes, limited variation in gene expression was observed between all of the three *T* (Supplementary Table S2.3).

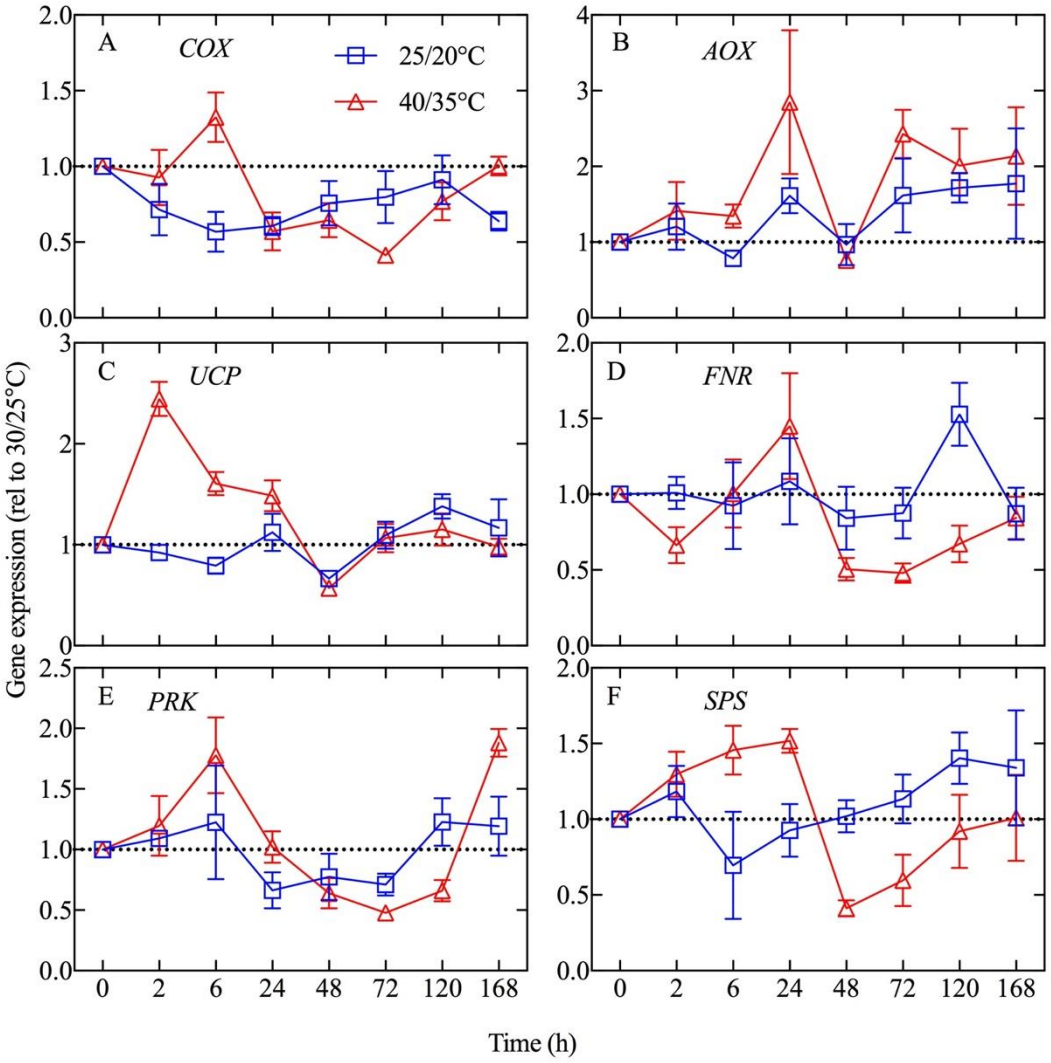

Figure 2.1. Quantitative PCR analysis of transcript abundance over the first 168 hours (7 days) after transfer of plants (PE leaves) from 30/25°C to 25/20°C or 40/35°C. Genes analysed were: the respiratory cytochrome c complex (*cox*) subunit II, alternative oxidase complex (*aox*) and uncoupling protein (*ucp*); the photosynthetic genes ferredoxin NADH reductase (*fnr*) and phosphoribulose kinase (*prk*); and the sugar metabolism gene sucrose phosphatase synthase (*sps*). The non-transferred 30/25°C control values were adjusted to 1 (dashed line) where 25/20°C values and 40/35°C values were normalised to at each time point. Data represents mean of 4 biological replicates ± SE and two-way ANOVA was carried out to test the differences between growth temperature and sampling time.

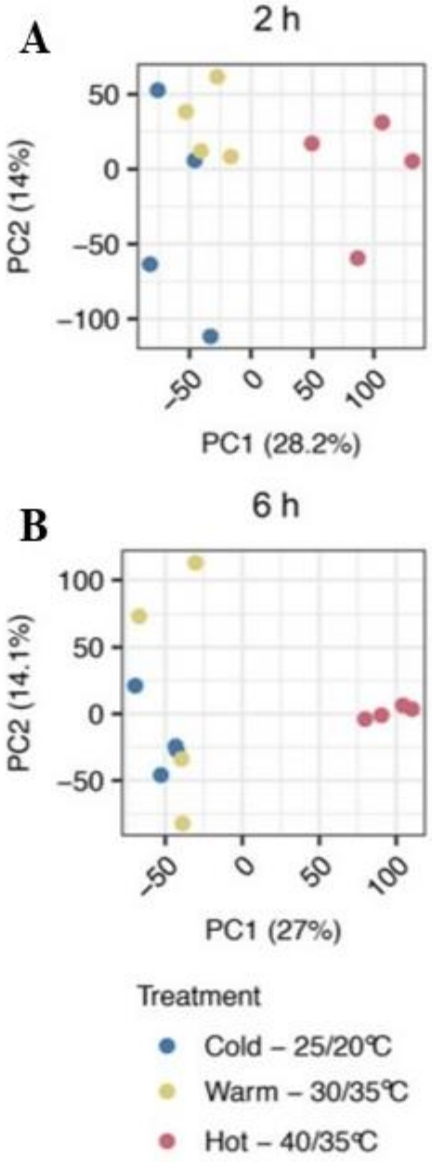

Figure 2.2. Principal component analysis of normalised RNA-seq expression values for each sample (PE leaves) following temperature treatment for (A) 2 hours and (B) 6 hours. Samples are coloured by treatment, day/night temperatures of 30/25°C (control), 40/35°C (hot), and 25/20°C (cold). The y-axis is principle component 1 (PC1) and the x axis is principle component 2 (PC2); the percent of variation explained by each axis is indicated. RNA-seq libraries were normalised using *edgeR* ("TMM" method) and *voom* transformation, scaled by unit variance and clustered using singular value decomposition.

To assess changes in the expression of individual genes, differentially expressed genes in the hot and cold samples were identified at each time point by comparison to the samples maintained under the warm control conditions [see Rashid et al. (2020) for Dataset S2]. There were very few genes differentially expressed in the cold conditions, six genes in total (Supplementary Table S2.3). By contrast there were 1,818 genes differentially expressed under hot conditions after two hours and 1,465 genes differentially expressed after six hours. After 24 hours, there were no differentially expressed genes under the hot conditions compared to the control plants. Comparing the genes differentially expressed after two hours and six hours of hot treatments, there was a significant overlap (Figure 2.3A-B). In total, 30% of the genes upregulated after six hours were also up regulated at two hours and 38% of genes downregulated after six hours were also downregulated at two hours. Many of the genes that were only significantly different in transcript abundance after six hours were already trending in the same direction at the two-hour time point, but the difference compared to the controls was not sufficient to pass the significance threshold (Figure 2.3C). Overall, these results show that there are significant short-term changes in transcript abundance in the leaves of rice plants exposed to high temperature. The expression profiles of samples exposed for two and six hours show consistent changes; however, some of the changes peak at two hours and others peak at six hours and most changes dissipate within 24 hours.

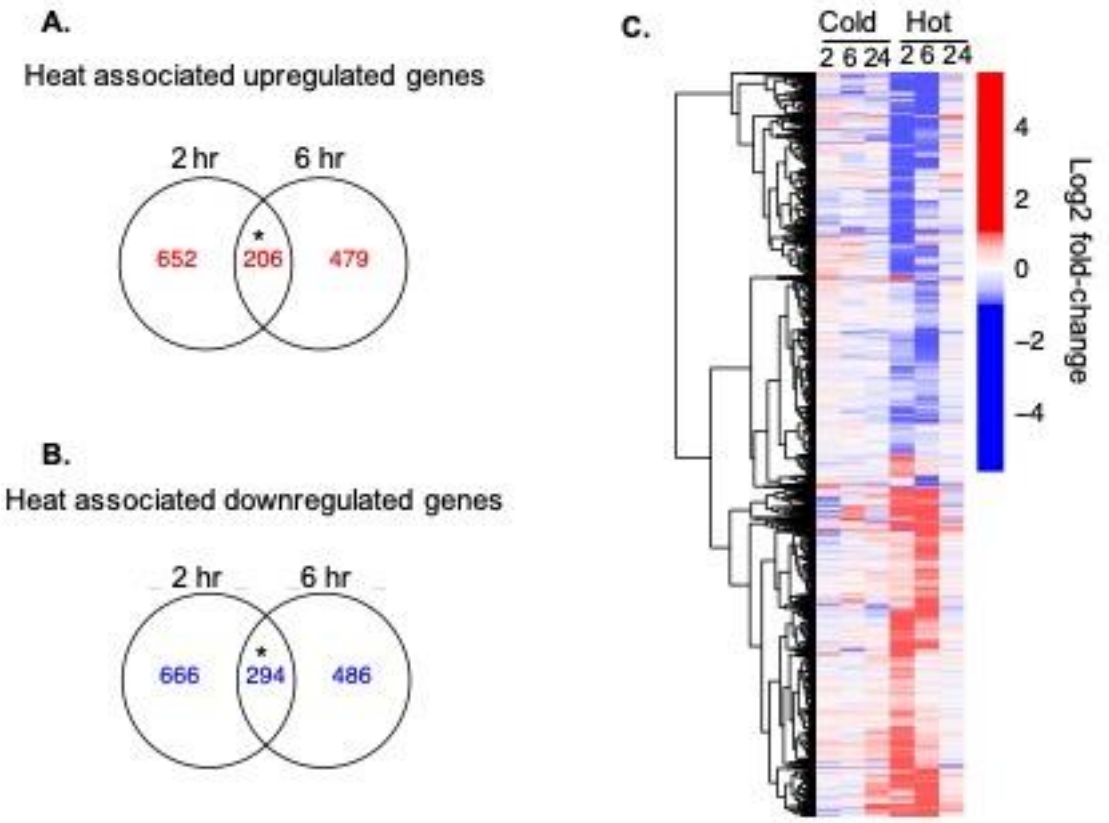

Figure 2.3. Identification of common and time point specific differentially expressed genes in PE leaves under heat treatment (40/35°C) (A and B). The overlap between genes differentially expressed at 2 and 6 hr under heat treatment for (A) upregulated genes and (B) downregulated genes. '*' indicates significant overlap $P < 0.001$, fisher's one-tailed exact test (hypergeometric). (C) Hierarchal clustering of differentially expressed genes. For each time point (2, 6 and 24 h) differentially expressed genes were determined for both the hot (40/35°C) and cold (25/20°C) temperature treatments relative to the warm (30/25°C) control conditions (false discovery rate < 0.05). For each differentially expressed gene, the relative fold change under each condition over the time series is then displayed on a log2 scale, red = upregulated, blue = downregulated.

In total, the hot treatment led to the up- and down-regulation of 1,337 and 1,446 genes, respectively. The qualitative MapMan analysis revealed that the expression of only a small number of photosynthetic/respiratory genes were affected (Supplementary Figures S2.1-2.3). To extract a list of high-confidence differentially expressed respiration related genes, a list of rice loci with homology to Arabidopsis respiration genes were manually curated [see Rashid et al. (2020) for Dataset S3]. Using this list, eight genes were differentially expressed at high temperature, with two genes down-regulated greater than 2-fold: *aox* and ATP-dependent phosphofructokinase (Table 2.1). The seemingly conflicting result of an initial increase in *aox* from the qPCR results, compared to the decline in *aox* during the same period from the RNA-seq results, can be explained by the qPCR being targeted to the *aox1a* isoform while the RNA-seq identified a decline in the *aox1c* isoform [see Rashid et al. (2020) for Dataset S3]. It is possible that the *aox1c* isoform is less tolerant of high temperatures and therefore is partially replaced by the *aox1a* isoform. In this context it is interesting that in Arabidopsis *aox1a* is the major stress-inducible isoform (Clifton et al., 2006; Giraud et al., 2008; Guy & Vanlerberghe, 2005). Since AOX operates as a non-covalently linked dimer (Siedow & Umbach, 2000), the change in the relative expressions of *aox1a* and *aox1c* isoforms may also indicate a change in the conformation of the AOX dimer, with a different mix of homo- and hetero-dimers in response to heat.

It is interesting that in addition to the increase in *aox* and *ucp* gene expression, the expression of an external NAD(P)H dehydrogenase also increased, while that of Complex II (catalytic subunit SDH2) decreased significantly (Table 2.1). Together these changes suggest an increase in non-phosphorylating electron transport occurred in response to exposure to higher *T*, at the expense of electron transport coupled to ATP synthesis, at least in the short term. The increase in external NAD(P)H dehydrogenase gene

expression may also indicate an increased need for mitochondrial oxidation of excess reductant produced in the chloroplast at higher *T*.

Table 2.1. Differential expression of respiration genes in PE leaves after exposure to *T* of 40/35°C relative to 30/25°C. Differential expression defined as false discovery rate < 0.05, marked as '*'. Electron transport chain (ETC), Pentose Phosphate Pathway (PPP). No TCA cycle genes were differentially expressed.

			Log2 fold-change	
Pathway	Gene_name	Locus	2 hours	6 hours
ETC	Complex II (Succinate dehydrogenase – catalytic subunit SDH2)	LOC_Os08g02640	-0.55*	-0.58*
ETC	External NAD(P)H dehydrogenase	LOC_Os06g47000	0.72*	0.44
ETC	Uncoupling protein	LOC_Os11g48040	0.81*	0.46
ETC	Alternative oxidase	LOC_Os02g47200	-0.99*	-1.91*
Glycolysis	ATP-dependent phosphofructokinase	LOC_Os01g53680	-0.11	-1.13*
Glycolysis	Phosphoglycerate kinase	LOC_Os02g07260	0.78*	0.39
Glycolysis	Enolase	LOC_Os10g08550	0.61*	0.34
PPP	Ribulose 5-phosphate 3-epimerase	LOC_Os09g32810	0.53*	0.87*

Given the relatively small effect of the heat treatment on the expression of respiration or photosynthesis related genes, Gene Ontology enrichment analysis were performed. This revealed a notable enrichment for genes involved in primary metabolism

(eg GO:0044238) and response to abiotic stimulus (eg GO:0050896), as well as many biosynthetic pathways (Figure 2.4).

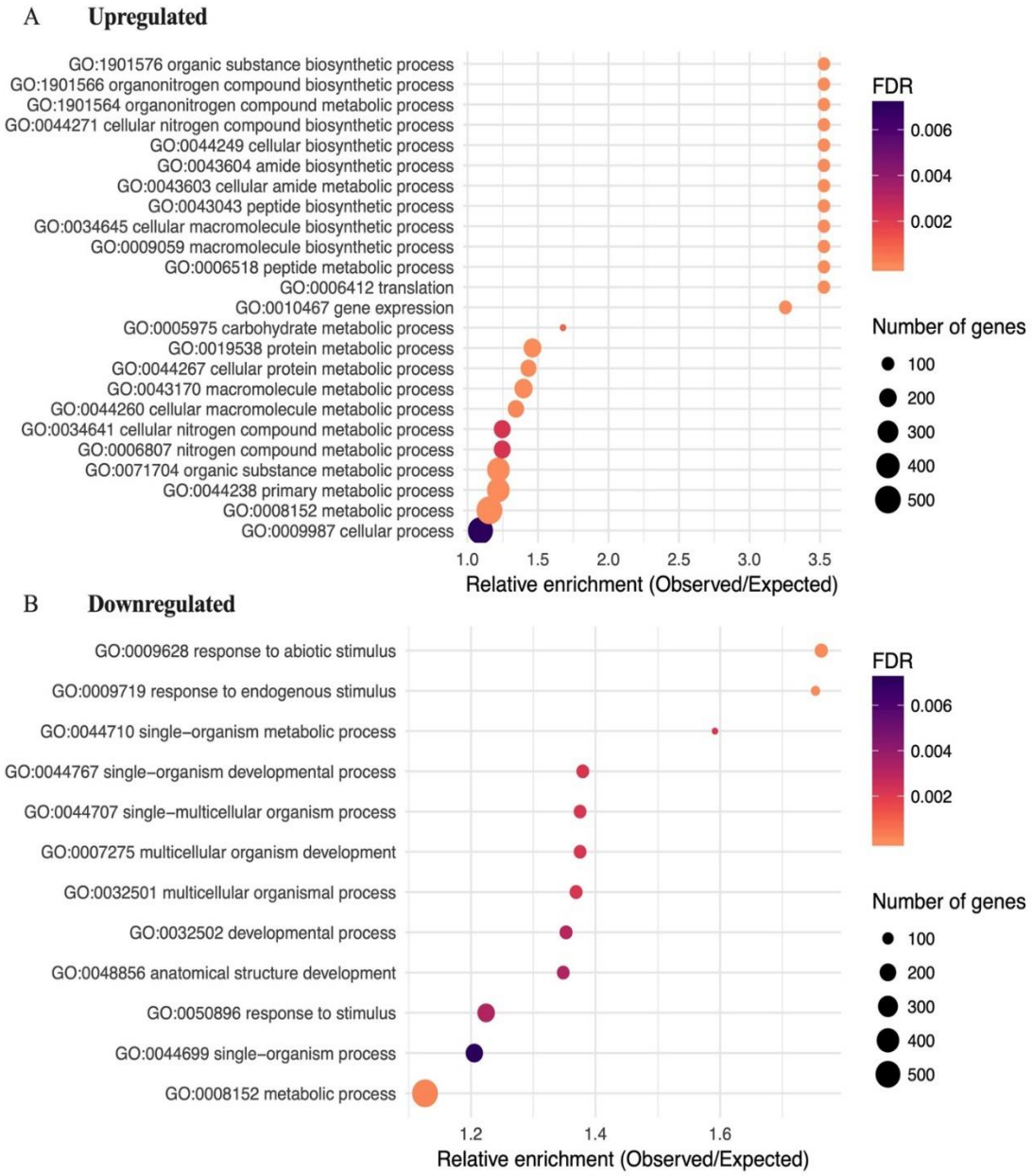

Figure 2.4. Gene ontology (GO) term enrichment among genes significantly (false discovery rate < 0.01) differentially (A) upregulated, or (B) downregulated genes after 2 h at 40/35°C. Ontological annotations downloaded from MSU and ontology enrichment tests performed with topGO in R using the Fisher standard test (on tailed fisher's exact test/hypergeometric test) with post hoc *P* value correction for multiple testing using the Benjamini & Hochberg method.

Protein abundance (expressed on a leaf area basis) of key mitochondrial electron transport components – cytochrome *c* oxidase (COX) subunit II, alternative oxidase (AOX) and uncoupling protein (UCP) – were determined by Western blots in PE leaves 6 h and one-day after *T*-transfer, and in newly developed (ND) leaves that formed under each prevailing growth *T* (Figure 2.5, Supplementary Figure S2.4). The cold (20/25°C) and hot (40/35°C) treatments did not affect the total protein concentration of leaves regardless the time-point (Supplementary Table S2.4). As was the case in the transcript abundance results noted earlier, there was a significant decline in COX subunit II protein abundance after 24 h, and in ND leaves when grown at 40/35°C compared to 25/20°C (Figure 2.5). The abundance of AOX and UCP protein did not vary in response to growth *T* or exposure time for either transferred or ND leaves, despite the initial spike in *aox* and *ucp* transcript abundance when transferred to 40/35°C (Figure 2.1). Interestingly two bands of AOX that varied relative to one another with temperature treatment were evident in the Western blot (Supplementary Figure S2.4). This is consistent with the fluctuations in expression of different *aox* transcript noted above (Figure 2.1). Patterns of protein abundance were similar when the analysis was standardised to dry mass or porin abundance (Figure S2.5). Porin is the most abundant protein in the mitochondrial outer membrane (Young et al., 2007), thus typically used as a proxy for mitochondrial surface area (Noguchi et al., 2005; Shane et al., 2004). There was a trend for the abundance of Rubisco to decline with the amount of time a leaf developed at 40/35°C (Figure 2.5D), although there were no statistically significant differences between *T* or developmental times.

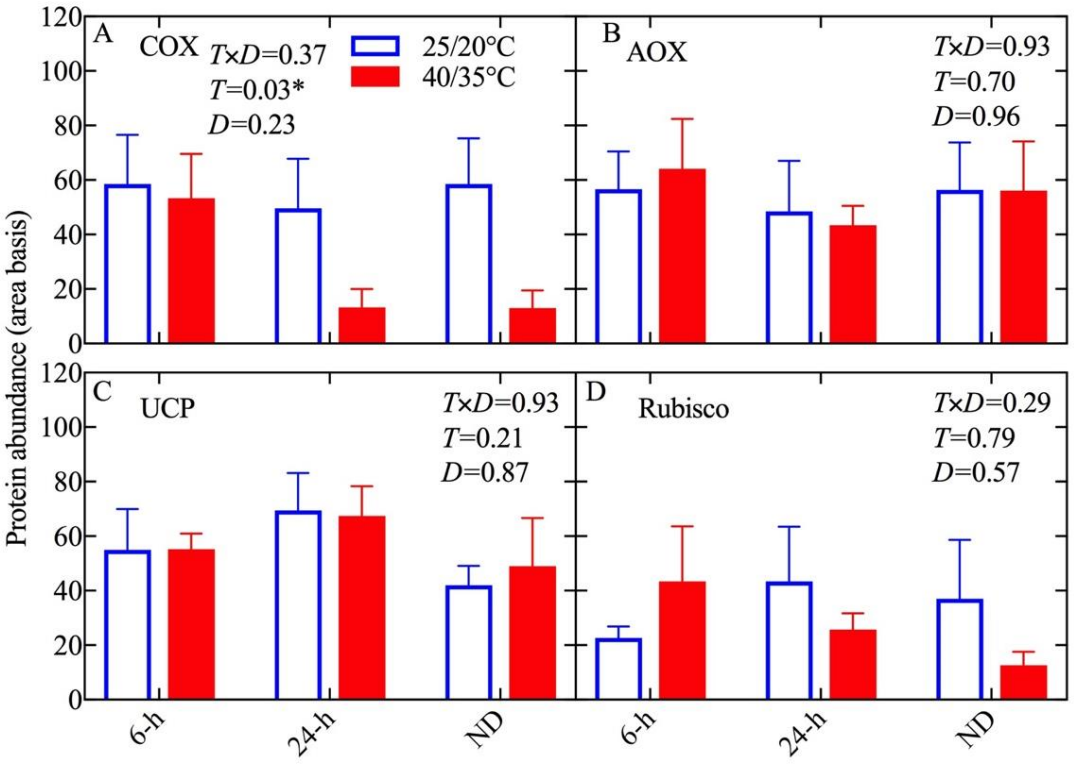

Figure 2.5. Abundance of mitochondrial electron transport chain proteins and Rubisco determined by Western blot analysis for rice leaves sampled at different developmental stages of; PE leaves six and 24 h after T transfer to 25/20°C or 40/35°C, and leaves newly developed (ND) post T-transfer. (A) Abundance of cytochrome *c* oxidase (COX) subunit II, (B) alternative oxidase (AOX), (C) uncoupling protein (UCP) and (D) Rubisco large subunit on a leaf area basis with data normalised by adjusting the largest value in each dataset to 100. Data represent mean ± SE of four independent western blots, with each blot representing leaf tissue from a separate plant. The *P*-values of a two-way ANOVA comparing temperature (*T*), developmental stage (*D*) and the interaction between the two (*T*×*D*) are reported on each graph. Representative blots are presented in Figure S2.4.

Leaf mass per area (LMA) and starch, glucose, fructose and sucrose concentration were measured in leaves one and seven days after *T*-transfer, and in ND leaves (Table 2.2). LMA did not change significantly in response to *T*, for either transferred PE leaves or ND leaves, similar to previous observations in rice over a similar *T* range (Nagai & Makino, 2009). However, ND leaves did exhibit significantly greater LMA than PE transferred leaves, suggesting an effect of leaf developmental stage on LMA. Transferred PE and subsequently formed ND leaves exhibited consistently lower starch concentration with increasing *T* and significantly lower starch with extended development times at the prevailing *T*. Unlike the dynamic responses in starch, soluble sugars were remarkably consistent in concentration for both PE and ND leaves, over the time-period and across *T*. Negative correlations between R_{dark} and soluble sugars were observed among leaves within each individual *T* treatment but not among the three *T* treatments (Supplementary Table S2.5).

2.4.2 CO_2 flux in responses to temperature

How molecular changes altered the physiological performance of rice carbon metabolism at differing growth *T* was investigated through gas-exchange analysis. Rates of A_n and R_{dark} are here presented on a dry mass (DM) basis, noting that the patterns are similar when expressed on a leaf area basis, reflecting the fact that growth *T* had no significant effect on LMA (Table 2.2). A substantial perturbation in both A_n and R_{dark} (using mid-sections of leaves placed in Licor 6400 3 x 2 cm chambers) occurred within the first 24 h for PE leaves being transferred to 40/35°C, with A_n falling and R_{dark} increasing when measured at the prevailing growth *T* (Figure 2.6). This was followed by a stabilisation at the new rate over the subsequent seven-days. By contrast, A_n and R_{dark} remained relatively constant at both 30/25°C and 25/20°C over a seven-day period. Interestingly, rates of R_{dark}

of the 30/25°C treated plants decreased with time, resulting in slightly lower rates of R_{dark} at *T* than that of the 25/20°C treated plants by day 7 – possibly reflecting temperature-dependent differences in leaf senescence rates.

Table 2.2. Leaf mass per unit area (LMA), starch and soluble sugars of PE leaves transferred from 30/25°C to 25/20°C or 40/35°C for one and seven days, and ND leaves at the prevailing *T*. Data represents mean of 3-4 biological replicates ± SE. The *F*-values and *P*-values of a two-way ANOVA comparing *T*, developmental stage (*D*) and any interaction (*I*) are reported with asterisks indicating significance at $P<0.05$.

		LMA (g m^{-2})	Starch (mg g^{-1} DM)	Soluble sugar (mg g^{-1} DM)
25/20°C	Day 1	20 ± 3	11.3 ± 1.9	13.5 ± 0.2
	Day 7	19 ± 2	5.5 ± 0.3	11.2 ± 0.1
	ND	30 ± 2	14.4 ± 3.0	11.1 ± 0.2
30/25°C	Day 1	19 ± 2	14.9 ± 2.1	13.0 ± 0.5
	Day 7	21 ± 2	4.9 ± 1.0	10.5 ± 0.2
	ND	28 ± 0.4	9.6 ± 1.6	11.0 ± 0.6
40/35°C	Day 1	18 ± 2	8.5 ± 0.4	11.9 ± 0.2
	Day 7	23 ± 2	3.5 ± 0.3	10.9 ± 0.3
	ND	29 ± 1	6.7 ± 0.5	11.6 ± 0.3
I		F=0.7, P=0.6	F=1.7, P=0.14	F=0.01, P=0.99
D		F=28, $P<0.001$*	F=13, $P<0.001$*	F=0.14, P=0.94
T		F=0.1, P=0.9	F=4.2, P=0.03*	*F=0.01*, P=0.99

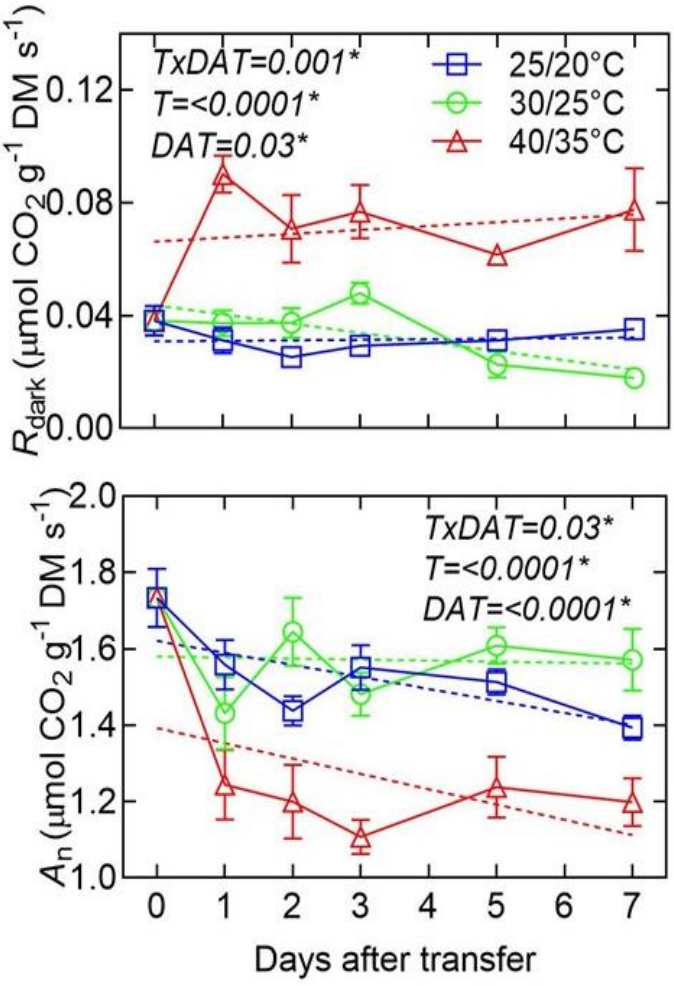

Figure 2.6. Rates of dry mass (DM) based (A) dark respiration (R_{dark}) and (B) net photosynthesis (A_n) of PE leaves, measured at the respective day-time growth temperature of each treatment just prior to (day 0), and 1, 2, 3, 5 and 7-days after transfer of control 30/25°C day/night grown plants to either 25/20°C, 40/35°C or maintained at 30/25°C. The *P*-values of a two-way ANOVA comparing temperature (*T*), days after transfer (*DAT*) and the interaction between the two (*T*×*DAT*) are reported on each graph. Dashed lines represent linear regression of R_{dark} or A_n against days after transfer at each temperature. Values are means of four biological replicates ± SE.

Short-term temperature response curves of entire ND leaves that formed at each prevailing growth *T* regime were quantified over a 20 to 60°C range using the Walz large leaf chamber (Figure 2.7). Over most of the range of measuring temperatures, leaves developed at 25/20°C exhibited higher rates of R_{dark} than the other two treatments; by contrast, rates were lowest in leaves developed at 40/35°C (Figure 2.7A). When normalised to 30°C the differences were less pronounced (Figure 2.7B), indicating that

while R_{dark} at a given measuring T was affected by growth T, the general shape of the R_{dark}-T curves remained largely similar across the three treatments. These observations are consistent with a Type II (changes in baseline) rather than Type I (changes in Q_{10}, the increase in R_{dark} with a 10°C increase in T) respiratory acclimation responses (Atkin & Tjoelker, 2003). Importantly, while respiratory thermal acclimation occurred, it was not sufficient to result in R_{dark} being homeostatic across the three growth T treatments. As a result, R_{dark} measured at the growth T (R_{growth}) was significantly higher in the hot-grown plants than the two other treatments (Table 2.3). Growth T also had a significant effect on the measuring T at which R_{dark} and A_n reached their maximum rates, with hot grown plants exhibiting higher T_{max} (R_{dark}) and T_{opt} (A_n) values (Table 2.3); the hot grown plants exhibited 4°C higher T_{max} and T_{opt} values than their cold-grown counterparts (Table 2.3) When measured at the prevailing growth T of each treatment, mass-based rates of light-saturated A_n (A_{growth}) were stable (i.e. homeostatic). The temperature at which PSII lost functionality (T_{crit}) also increased with growth T (being 3.8°C higher in the hot-grown plants compared to those grown at 25/20°C), although the differences were not statistically significant at P<0.05 (Table 2.2). The high degree of thermal acclimation exhibited by photosynthesis resulted the ratio of R_{dark} to A_n being lowest in the hot-grown plants, particularly at high measuring T (Figure 2.8A); at a measuring T of 40°C, hot-acclimated plants exhibited R_{dark}/A_n ratios that were 5% lower than their cold-grown counterparts. Further evidence that rice acclimated to the hot conditions is seen in the fact that leaf elongation rates - taken over the day and night period - were higher for the 40/35°C grown plants across all time points (Figure 2.8B). Interestingly, A_n of PE leaves shown in Figure 2.6 exhibited rates of CO_2 fixation ranging between 1.2 and 1.8 μmol g^{-1} DM s^{-1} while rates were close to 0.6 μmol g^{-1} DM s^{-1} for the ND leaves shown in Figure 2.7. The former was based on measurements made using mid-leaf sections placed in a 6

cm^2 chamber, while the latter was on entire leaves placed in a 14 x 10 cm Walz chamber; presumably, measurements made using the entire leaf have lower rates of A_n, possibility due to entire leaves having a lower proportion of mesophyll cells per unit of area or DM.

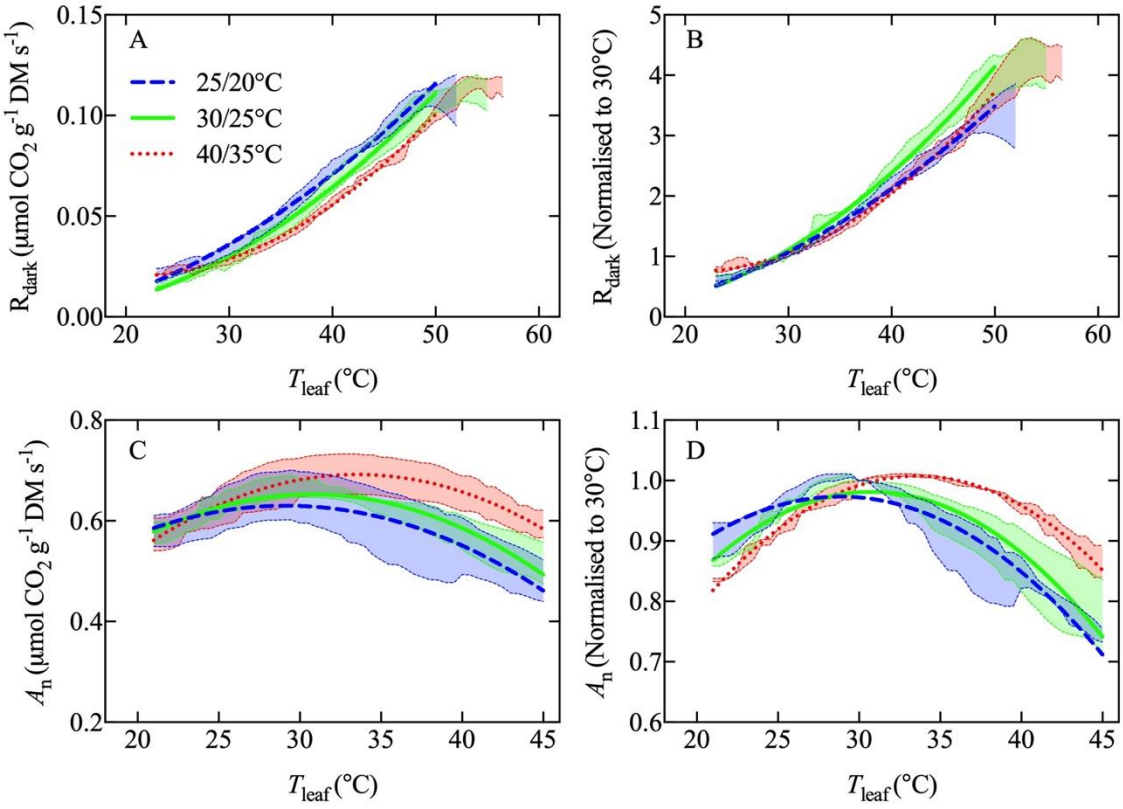

Figure 2.7. Temperature-response curves (A-B) of dark respiration (R_{dark}) and (C-D) net photosynthesis (A_n) of ND leaves, on a dry mass (DM) basis. Values are absolute (A-C) or normalised to values at 30°C (B-D). Measurements were made on whole newly-developed (ND) leaves growing for 21-d at day/night temperatures of 25/20°C, 30/25°C or 40/35°C. Curves fitted to R_{dark} and A_n are quadratic functions. Calculated acclimation parameters from the curves are presented in Table 2.3. Rates were recorded every 30 sec as leaves were heated at 1°C per minute. Filled area represent standard error of three to four biological replicates.

Table 2.3. Summary of key photosynthetic and respiratory parameters generated from temperature-response curves of ND leaves in Figure 2.7. Parameters are: leaf mass per area; the temperature at which R_{dark} and A_n exhibited maximum rates (T_{max} and T_{opt}, respectively); the maximum rates of R_{dark} and A_n reached (R_{max} and A_{opt}, respectively); rates of R_{dark} and A_n at the prevailing growth temperature (R_{growth} and A_{growth}, respectively); and the temperature at which PSII lost functionality as determined by an increase in basal fluorescence (T_{crit}). Data represents means of three or four biological replicates ± SE and statistical data (*F*-value and *P*-value) based on one-way ANOVA of temperature treatment effect. Superscript letters show significant differences between the *T* treatments according to a Tukey test.

	25/20°C	30/25°C	40/30°C	*F*	*P*
LMA (g m^{-2})	33±2	30±2	35±3	1.4	0.31
T_{max} (°C)	51±1^{a}	54±1^{a,b}	55±1^{b}	4.7	0.04*
T_{opt} (°C)	29±1^{a}	31±1^{a,b}	33±0.3^{b}	6.1	0.04*
R_{max} (nmol g^{-1} DM s^{-1})	120±5	117±6	121±2	0.16	0.86
A_{opt} (μmol g^{-1} DM s^{-1})	0.65±0.05	0.67±0.02	0.69±0.04	0.28	0.76
R_{growth} (nmol g^{-1} DM s^{-1})	24±3 ^{a}	27±3 ^{a}	57±2^{b}	35	<0.001*
A_{growth} (μmol m^{-2} s^{-1})	0.62±0.04	0.67±0.02	0.65±0.04	0.52	0.62
T_{crit} (°C)	46.0±0.6	46.9±0.9	49.8±1.5	3.726	0.089

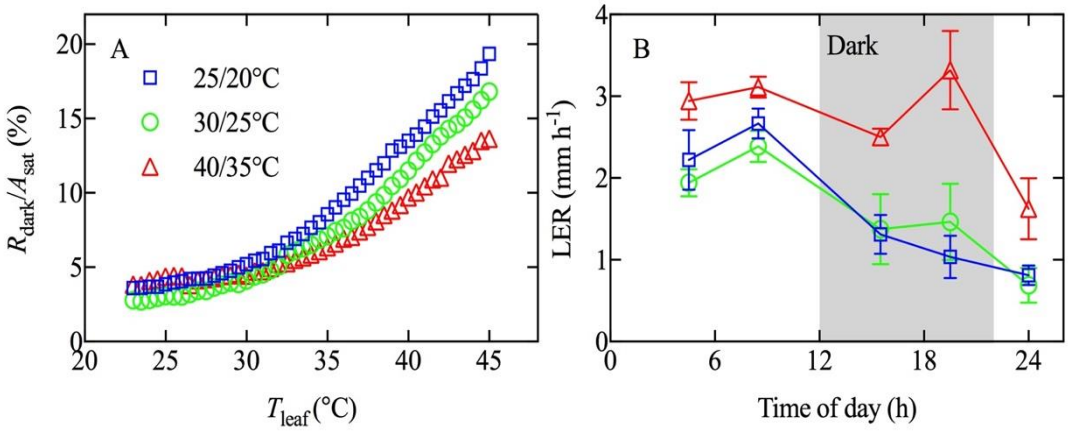

Figure 2.8. The percentage of dark respiration (R_{dark}) relative to light-saturated net assimilation (A_n) (A), and leaf elongation rates (LER) over a 24 h day/night cycle (B), for ND leaves growing for 21-days at day/night temperatures of 25/20°C, 30/25°C or 40/35°C. For the R_{dark}/A_n ratio values are calculated from the absolute means presented in Figure 2.7. For LER the dark (night) period of the 24 h cycle is shaded in grey and values are the means ± SE of four plant replicates.

2.5 Discussion

Our study investigated the response of photosynthetic and respiratory metabolism to short- and long-term changes in growth temperature, using exposure to three growth temperatures – the highest of which is indicative of heat wave temperatures – to explore: (1) the extent of thermal acclimation of photosynthesis and respiration; and (2) what underlying changes in gene expression and protein abundance occur during the acclimation process. The results demonstrate that the process of acclimation begins with abrupt changes in gene expression of PE leaves within hours of heat exposure, followed by a return to homeostatic gene expression after 24 hours of treatment. Importantly, the abundance of the key energy-conserving respiratory protein, COX, declined in abundance when pre-existing leaves were heat treated for 24 hours, with this phenotype being maintained in ND leaves formed at 40/35°C. This decline in COX was linked to a slight decline in overall rates of R_{dark}. Finally, the results support the hypothesis that acclimation is asynchronous in rice, but contrary to my original expectation, light-saturated A_n

acclimated to a greater extent than R_{dark}, further supporting the conclusion of strong thermal acclimation by A_n in ND leaves, with the degree of homeostasis in ND leaves (Figure 2.7) being much greater than was the case for PE leaves (Figure 2.6). The ability to maintain photosynthetic carbon gain at 40°C is likely to be of crucial importance in helping rice maintain growth during heat-wave conditions.

2.5.1 Acclimation to changes in temperature are rapid and involve a multitude of genes

There was a substantive change in the gene expression profile of rice leaves shifted from 30°C to 40°C within the first 24 h of transfer (Figure2.1, 2.2, 2.3 and 2.4). As might be expected, the largest number of gene perturbations were in primary and cellular metabolic processes (Figure 2.4). This metabolic gene response aligns with the instability in R_{dark} and A_n fluxes over the initial 24 h *T*-transfer (Figure 2.6), which would have contributed to a metabolic imbalance through changes in assimilate supply and demand. Interestingly, the most responsive upregulated genes to the initial exposure to heat were in the biosynthetic processes (Figure 2.4) suggesting a stimulation of growth, aligning with the longer-term increase in leaf elongation rates for the 40/35°C grown plants.

When analysed in more detail, I observed heat stimulation of the genes linked to energy dissipation (*aox* and *ucp*) over the first 24 h of 40°C heat exposure (Figure 2.1, Table 2.1). AOX and UCP are associated with diverting electrons from formation of proton gradients and subsequent ATP synthesis (Krauss et al., 2005; Vanlerberghe, 2013). Past work has shown that overexpressing *aox* in young rice seedlings imparts a benefit on growth when exposed to a growth *T* of 37°C for eight days, attributed to reducing excessive proton motive force and subsequent reactive oxygen species (Murakami & Toriyama, 2008). Given that AOX and UCP both divert electrons away from ATP synthesis under conditions of high reductant supply, the rapid upregulation of these genes

following the initial changes in growth T – with rapid stimulation of R_{dark} and presumably greater reduction of ubiquinone pools (UQ) – indicates that there may have been a temporary imbalance between NAD(P)H supply and demand for ATP. The initial increases in gene expression of *aox* and *ucp* (Figure 2.1) did not translate into increased total AOX and UCP protein abundance (Figure 2.5). However, qPCR results indicate upregulation of the *aox1a* isoform, responsive to abiotic stress in Arabidopsis mitochondria (Clifton et al., 2006; Shapiguzov et al., 2019), while over the same period RNA-seq analysis indicated a significant decline in a separate *aox1c* isoform. This suggests that AOX shifted to a more heat-tolerant conformation at 40/35°C, at least when the initial shock was imposed. This is an illustration that enzyme isoforms can be an important part of abiotic stress responses that can be easily overlooked when only considering total protein abundance.

The limited gene induction when leaves were transferred from 30 to 25°C suggests a shift to this colder growth T did not perturb metabolic processes in rice leaves significantly, supported by the limited response of rates of R_{dark} or A_n exhibited by PE leaves exposed to the cold (Figure 2.6). However, sustained exposure to the cold treatment did result in changes in metabolic rates, as shown by the fact that rates of R_{dark} at a given T were higher in the ND leaves from cold-grown plants and by the homeostasis of photosynthetic rates when measured at the prevailing growth T (A_{growth}) of each treatment (Table 2.3, Figure 2.7). Thus, while the initial gene response and changes in metabolic rates were limited when 30°C grown leaves were exposed to the cooler T, the fact that there were adjustments in ND leaves suggests that some perturbation and readjustment of energy balances occurred.

2.5.2 The most evident longer-term acclimation response is reduced COX abundance

Data in Figure 2.5 represent mean (±SE) of four independent western blots. Although the data were normalised by adjusting the most intense replicate of the samples to 100, there was a substantial decline in COX subunit II when grown at 40/35°C. I assume the changes observed in this COX subunit reflex changes in abundance of the entire complex. A decline in the abundance of COX was the clearest biochemical response to increasing growth *T*, both in PE and ND leaves. The matching results using leaf area, dry mass or porin abundance indicates that the decline in COX abundance with increased temperature was not likely as a result of changes in LMA or reduced mitochondria per unit area of leaf. Porin is a voltage-dependent channel protein located at the outer membrane of mitochondria and is widely used as a proxy for mitochondrial surface area due to its stability under a wide range of environmental conditions (Noguchi et al., 2005; Shane et al., 2004). This decline in COX abundance has been reported for rice roots when grown at a 25°C relative to 15°C (Kurimoto et al., 2004b). Conversely, COX abundance increased in *Arabidopsis thaliana* leaves grown at 5°C relative to 21°C (Armstrong et al., 2008). In all these cases, COX protein abundance and rates of respiration at a given measuring *T* (including the results shown in Figure 2.5 and 2.7) decreased when growth *T* increased, suggesting that thermal acclimation results in changes not only in overall rates of respiration but also in the capacity to produce ATP. The acclimation response was rapid as COX declined in abundance by 24 h after 40°C *T* transfer in PE leaves (Figure 2.5).

The decline in COX abundance with hotter growth *T* is intriguing. If COX activity became rate-limiting, it is likely that more ROS would be produced as the UQ pool would quickly become over-reduced. However, other reports suggest that the UQ redox state is relatively stable, including during changes in *T*, despite faster R_{dark} (Covey-Crump et al., 2007; Wagner & Wagner, 1995). If we assume that UQ redox poise was also stable during

the faster R_{dark} at the hottest growth *T* in my experiments, there are two possible explanations. (1) The absolute flux of electrons through COX actually increased despite the decrease in protein abundance. This could be due to COX capacity being far greater than the capacity of the overall mETC. But since increasing *T*s stimulates the relative activity of enzymes (Copeland, 2004), it is possible that the smaller amount of COX protein had faster activity. In other words, the plants could make do with less COX at hotter *T*. (2) Alternatively, activation of AOX at the higher *T* may have occurred to supplement COX activity thereby preventing overload of the UQ pool. (3) Another possibility is that a decrease in COX could simply cause more electrons to be diverted through AOX, which may or may not require further activation of the enzyme. Measuring *T*-dependent *in vivo* O^{18} fluxes through COX and AOX, as well as leaf ATP concentrations are required to determine terminal oxidase activity and ATP synthesis. Understanding what, if any, biological benefit or harm arises from synthesising less COX at warmer growth *T* is another important consideration.

2.5.3 Acclimation of R_{dark} and A_n is asynchronous in rice

The R_{dark}/A_n ratio increased with short-term increases in measuring *T* (Figure 2.8), reflecting the fact that R_{dark} is more temperature dependent than is A_n. R_{dark}/A_n ratios were similar in 25 and 30°C grown leaves, when measured at the prevailing growth *T* of each treatment (i.e. R_{dark}/A_n was homeostatic). Thus, the acclimation process led to the balance between carbon gain and release being maintained across this range of growth *T*s (Figure 2.8). Acclimation was not, however, sufficient to maintain homeostasis of R_{dark}/A_n in 40°C grown plants (Figure 2.8). Similar results of R_{dark}/A_n ratios in leaves and whole plants remaining relatively stable over moderate but not extensively higher *T* have been reported (Atkin et al., 2006b, 2007; Campbell et al., 2007; Drake et al., 2016; Loveys et

al., 2003). Where my results differ from those past studies is my finding that in rice, homeostasis of R_{dark}/A_n is largely the result of maintenance of A_n more than through a marked reduction in rates of R_{dark}; in the above cited studies, homeostasis of R_{dark}/A_n was largely mediated through a greater proportional reduction in R_{dark} than maintenance of A_n. In fact, for many plant functional types, including temperate grasses, R_{dark} acclimates to a greater extent than A_n (Campbell et al., 2007; Ow et al., 2008; Way & Oren, 2010; Way & Sage, 2008; Yamori et al., 2005). In this context, it should be noted that the previous studies are of species from temperate rather than tropical habitats, raising the question of whether tropical grasses such as rice have asynchronous acclimation favouring A_n.

As noted earlier, in recent years, rice yields have declined in response to increased daily mean temperatures, with the decline being more strongly correlated with increasing night rather than day temperatures (Peng et al., 2004; Welch et al., 2010). My finding that A_{growth} is homeostatic across a 15°C range of growth T, whereas R_{growth} is not (Table 2.3) – underpinned by greater acclimation of photosynthesis than respiration – suggests that one reason why yields are declining with increasing night temperatures is because high temperatures stimulate respiratory CO_2 release. This would have a negative effect on daily net carbon gain, and thus the ability to accumulate biomass in the lead up to anthesis.

2.5.4 Potential implications of rice leaf acclimation and starch concentration on crop yield

The approach used in this chapter for determining R_{dark} requires a caveat. Leaves (which thereafter were also used for determination of starch and soluble sugar concentrations) were dark-adapted for at least 30 minutes to avoid light-enhanced R_{dark} (Atkin et al., 2000b; Padmasree et al., 2002), but were still collected during the day period. This draws concerns about the circadian impact on R_{dark} and starch turnover. At night,

when plants are not photosynthesizing, starch is broken down to maintain sugar supply for R_{dark} and growth. This would not have been captured when measuring and collecting samples in the day. However, as dark-adapting leaves instead of collecting at night is the most common approach of measuring R_{dark} (and R_{dark}-related traits), I chose to be consistent with this methodology. Considering measurements took place during the light period, R_{dark} was measured at the prevailing day temperature of each growth treatment.

I found that the soluble sugar concentration of leaves was remarkably consistent, irrespective of growth *T* or developmental time at each growth *T* (Table 2.2). Maintaining soluble sugar homeostasis is an important physiological requirement for many plant species, achieved through balancing CO_2 uptake and release in source leaves with sugar export to sink tissues (Rolland et al., 2002). Homeostasis of sucrose concentration in rice leaves has been observed even when carbon demand by sink tissues is limited [e.g. reduced partitioning of sugars to grain (Wang et al., 2008)]. In my study, homeostasis of soluble sugar concentrations occurred even at 40°C, where rates of R_{growth} where significantly higher than in plants at the two cooler growth *T*. Associated with the maintenance of sugar concentration was a *T*-dependent decline in starch concentration, both in PE and ND leaves (Table 2.2). For PE leaves exposed to 40°C, assimilate supply declined, caused by a marked increase in R_{dark} and decline in A_n (Figure 2.6). Starch concentration also significantly declined with developmental time, whereas soluble sugar concentrations remained unaffected by development (Table 2.2). It seems likely, therefore, that the reason soluble sugars did not significantly decline at warmer *T* for PE leaves - even though assimilate supply fell - was due to greater drawdown in starch concentration to maintain soluble sugar concentrations (i.e. a reliance on stored assimilate). Other studies [e.g. on the temperate tree *Populus tremula* (Hüve et al., 2012)]. have highlighted the importance of starch degradation in maintaining sugar

concentrations, particularly under conditions that stimulate CO_2 release by respiration. Interestingly, in my study, ND leaves exhibited reduced starch concentration while also maintaining assimilate supply; one explanation for this might be that the decline in starch and maintenance of sugars of ND leaves was linked to the increased leaf elongation rates I observed for 40°C ND leaves (Figure 2.8B), with increased growth (i.e. sink demand) necessitating a greater supply of sugars mediated by the starch pool (Stitt & Zeeman, 2012).

The decline in starch concentration for PE and ND leaves at 40°C has interesting implications on rice development and yield. Starch is stored in the stems in the late vegetative stage of rice, and accounts for a large proportion of carbon accrued to seeds, a process that is detrimentally affected by heat stress (Blum et al., 1994; Impa et al., 2019; Morita & Nakano, 2011; Yang & Zhang, 2005). Other studies using the IR64 cultivar exposed to hot night temperatures have shown an increase in R_{dark} and associated cost to vegetative growth and starch content of panicles, ultimately reducing yield (Bahuguna et al., 2017; Glaubitz et al., 2014). The reduced storage of leaf starch concentration with increasing T that I observed at the vegetative stage – assuming it was not transported to stems – would suggest reduced potential for the storage of starch in stems and a penalty to yield of rice growing in warmer environments. This would be particularly true for rice plants exposed to transient T – such as during heat waves – as I postulate the reduction in starch for PE leaves was due to a reduction in assimilate acquisition due to stimulated R_{dark} and supressed A_n. However, while ND leaves did show reduced starch concentration, this was not as a result of reduced assimilate acquisition but most likely associated with an increase in growth rates. Thus, it is likely that rice will experience different limitations on yield depending on the duration of changes to T, with shorter-term exposure to rising T – over a period in which tissue cannot develop anew – likely leading to a greater

suppression of yield than leaves developed under the prevailing growth *T*. Rice may even experience increased yield with sustained mild warming of both night and particularly day *T*. However, yield potential of the latter is dependent on whether heat-dependent changes in growth at the vegetative stage of rice positively contributes to yield, which may be true (Glaubitz et al., 2014; Scafaro et al., 2018), and not simply accelerate development and shorten the time to flowering.

2.6 Conclusions

Overall, the results I present here demonstrate that both leaf respiration and photosynthesis can acclimate in rice but the extent of acclimation is asynchronous and dependent on the timeframe of *T* exposure. Warmer growth *T* of 40°C relative to 25°C will have a greater impact on rice CO_2 flux, metabolic pathways, starch concentration and ultimately growth. Consequently, rice growing in a warmer climate with more extreme heating events will likely experience *T*-dependent alterations in growth and yield. The duration and intensity of *T* changes, together with complex interactions between assimilate acquisition, storage and utilisation will determine if this warmer environment will be beneficial or detrimental to rice productivity over the coming decades. I suggest that enhancing the acclimation capacity of R_{dark} for rice at warmer growth *T* – potentially through COX, AOX and UCP regulation – could be a key target for improving rice productivity in a warmer world.

Chapter 3 – Diel and temperature driven variation of dark respiration and metabolites in rice leaves

3.1 Abstract

Plant respiration in the dark (R_{dark}) and its acclimation to growth temperature are often measured at only one time point during the day. However, it is not clear whether acclimation of R_{dark} is influenced by the diel cycle. To examine this, I grew rice at 25/20°C (day/night), 30/25°C and 40/35°C, measuring R_{dark} of leaves and associated changes in metabolites at five time points spanning a single 24-hour period. Independent of growth temperature I observed acclimation capacity varying significantly over the diel cycle. Acclimation of R_{dark} was strongest at the end of the day, but weak at night or in the early morning. As expected starch was depleted during the night, yet surprisingly regardless of the growth temperature. Soluble sugars by contrast, were significantly reduced in cold-grown leaves throughout the diel cycle. Amino acids were highly responsive to both the diel cycle and growth temperature, particularly the shikimate pathway derived aromatic amino acids. In general, many amino acids were negatively correlated with carbohydrates and organic acids of the tricarboxylic acid (TCA) cycle, particularly at night and at warmer growth temperatures. There was minimal interaction between acclimation, the diel cycle and organic intermediates of the TCA cycle, which I attribute to light-dependent regulatory control of TCA enzyme activities that seem to be independent of growth temperature. The amino-acid serine and amino acid breakdown product putrescine correlated significantly with both temperature and light driven adjustments in R_{dark} Collectively, this study shows dynamic changes in TCA metabolite levels that likely coordinate energy demand with temperature and diurnal cycle to R_{dark}.

3.2 Introduction

Leaf respiration in the dark (R_{dark}) provides plants with energy (i.e. ATP and reducing equivalents) and carbon skeletons needed to support growth and cellular maintenance (Atkin & Macherel, 2009; Gonzalez-Meler et al., 2004; Lambers et al., 2005; Noguchi & Yoshida, 2008). It is well recognised that R_{dark} increases exponentially with short-term (i.e. seconds-minutes) increases in temperature (Amthor, 2000; Atkin et al., 2005c; Reich et al., 2016; Smith & Dukes, 2013). When grown at different temperatures, over sustained periods (e.g. days to months), leaf R_{dark} often acclimates. Acclimation results in higher rates of R_{dark} at a common measuring temperature in cold-grown plants compared to plants grown at warmer temperatures (Arnone & Körner, 1997; Atkin et al., 2005b; Slot & Kitajima, 2015). Importantly, studies assessing acclimation in this form invariably rely on measurements made at a single time point in a day, with an implicit assumption being that the degree of acclimation is constant through a 24-hour diel cycle. This assumption remains untested, even though we know that carbon gain by photosynthesis only occurs during the day, and is absent at night. A consequence of diel variations in photosynthetic CO_2 uptake is that there is likely to be in variations in overall carbon supply to cellular metabolism, including fluctuations in the levels of metabolites involved in glycolysis, the tricarboxylic acid (TCA) cycle, the shikimate pathway and several other related metabolic networks (Gibon et al., 2009; Hurry et al., 2005; Urbanczyk-Wochniak et al., 2005). Given that rates of R_{dark} can vary in response to changes in substrate supply and demand for respiratory products, and that these factors vary between day and night, it is important that interactions between time of day and the effect of growth temperature on R_{dark} be assessed. This interaction between the diel cycle and respiratory acclimation is important if a realistic understanding of global carbon fluxes, and crop yield responses to temperature is to be achieved.

Evidence for substrate-dependent changes in R_{dark} is inconclusive. Glucose and sucrose availability for glycolysis correlates with respiration rates (Griffin et al., 2002; Ogren, 2000), and higher sugar levels are linked to faster respiration rates in leaves of *Quercus rubra* (Whitehead et al., 2004), *Spinacia oleracea* and *Alocasia macrorrhiza* (Noguchi et al., 1996). However, variations in the concentration of sugar substrates for glycolysis do not necessarily correlate with variations in the rate of R_{dark}. For example, sugar levels were not correlated to R_{dark} in a study on two alpine perennials, *Bisorta bistortoides* and *Campanula rotundifolia* (McCutchan & Monson, 2001). Moreover, while thermal acclimation often results in changes in temperature-normalized rates of R_{dark}, such changes can occur without concomitant changes in concentrations of soluble sugars. Indeed, sugar concentrations are often relatively constant across a range of environmental treatments, with this constancy linked to synthesis and degradation of starch (Hüve et al., 2012). During the day, carbon assimilated by photosynthesis is typically stored as starch in leaves if not immediately converted to sugars (Pilkington et al., 2015; Smith & Stitt, 2007). Starch turnover is regulated by a circadian clock (Graf et al., 2010; Graf & Smith, 2011) such that starch is broken down in a near-linear manner during the night to provide a relatively constant supply of sugars for R_{dark} and growth when photosynthesis is not occurring. Approximately 90-95% of the starch is remobilized by dawn in metabolic processes required for plant growth and maintenance (Graf et al., 2010; Pilkington et al., 2015; Smith & Stitt, 2007). In cases where day-time photosynthesis and night-time starch remobilization results in constant sugar concentrations over a 24-hour cycle, any diel variation in R_{dark} is unlikely to be due to changes in sugar availability. Nevertheless, rates of R_{dark} could still be affected by diel changes in availability of other metabolites which can potentially feed respiration, specifically amino acids, proteins, organic acids and/or lipids (O'Leary et al., 2011;

O'Leary et al., 2017). As described by O'Leary et al. (2017), such metabolites may drive respiration through further movement of carbon via the TCA cycle, providing reductant for ATP synthesis via oxidative phosphorylation, and the provision of carbon skeletons required for nitrogen (N) assimilation into amino acids or as biosynthetic precursors. Thus, when assessing the interactive effect of growth temperature and time of day on leaf R_{dark}, attention needs to be given to substrate-product relationships across the wider respiratory network. Doing so may enable identification of metabolites that correlate strongly with respiration, particularly in response to contrasting growth temperatures and the diel cycle.

Metabolomics may provide insights into factors underpinning variability in fluxes through the respiratory pathways, and how supply- and demand-driven R_{dark} is coordinated with diel and temperature dependent stimuli. The importance of metabolites is evident in a temperature-related experiment by Sicher (2015) on leaflets of soybean; in that study, 28 of 43 total metabolites explored were affected when plants experienced an increase in growth temperature from 28/20°C to 36/28°C. With the exception of raffinose and γ-aminobutyric acid (GABA), most of the organic and amino acids decreased in response to elevated growth temperature. In wheat (*Triticum aestivum*) leaves, the abundance of fumarate and alanine increased in response to warmer night temperatures, while glutamine, glutamate and GABA remained constant (Impa et al., 2019). In rice, Glaubitz et al. (2014) found that high night temperature treatment [day (30°C) and night (28°C)] increased the abundance of amino and organic acids in temperature-sensitive rice cultivars (e.g. DR2 and M202), but resulted in little to no change in amino/organic acids of intermediate (e.g. IR64 and IRRI123) and temperature-tolerant cultivars (e.g. IR72 and Taipei309). As well as temperature, amino acids change in response to the diel cycle. For example, Urbanczyk-Wochniak et al. (2005) found differences in amino acid levels

between light and dark periods in potato (*Solanum tuberosum*) leaves, in which most of the amino acids increased in the presence of light, and decreased in the dark. The central role of the TCA cycle in respiration suggests that the strong response of these metabolites to temperature and light are likely to be associated with perturbations in respiration.

Diel changes in primary TCA cycle intermediates have been observed previously. Firstly, there is an accumulation of malate and fumarate during the day, followed by accumulation of citrate, aconitate and succinate at night (Gibon et al., 2006; Watanabe et al., 2014). Light suppresses the pyruvate dehydrogenase complex (PDC) (Tcherkez et al., 2009). PDC catalyses the decarboxylation of pyruvate and controls entry of carbon into the TCA cycle (Tovar-Méndez et al., 2003). Light dependent changes in PDC activity would contribute to differences in TCA cycle intermediates between the day and night. In some cases, the TCA cycle can operate in a reverse direction by phosphorylation of light-induced phosphoenolpyruvate carboxylate (PEPC), converting oxaloacetate to malate and fumarate (Araújo et al., 2012; Marsh et al., 2003; Sullivan et al., 2004). In this pathway, PEPC generally replenishes the TCA cycle intermediates that are depleted when α-ketoglutarate is used for amino acid synthesis. Measuring temperature can also alter the abundance of TCA cycle intermediates. For example, increases in the concentration of α-ketoglutarate, fumarate, malate and citrate can occur when Arabidopsis leaves are shifted from 20°C to 4°C (Cook et al., 2004). In a study by Florian et al. (2014), Arabidopsis treated at 15°C relative to 20°C, accumulated succinate, fumarate and malate at the beginning of the day but similar declines during the light period. Further, metabolites that typically vary diurnally - such as alanine, phenylalanine and glutamine - maintain their diurnal oscillations even when leaves are chilled at 4°C (Espinoza et al., 2010). Taken together, the above studies point to inter and intra-specific differences in how metabolite levels respond to both temperature and the diurnal cycle, with responses likely to be

linked to the intrinsic ability of a given genotype to function under the prevailing growth environment. Whether important metabolites of respiration change in response to temperature and light/dark cycles independently of one another, or there is interaction between the two processes is yet to be explored.

In the current study, I investigated whether there is an interaction between thermal acclimation of respiratory metabolism of rice (*Oryza sativa*, cultivar IR64) and the day/night cycle. Rice is one of the most important food crops, with declining rice yields being increasingly linked to rising growth temperatures (Wassmann et al., 2009), with the effect of growth temperature on leaf metabolism influencing rates of net carbon gain in the lead up to anthesis. It is thus vital that we develop a more holistic understanding of how growth temperature effects respiratory metabolism in rice, both during the day and night. My study used metabolite profiling to assess how growth temperature and the diel cycle might influence the flow of carbon through individual steps of the respiratory network. I specifically wanted to understand: (1) if rates of R_{dark} vary over a day, and if so, if the extent of diel variation is influenced by growth temperature; (2) if diel variations in dark respiration are linked to concomitant changes in substrate availability; and (3) the extent to which growth temperature and day/night cycle influence the metabolome profile and whether adjustments in the abundance of individual metabolites provide insights into how the growth environment influences regulatory control of respiratory metabolism. I hypothesised that: (1) respiration varies throughout the day/night cycle with hot grown plants exhibiting greater diel variation than colder grown plants; (2) associated with the greater diel variation in hot is the diel changes in substrate availability along respiratory pathway; and (3) growth temperature and day/night cycle influence the variations in respiratory metabolomic profile with higher level of metabolites in glycolysis and TCA cycle in cold compared to warmer temperature.

3.3 Materials and Methods

3.3.1 Plant materials and temperature treatments

Rice (*Oryza sativa*) cultivar IR64 was grown hydroponically in a glasshouse facility at the Australian National University in Canberra, Australia. The hydroponic solution (16 mL solution in each tank) consisted of NH_4NO_3 (1.4 mM), $NaH_2PO_4.2H_2O$ (0.6 mM), K_2SO_4 (0.5 mM), $CaCl_2.2H_2O$ (0.2 mM), $MgSO_4.7H_2O$ (0.8 mM) and micronutrients: Fe-EDTA (0.07 mM), H_3BO_3 (0.037 mM), $MnCl_2.4H_2O$ (0.009 mM), $ZnSO_4.7H_2O$ (0.00075 mM), $CuSO_4.5H_2O$ (0.0003 mM), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.0001 mM), NH_4VO_3 (0.000138 mM) and Na_2SiO_3 (0.0012963 mM) (Supplementary Table S2.1). The nutrient solution was replaced weekly. Sulphuric acid or sodium hydroxide were used to adjust the pH to between 5-6, with pH monitored daily using a portable pH meter (Rowe Scientific Pty. Ltd., NSW, AU). The hydroponic solution was aerated continuously using Infinity AP-950 air pumps (Kong's Pty. Ltd., Ingleburn, AU). Twelve hydroponic tanks holding a maximum 20 plants were placed in a temperature-controlled glasshouse at 30°C during the day and 25°C (30/25°C) at night under a natural photoperiod. The sunset and sunrise times were 19:30 and 06:30 ± 15 min, respectively. Photosynthetically active radiation (PAR) ranged from 400 and 1200 µmol quanta m^{-2} s^{-1}. After two weeks at 30/25°C, eight tanks were randomly chosen and shifted to adjacent glasshouse rooms set to 25/20°C or 40/35°C temperature regimes (four tanks in each room). Another four tanks were maintained in the original 30/25°C room as controls. Each tank was considered as a pot replicate. Sampling for all experiments unless stated otherwise occurred over a single 24 hour period at 04:30, 08:30, 13:00, 17:30 and 21:30 hours. All leaf samples for respiratory and metabolite profiling were taken from young fully expanded leaves which developed under the prevailing temperature regime.

3.3.2 Dark respiration measurements

Rates of dark respiration (R_{dark}) were measured using a high-throughput fluorophore Q2 O_2-sensor system (Astec Global, Maarssen, The Netherlands) as previously described (Coast et al., 2019; O'Leary et al., 2017; Scafaro et al., 2017a). Measurements occurred on day seven and eight after temperature transfer. Four independent plants, each from a separate tank, from each growth temperature treatment were placed in a container with a small volume of hydroponic solution and brought back to the laboratory to be processed. Two separate 2 cm^2 sections from the middle part of the lamina were cut and placed in separate 2 mL capacity Q2 sampling tubes. The tubes were then sealed with specialised caps containing a fluorescent metal organic dye that sensitive to O_2 quenching (Supplementary Figure S3.1). The Q2 measurements began within 30 min of leaf sectioning. A blue-spectrum LED excitation, followed by a red spectrum emission (approximately 480 nm and 580 nm, respectively) pulse onto the top surface of caps. The phenomenon of O_2 quenching allows the O_2 dependent decay in fluorescence signal to be quantified and eventually converted to absolute values of dark respiration rates (Scafaro et al., 2017a). One sample tube was measured at a common temperature of 30°C, and the other at the prevailing growth temperature. The system was covered with black cloth during the 3 h measurements to maintain darkness. At the cessation of measurements, leaf sections were collected, stored in paper bags, oven-dried at 70°C and weighed to obtain dry mass. The dried leaf materials were subsequently used in the determination of nitrogen concentration as described below. For metabolite profiling over the diurnal cycle, leaves from the same plants chosen for respiration were harvested in the glasshouse on day seven and eight after temperature transfer (the same day as dark respiration

measurements were conducted), immediately frozen in liquid N_2 and stored at minus 80°C.

For the short-term simultaneous measurements of respiration and metabolites, plants were grown for 14 days at the transferred temperatures and respiration measured at a common 30°C, starting at 13:00. Leaf sections were run on the Q2 and snap froze every 30 min over a three-hour period for metabolite profiling, with six plant replicates per sampling point.

3.3.3 Determination of soluble sugars, starch and nitrogen concentrations

To determine the concentrations of soluble sugars and starch, frozen rice leaf sections were lyophilised and ground to a fine powder and 5-10 mg placed in a 2 mL microfuge tube. 500 μL of 80% (v/v) ethanol was added and vigorously vortexed for 20 s. The tissues were incubated at 80°C while being centrifuged at 3000 x g for 20 min. After a further five minutes of centrifugation at 24,000 x g, the resulting supernatant and pellet were separated. This procedure was repeated twice, pooling supernatant and pellet, and used for determination of the concentrations of soluble sugars and starch using a Fructose Assay Kit (Sigma-Aldrich) and a Total Starch Assay Kit (Megazyme), respectively, following manufacturer's instructions. A standard curve for soluble sugars was generated using a series of known concentrations of sucrose, glucose and fructose (Sigma-Aldrich). Measurements were collected using a microtitre plate reader (Infinite® M1000 Pro; Tecan).

To determine the concentration of nitrogen (N), approximately 1-2 mg dried rice leaf sections were ground and placed in a tin-made capsule. The analysis was conducted using combustion, combining an elemental analyser 15 (Heraeus CHN-O Rapid), a Finnigan MAT Trapping box HT and a Finnigan MAT mass spectrometer (delta D) with

a dual inlet at a precision of 0.1 ‰. N concentrations on leaf dry mass basis were calculated based on (Gebauer & Schulze, 1991).

3.3.4 Gas Chromatography-Mass Spectrometry (GC-MS) metabolite analysis

To quantify metabolites involved in carbon metabolism a mass spectrometry approach using GC-MS metabolite profiling was conducted. The extraction was carried out according to procedure previously described in Che-Othman et al. (2019) with some modifications. Frozen leaf tissue was ground to powder and approximately 25 mg transferred into frozen 2 mL microfuge tubes. 500 µL of cold extraction buffer was added, and incubated in a Thermomixer for 20 min at 75^{o}C at 1400 rpm. All tubes were then centrifuged at 16,000 x g for 10 min at room temperature. 60 µL of supernatant was dried down in 200 µL glass insert for derivatization prior to Metabolite profiling using Gas Chromatography-Mass Spectrometry (GC-MS). The derivatisation was performed using the MPS2 XL-Twister autosampler (Gerstel GmbH & Co. KG). Derivatised sample (1 uL) was immediately vaporised in the inlet at 250˚C and injected onto the chromatography column in split-less mode. Helium was used as carrier gas. Compounds were eluted by the following temperature gradient: hold for 1 min at 70˚C then ramp with 7˚C/min to 325˚C and hold for 3.5 min. The ion transfer line was heated to 280˚C and the ion source and quadrupole were at 150˚C and 230˚C respectively. The resulting peaks were analysed using MassHunter Workstation Software Quantitative Analysis Version B.07.1 / Build 7.1.524.0 for GCMS (Agilent Technologies). The integrated area of the quantifier ion for each peak was compared between samples after normalisation. Metabolites were normalised against the weighted and averaged signal of 3 internal standards followed by weighting against the average measured signal across all samples for each compound before statistical analysis was performed.

3.3.5 Statistical analyses

Data were analysed using one-way analyses of variance (ANOVA), two-way ANOVA, principal component analysis (PCA), weighted correlation network analysis, permutational multivariate analysis of variance and multiple comparison tests using the SigmaPlot v11.0 software (Systat Software Inc) and/or R v3.4.4 software (R Core Team, 2018) with RStudio. In R v3.4.4 software, data preparation and plotting were done with packages tidyverse (Wickham, 2017) and factoextra (Kassambara & Mundt, 2017). The PCA was carried out with R packages 'FactoMineR' (Le et al., 2008) and ade4 (Dray & Dufour, 2007). Metabolite levels were centred and scaled with standard score. Significance testing was done with R package 'vegan' (Oksanen et al., 2018). Weighted correlation network analysis was conducted using R package 'WGCNA' (Langfelder & Horvath, 2008). Permutational multivariate analysis of variance was done using Euclidean distance matrices of metabolites compared pairwise (Anderson, 2001; McArdle & Anderson, 2001). The analysis produces test statistic analogous to Fisher's *F*-ratio, and calculates *P*-value through permutations.

3.4 Results

3.4.1 Diurnal variations in acclimation of R_{dark}

Measurements were made of R_{dark} at separate time points over a diel period to determine the extent to which R_{dark} varies throughout the diurnal cycle and with growth temperature (Figure 3.1). Measurements made at the prevailing growth temperature revealed significantly faster rates in the hottest treatment (40°C) and slower R_{dark} at cooler growth temperatures (Table 3.1; Figure 3.1A). Rates of R_{dark} at the prevailing growth temperature also varied with time of day (Table 3.1; Figure 3.1A), with rates typically being at their lowest in the early evening (noting that night temperatures were 5°C lower than those

during the day). The effect of growth temperature on prevailing rates of R_{dark} was consistent through the day (i.e. no significant growth temperature x time interaction (Table 3.1).

R_{dark} measured at a common temperature of 30°C significantly differed among the growth treatments (Table 3.1), with faster and slower R_{dark} for the 25/20°C and 40/35°C grown plants, respectively (Figure 3.1B). This matched my previous findings of slight but significant rice leaf R_{dark} acclimation in measurements made in the middle of the day period (10:00-14:00 hours; Chapter 2). The differences among the three growth temperatures were greater in measurements made at 17:30 hours (2 h before sunset) compared to those during the night (Figure 3.1B), albeit without a significant interaction between growth temperature and time of day (Table 3.1). Taken together, these results reveal that while prevailing rates of R_{dark} at each growth temperature where greater in hot vs warm/cool treatments (Figure 3.1A), acclimation did occur, as shown by the fact that the hot-grown plants exhibit lower temperature-normalized rates of R_{dark}.

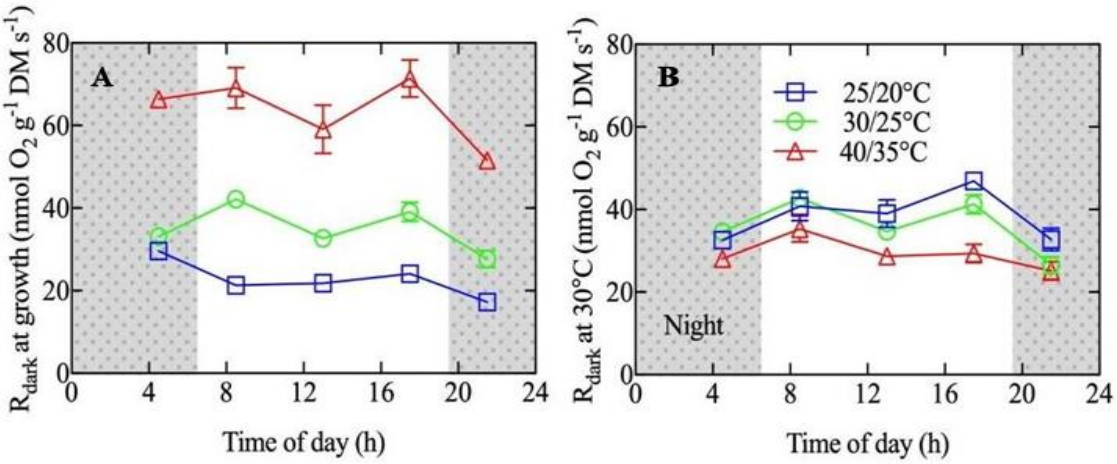

Figure 3.1. Dark respiration (R_{dark}) of rice leaves on a dry mass basis measured over the diurnal cycle at; (A) the respective day-time growth temperature of each treatment; and (B), a common temperature of 30°C. Measurements were conducted on rice leaves grown at 25/20°C (blue squares), 30/25°C (control; green circles) and 40/35°C (red triangles). Area shaded in grey represents the night-time. Data represents means of four biological replicates ± SE.

Table 3.1. Two-way ANOVA of growth temperature and time effects on R_{dark} measured at the prevailing day-time growth temperature and at a common temperature of 30°C. Asterisks (*) denote statistically significant difference (P=<0.05) among the levels of temperature treatments and time points.

	Measured at the prevailing day-time growth temperature		Measured at 30°C	
	F	*P*	*F*	*P*
Growth temperature	254.589	<0.001*	22.628	<0.001*
Time	10.958	<0.001*	14.474	<0.001*
Growth temperature x Time	1.344	0.247	2.031	0.065

3.4.2 Diurnal variations in starch, soluble sugar and N concentration

To assess if the growth temperature and diurnal changes in R_{dark} measured at 30°C were associated with concomitant changes in leaf chemistry, I analysed leaf soluble sugar, starch, and N concentration (Figure 3.2). Leaf soluble sugars remained constant throughout the day and total soluble sugar concentration was slightly but significantly affected by growth temperature being lower in the 25/20°C grown leaves, mainly due to lower glucose and fructose levels (Table 3.2; Figure 3.2A-D). This suggests that the acclimation of R_{dark} measured at 30°C for 25/20°C and 40/35°C grown rice was not associated with changes in sugar substrate availability. Unlike soluble sugars, starch concentration varied diurnally irrespective of growth temperatures, decreasing through the night and increasing through the day, with a minimum value near sunrise and maximum value near sunset (Figure 3.2E). Growth temperature had no significant effect on starch concentration (Table 3.2). Low growth temperature significantly increased N concentration (Figure 3.2F), with plants grown at 25/20°C exhibiting higher values throughout the 24-h cycle than their warmer grown counterpart plants.

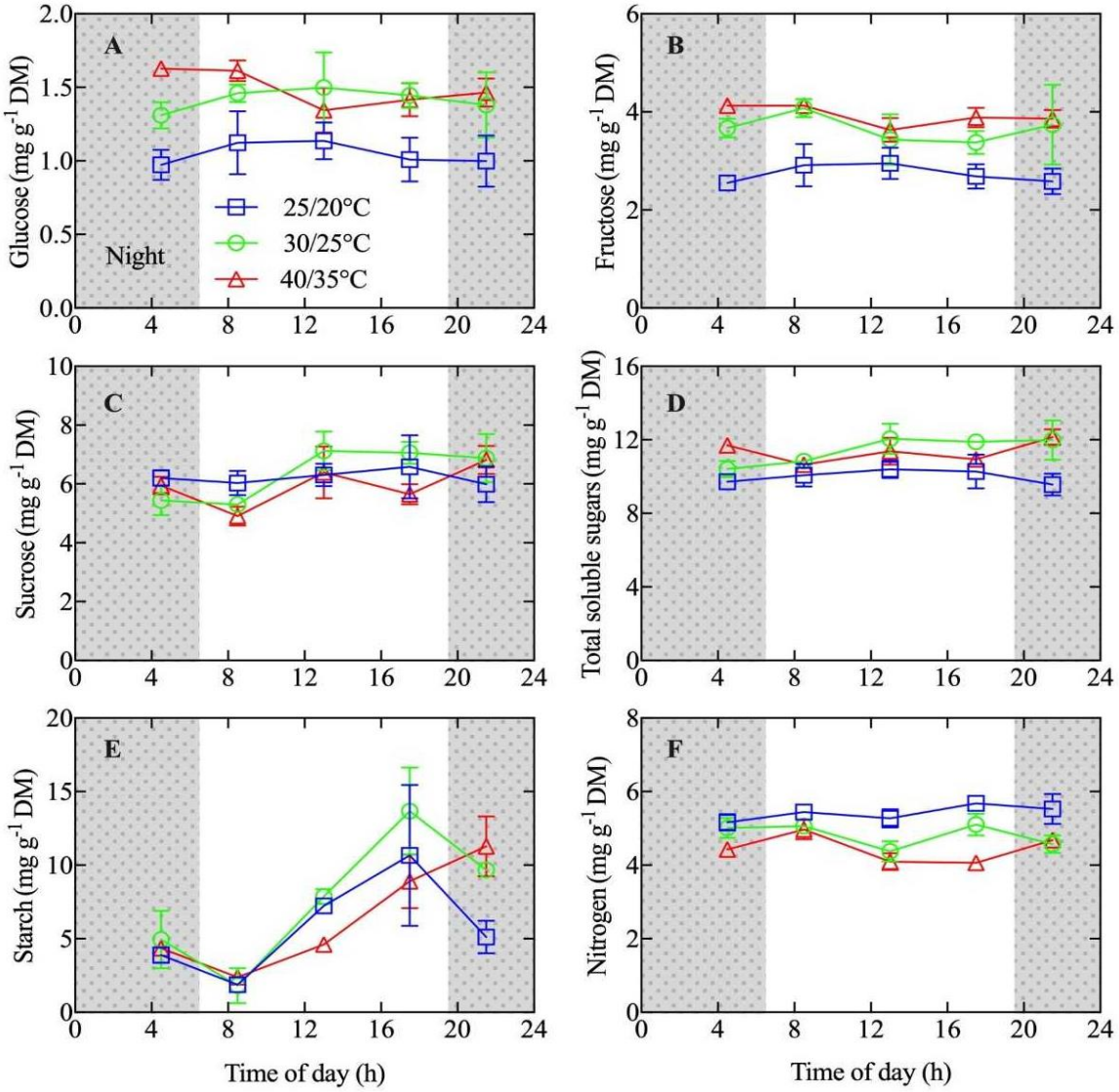

Figure 3.2. Rice leaf concentrations of glucose (A), fructose (B), sucrose (C), total soluble sugar (D), starch (E), and nitrogen (F). Leaves were harvested from plants grown at different temperatures of 25/20°C (blue squares), 30/25°C (control; green circles) and 40/35°C (red triangles). Area shaded in grey represents the night-time. Data represents mean of three biological replicates ± SE.

Table 3.2. Two-way ANOVA of temperature and diurnal effects on the concentrations of glucose, fructose, sucrose, total soluble sugar, starch and nitrogen in rice leaves. Asterisks (*) denote statistically significant difference (P=<0.05) among the levels of temperature treatments and time points.

	Temperature		Diurnal		Temperature x Diurnal	
	F	*P*	*F*	*P*	*F*	*P*
Glucose	14.089	<0.001*	0.334	0.853	0.497	0.849
Fructose	17.945	<0.001*	0.677	0.613	0.377	0.924
Sucrose	0.717	0.497	2.564	0.059	0.960	0.485
Total soluble sugar	4.126	0.026*	0.470	0.757	0.434	0.891
Starch	1.425	0.256	12.024	<0.001*	1.192	0.337
Nitrogen	24.18	<0.001*	2.908	0.032*	1.913	0.083

3.4.3 Metabolite profiles

I explored how the diurnal cycle and growth temperature affected the metabolite profile of rice leaves. I conducted principal component analysis (PCA) to investigate correlations among metabolites (Figure 3.3). The most striking general observation was that many branched chain and aromatic amino acids increased in abundance during the night and in response to warmer temperatures Associated with this was a negative correlation between amino acids and carbohydrates/organic acids (Figure 3.3A).. There were exceptions to this divergence in how metabolite classes responded to time of day/growth temperature. For example, short-chained molecules such as alanine, glycine and serine, as well as the N side chain amino acids glutamine and asparagine, were more responsive to day and cooler temperatures. Moreover, aconitate and citrate were not as negatively correlated with amino acids in response to warmer nights. The correlations did not align with specific metabolic pathways (i.e. glycolysis, TCA cycle, shikimate pathway, amino acid synthesis) (Supplementary Figure S2.1). For example, a change in the TCA cycle intermediate, aconitate, was not preceded by a change in the antecedent metabolite citrate,

nor were changes in aconitate followed by the changes in subsequent metabolites succinate, fumarate, and malate. For most metabolites, there was no interaction between growth temperature and time of the day, with the exception being tyrosine and tryptophan, products of the shikimate pathway (Table 3.3), suggesting that time of the day is most likely unrelated to the metabolite responses to growth temperature treatment.

I used weighted correlation network analysis (Figure 3.3A and Figure 3.4) to determine if metabolite responses correlated to respiration rates presented in Figure 3.1. The weighted correlation network analysis is based on networks of connected nodes with scale-free topology, a structure of networks that arises as a result of adding new nodes to existing nodes and is thought to reflect the evolution of biological systems such as metabolic networks (Jeong et al., 2000). Similar methods have been successfully used to analyse biological networks such as gene co-expression (Stuart et al., 2003) and protein-protein interactions (Jeong et al., 2001). The analysis identified central metabolites that are relatively well connected to changes in R_{dark} at 30°C due to growth temperature, the diel period, or both. R_{dark} was related to growth temperature through the metabolites' xylitol, aconitate, and erythritol (indicated by red boxes in Figure 3.3A). R_{dark} was related to diurnal changes alone through the metabolite, succinate (purple box in Figure 3.3A). The metabolites' related to R_{dark} through both temperature and time, were serine, putrescine, and fructose-6-phosphate (blue boxes in Figure 3.3A). The individual correlations of each central metabolite are presented in Figure 3.4 and Table 3.4. Thus, while variations in R_{dark} were not associated with concomitant variations in the primary substrates feeding into glycolysis, the results do point to light and growth temperature mediated changes in a range of metabolites being linked to variations in R_{dark}.

Principal component analysis (Figure 3.3B) revealed that the day/night phase explained 10% variation in metabolite levels (P<0.001) (Supplementary Figure S3.2).

While both principal components contributed to diurnal variations in metabolites, the first principal component (Dim1) played a stronger role than the second principal component (Dim2), with day samplings (i.e. 08:30, 13:00 and 17:30 hours) being clustered together to the right of Dim1 axis, whereas night samplings (i.e. 21:30 and 04:30 hours) were grouped in the left of Dim1 axis.

Principal component planes Dim1 and Dim2 in Figure 3.3C indicate responses of metabolites to growth temperature. Growth temperature explained 17% of overall variation in metabolite levels (P<0.001) (Supplementary Figure S3.2). Metabolites of different growth temperatures separated along both principal components, with the first principal component (Dim1) playing a stronger role than the second principal component (Dim2): 40/35°C grown plants were grouped together in the left part of Dim1 axis, whereas, 25/20°C grown plants in the right part of Dim1 axis.

Table 3.3. Two-way ANOVA of temperature and diurnal effects on the metabolite level of rice leaves grown at different temperatures. Asterisks (*) denote statistically significant difference (P=<0.05) among the levels of temperature treatments and time points.

Metabolite	*P*-value		
	Temperature	Diurnal	Temperature x Diurnal
Alanine	0.1130	5.25×10^{-7}*	0.2665
Valine	0.2452	0.0242*	0.4354
Isoleucine	0.0281*	0.2110	0.3534
Proline	0.0116*	0.7936	0.2217
Succinate	0.0108*	7.28×10^{-9}*	0.6848
Fumarate	0.0363*	0.0007*	0.9844
Threonine	0.9219	0.0747	0.1243
Malate	0.1522	2.22×10^{-13}*	0.2691
Aspartic acid	0.0829	1.07×10^{-6}*	0.2295
4-Aminobutyric acid	0.0028*	0.9579	0.5610
α-ketoglutarate	3.32×10^{-5}*	$<2.2 \times 10^{-16}$*	0.1675
Phenylalanine	3.54×10^{-6}*	0.0003*	0.0741
Asparagine	0.9157	0.2746	0.2260
Putrescine	2.37×10^{-5}*	0.1720	0.4763
Aconitate	3.75×10^{-9}*	0.0016*	0.6774
Glutamine	1.04×10^{-5}*	0.0279*	0.3447
Shikimic acid	0.5227	0.1504	0.4783
Citrate	0.0919	8.19×10^{-5}*	0.7641
Fructose	0.1620	0.1418	0.8123
Glucose	0.4284	0.0189*	0.9153
Lysine	1.36×10^{-6}*	2.04×10^{-5}*	0.4090
Tyrosine	2.01×10^{-8}*	5.83×10^{-12}*	0.0092*
Tryptophan	0.2963	1.35×10^{-5}*	0.0166*
Fructose-6-phosphate	4.74×10^{-14}	1.59×10^{-7}*	0.5368
Glucose-6-phosphate	5.62×10^{-12}	5.18×10^{-6}*	0.9891
Sucrose	2.66×10^{-5}*	0.0287*	0.7049

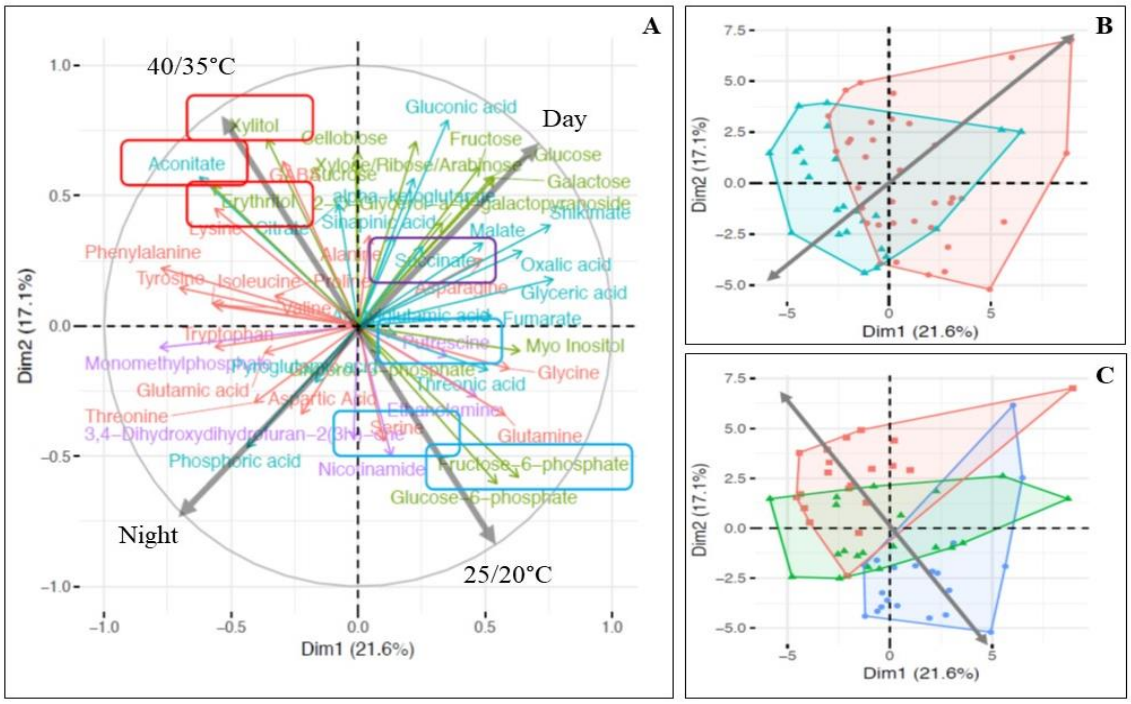

Figure 3.3. Principal component analyses (PCA) portraying relationships among metabolites in relation to the diurnal cycle and growth temperature. (A) Each thin arrow represents a metabolite, and for each arrow, the direction and length from the centre represent a correlation with principle components (x- and y-axis) and the quality of representation (loading) on the principle component plane. The thicker grey arrows indicate the component planes of sampling time (day or night) and growth temperature (25/20°C or 40/35°C) as labelled. Different colours of metabolites and their associated arrows denote different metabolite classes; amino acids in red, carbohydrates in green, organic acids in blue, and others in purple. Respiration rates (at a common temperature of 30°C) were correlated with metabolites that responded to growth temperature (red boxes), changed diurnally (purple box), and responded to both (blue boxes). Note – only metabolites with strong correlations are shown. (B) Metabolite responses in the first and second dims of rice leaves to sampling time (day/night), with day indicated in red and night in blue. (C) Metabolite responses in the first and second dims of rice leaves to growth temperature with 25/20°C in blue, 30/25°C in green and 40/35°C in red.

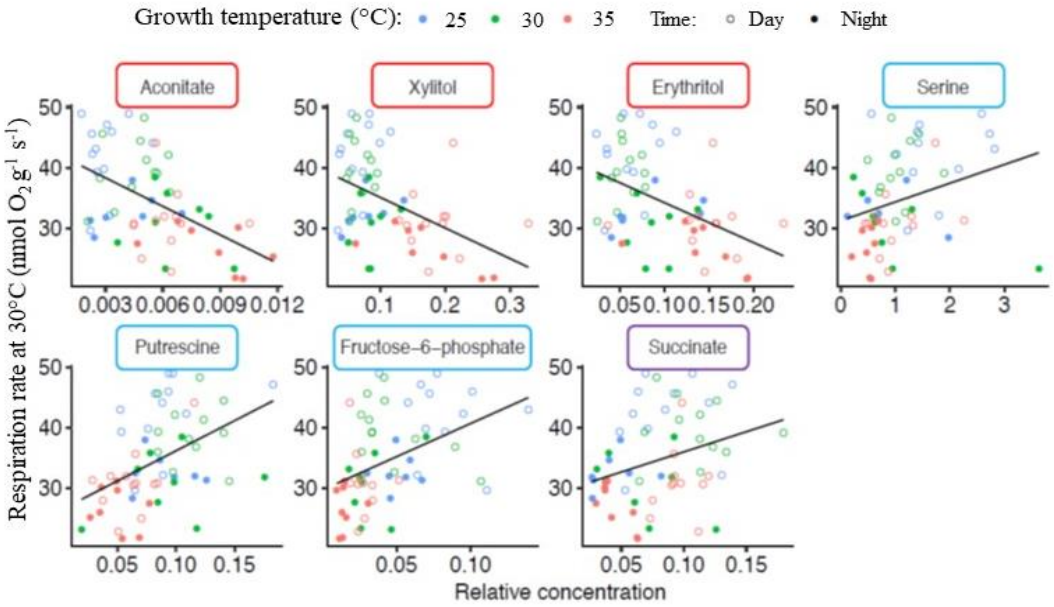

Figure 3.4. Metabolites that significantly correlated with R_{dark} at 30°C identified through weighted correlation network analysis. For aconitate, xylitol and erythritol, correlations were strongest when considering growth temperature alone. For serine, putrescine and fructose-6-phosphate, correlations were strongest when considering both growth temperature and the diel cycle. Succinate correlated to the greatest extent with the day/night cycle alone.

Table 3.4. Results of linear regression on R_{dark} measured at 30°C and key metabolites identified in weighted correlation network analysis as presented in Figure 3.4.

Metabolite	*P*-value	R^2
Aconitate	3.18×10^{-5}	0.25
Xylitol	4.0×10^{-4}	0.18
Erythritol	2.81×10^{-4}	0.19
Serine	0.024	0.07
Putrescine	2.08×10^{-4}	0.20
Fructose-6-phosphate	8.84×10^{-4}	0.16
Succinate	0.016	0.08

3.4.4 Metabolite profiles: effect of the diurnal cycle

Since there was no interaction between diurnal cycle and growth temperature influencing the concentrations of most metabolites apart from tryptophan and tyrosine (Table 3.3), I

performed a one-way ANOVA exploring diurnal cycle changes in metabolites of the respiratory pathways, independent of the growth temperature, and displayed these changes in relation to the respiratory pathway (Figure 3.5). The levels of most of the TCA cycle intermediates were affected diurnally. The levels of glucose-6-phosphate and fructose-6-phosphate, intermediate substrates at the beginning of the glycolysis pathway, varied significantly through the 24 hour cycle, with abundances higher in the day prior to the onset of night (Figure 3.5). Citrate accumulated at night, α-ketoglutarate accumulated during the day, and malate depleted during the day. The amino acid alanine was more abundant during the day and depleted at night, but aspartic acid was reduced in the day and more abundant at night. Similarly, tryptophan, phenylalanine and tyrosine, aromatic amino acid products of the shikimate pathway, significantly increased at night (Figure 3.5).

3.4.5 Metabolite profiles: effect of growth temperature treatments

In contrast to the diurnal cycle, less metabolites in the respiratory pathways were significantly altered by growth temperature alone. This may be a consequence of the diurnal cycle having both an irradiance and temperature component. Metabolic profiling analysis of the growth temperature treatments independent of the diurnal cycle (Figure 3.6) showed subtle changes in soluble sugar substrates of glycolysis, as was the case with the enzymatic assay of total soluble sugars (Figure 3.2 and Table 3.2). Again, the colder growth temperature seemed to reduce glucose and fructose levels, although not to a significant level. Although subtle changes were observed, collectively, the inputs into glycolysis were relatively stable across growth temperatures. By contrast, increasing growth temperature greatly reduced glucose-6-phosphate and fructose-6-phosphate. As with diurnal cycle, phenylalanine and tyrosine were significantly affected by temperature with lower abundance in the colder grown rice. Primary metabolites of the TCA cycle did

not significantly differ with growth temperature apart from aconitate which increased with growth temperature. Many amino acid pools generated from TCA intermediates significantly changed in response to growth temperature, with lysine and GABA increasing at the hottest temperature of 40/35°C, proline and isoleucine decreasing at the lowest temperature of 25/20°C, and glutamine increasing at 25/20°C.

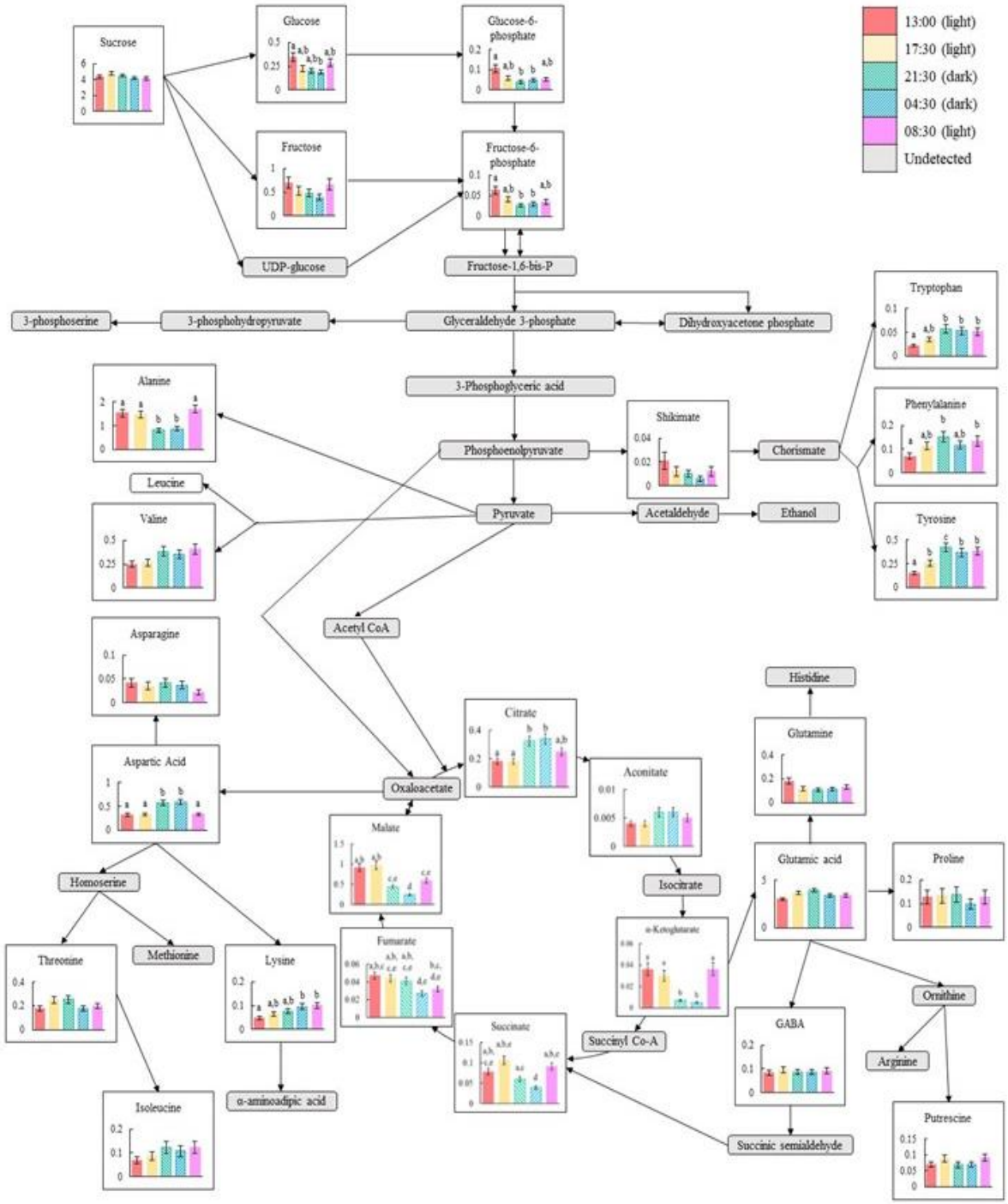

Figure 3.5. Effect of the diurnal cycle on the relative abundance levels of metabolites in rice leaves associated with carbohydrates, glycolysis, amino acids and the tricarboxylic acid (TCA) cycle. Rice leaves were sampled at five different time points throughout light/dark cycle: 13:00 (red), 17:30 (yellow), 21:30 (green), 04:30 (blue) and 08:30 (purple) hours. Data represents mean of four biological replicates. One-way ANOVA and post hoc test were conducted and different letters show significant differences according to Tukey test (P=<0.05).

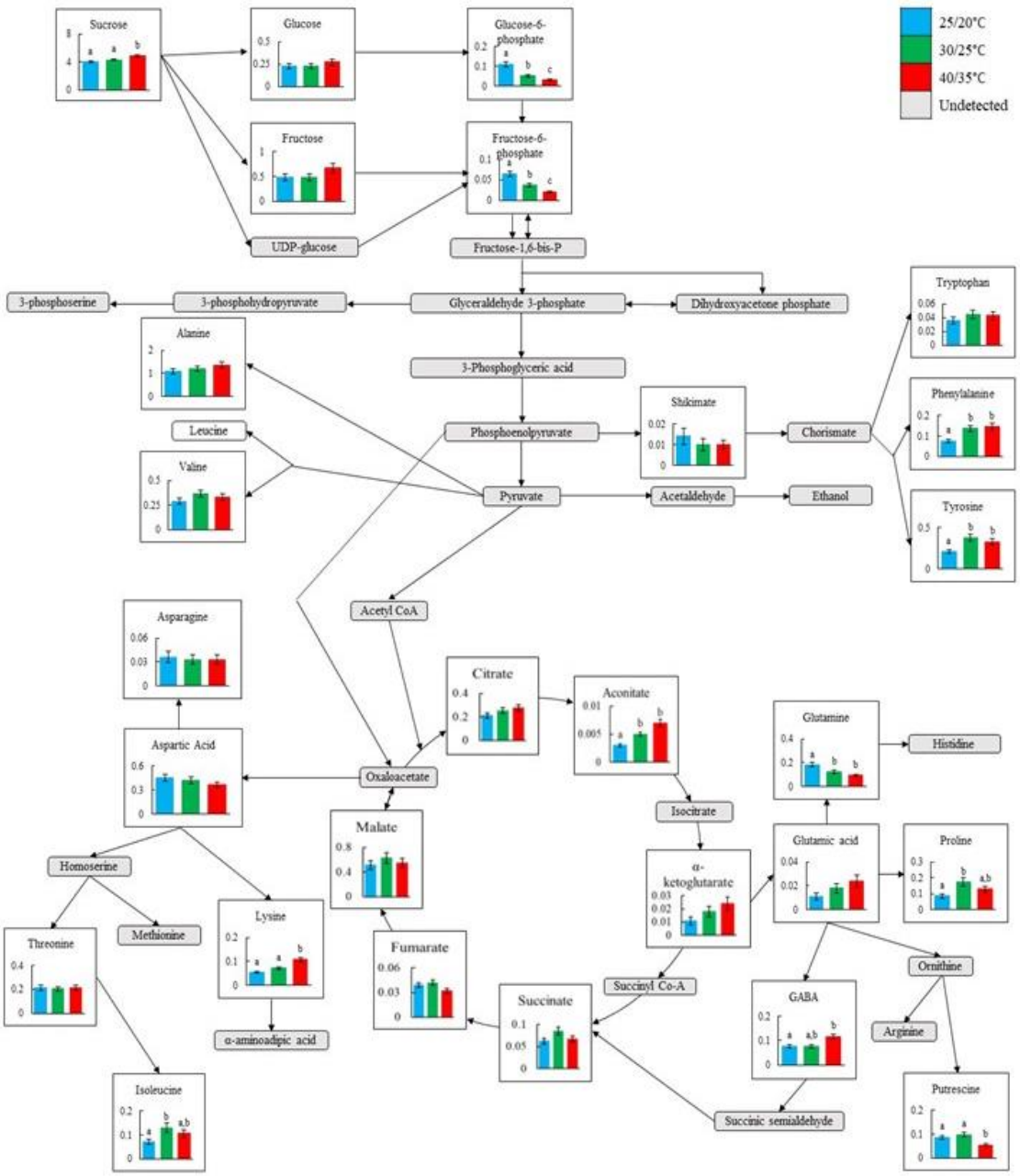

Figure 3.6. Effect of growth temperature treatments on the relative abundance levels of metabolites in rice leaves associated with carbohydrate metabolism, glycolysis, amino acid metabolism and tricarboxylic acid (TCA) cycle. Measurements were conducted on rice leaves grown in 25/20°C (blue), 30/25°C (green) and 40/35°C (red). Data represents mean of four biological replicates. One-way ANOVA and post hoc test were conducted and different letters show significant differences according to Tukey test (P=<0.05).

3.4.6 Short-term simultaneous measurements of respiration and metabolites

To determine how metabolites and respiratory rates aligned on a shorter timeframe I conducted simultaneous measurements of metabolite levels and respiration dependent O_2 consumption. Measurements were made on excised leaf sections placed in sealed tubes in the dark. Multiplicative changes in metabolite were measured prior to respiration measurements, soon after being dark adapted, as well as 30 mins, 1 h, 1.5 h, 2 h, 2.5 h and 3 h into the O_2 consumption recordings, corresponding to the O_2 consumption timeframe used to generate dark respiration rates. At the final time point of 3 h, most of amino acids arising from primary carbon metabolism increased in abundance (Figure 3.7). For example, isoleucine increased the most with 9-fold increase in abundance. Other amino acids including alanine, valine, proline, phenylalanine, lysine, tyrosine, glycine, aspartic acid, GABA, putrescine and tryptophan changed by 0.3- to 5.8-fold. For organic acids and TCA cycle intermediates (except succinate and citrate), the levels remain fairly constant even after 3 h of dark adaptation. Succinate and citrate decreased in abundance to approximately half of their original levels. Carbohydrates at the initial steps of glycolysis such as fructose, glucose and fructose-6-phosphate increased by 1.7- to 2.5-fold. Glucose-6-phosphate abundance was fairly stable throughout the experiment.

Compound	0 min	30 mins	1 h	1. 5 h	2 h	2.5 h	3 h
Alanine	1.0	1.0	1.0	1.3	1.5	1.7	1.8
Valine	1.0	1.2	1.4	2.1	2.8	3.4	4.0
Isoleucine	1.0	1.5	2.1	3.6	5.3	7.2	9.0
Proline	1.0	1.3	1.4	2.2	3.5	4.9	5.8
Glycine	1.0	0.9	0.9	0.8	0.8	0.9	0.9
Succinate	1.0	0.8	0.9	0.8	0.7	0.6	0.5
Fumarate	1.0	1.1	1.0	0.9	0.9	0.8	0.8
Serine	1.0	1.3	1.4	1.3	1.5	1.6	1.9
Threonine	1.0	1.2	1.3	1.5	1.7	1.8	1.9
Malate	1.0	0.9	1.0	0.9	0.9	1.0	0.8
Aspartic Acid	1.0	0.9	0.9	0.9	1.0	0.9	0.7
GABA	1.0	0.8	0.9	0.7	0.9	0.5	0.8
Phenylalanine	1.0	1.3	1.5	2.4	3.3	4.0	4.6
Asparagine	1.0	1.5	1.3	1.2	1.5	1.5	1.8
Putrescine	1.0	0.9	0.9	0.9	0.8	0.8	0.7
Aconitate	1.0	1.1	1.4	1.1	1.2	1.1	0.9
Glutamine	1.0	1.2	1.1	1.1	1.8	2.2	2.5
Shikimic acid	1.0	1.3	1.4	1.4	1.2	1.5	1.0
Citrate	1.0	1.1	1.3	1.0	0.9	0.8	0.6
Fructose	1.0	1.0	1.2	2.2	2.4	3.0	2.5
Glucose	1.0	1.1	1.5	2.2	2.2	2.6	2.2
Lysine	1.0	1.3	1.5	2.5	3.4	4.2	5.0
Tyrosine	1.0	1.5	1.8	2.8	3.8	4.7	5.1
Tryptophan	1.0	1.1	1.1	1.0	1.0	0.9	0.8
Fructose-6-phosphate	1.0	0.9	1.0	1.2	1.5	1.5	1.7
Glucose-6-phosphate	1.0	1.1	1.4	1.2	1.3	1.2	1.1

Figure 3.7. Changes in the levels of metabolites from excised leaf sections with short-term exposure to the dark. The values are the multiplicative changes between each time point relative to the initial 0-min control.

3.5 Discussion

Our study has shown a role of metabolites – particularly amino acids – in the acclimation response of respiration, both in the short and longer-term. Metabolites derived from glycolysis and the TCA cycle (xylitol, erythritol, serine, and putrescine) were strongly correlated with adjustments in R_{dark} to temperature and the day/night cycle. Less unexpectedly, primary metabolites of glycolysis (fructose-6-phosphate) and the TCA cycle (aconitate and succinate) were associated with adjustments in R_{dark} (Figure 3.4 and Table 3.4). Many of the metabolites that did change in abundance to growth temperature or the diel cycle did so for one and not the other (Table 3.1), supporting separate influences of growth temperature and diel perturbations on respiration. The limited interaction between metabolite responses to growth temperature and a day/night cycle was evident in respiratory flux measurements, with the acclimation phenotype largely been held across the diurnal cycle (Figure 3.3B). More metabolites in the respiratory pathway were altered by diurnal cycle, compared to growth temperature, likely as a consequence of the diurnal cycle having both an irradiance and temperature component. Of interest, the shikimate derived aromatic amino acids were the only metabolites that significantly adjusted in relative abundance to both growth temperature and the diel period. My results provide important and unexpected insight into what are the likely metabolic determinants of respiratory acclimation and its response to the day/night cycle.

3.5.1 The importance of considering the diel cycle when determining temperature acclimation

R_{dark} was significantly different over the diurnal cycle, potentially less so for rice acclimated to a hotter temperature of 40/35°C, although the interaction was not significant (P=0.065; Table 3.1). The time of day when samples are measured will therefore

influence the extent of observed R_{dark} acclimation to growth temperature. Thus, R_{dark} measured at different time points of the day should be compared with caution. Most studies exploring the extent of respiratory acclimation, both *in* and *ex situ*, take account of factors such as nutrient availability, geographical location and soil type, but little attention is paid to what time of the day/night cycle measurements are collected (Kattge et al., 2011). It might not be practical to measure R_{dark} at multiple time points throughout a day in large scale studies. However, it is important to be aware that variation in respiration throughout the diel cycle does occur and can be influenced, although weakly, by temperature.

Of further consideration is mitochondrial respiration in the light (R_{light}). R_{light} is suppressed compared to the dark by as much or more than 50% in many plant species (Atkin et al., 1998; Atkin et al., 2000b; Tcherkez et al., 2005), including rice (Scafaro et al., 2012), and more so at warmer temperatures (Way & Yamori, 2014). Light dependent differences in respiratory flux are logical considering the rapid changes in metabolite pools I observed within minutes to hours of dark exposure (Figure 3.7). The mismatch of extracting metabolites from light exposed leaves while measuring R_{dark} rather than R_{light} would have added a confounding effect to my association analysis between diurnal respiration and metabolites, which must be kept in mind. However, decoupling respiratory from photosynthetic fluxes is difficult and reduces the accuracy and ease of measuring R_{light}, and thus, despite its limitations, R_{dark} is usually taken as a surrogate for R_{light}, as I did in this study.

3.5.2 Specific metabolites that correlate with R_{dark}

The sugar alcohols xylitol and erythritol and the TCA organic acid aconitate were the metabolites that had the strongest correlation between growth T and changes in R_{dark} at 30°C (Figure 3.4 and Table 3.4). In a recent study by Impa et al. (2019), warmer nights

led to an increase in the sugar alcohol myo-inositol in spikes of a relatively heat tolerant wheat cultivar. The authors attribute this to osmolyte protection. The increase in xylitol and erythritol that I observed (Figure 3.4 and Table 3.4) is likely to be a similar osmolyte protection response to heat. Increases in these sugar alcohols and reduced R_{dark} at 30°C may be independent responses to increased *T*. Alternatively, the accumulation of osmolytes for heat protection may in some way suppress respiratory requirements of the leaf. Stabilisation of membranes as a result of osmolyte protection during heat acclimation is postulated (Gauthier et al., 2014; Sung et al., 2003). As such, a greater maintenance of proton motive forces, through reduced membrane leakiness, is a conceivable way in which respiratory costs could be reduced with increases in xylitol and erythritol.

Succinate was the metabolite with the strongest correlation between diel variations in metabolite levels and R_{dark} at 30°C (Figure 3.4 and Table 3.4), being more depleted prior to sunrise and post sunset (Figure 3.5). Succinate may therefore be a key control point in coordinating the TCA cycle with respiratory adjustments over a day/night cycle. This is likely associated with the light dependent regulation of succinate dehydrogenase activity and its influence over succinate content (Daloso et al., 2015; Eprintsev et al., 2013).

The amino acids serine, the amino acid breakdown product putrescine, and the phosphorylated sugar fructose-6-phosphate were the metabolites with strongest correlation between R_{dark} at 30°C over changes in growth temperature and the diel cycle (Figure 3.4 and Table 3.4). It is interesting that an amino acid and associated amino acid breakdown compound correlate as strongly as a sugar substrate of glycolysis. The correlation between fructose-6-phosphate and R_{dark} at 30°C, both over the diel cycle, and growth temperature, is understandable as this initial substrate of glycolysis is a regulatory

point of respiration (Plaxton & Podestá, 2006; Stitt, 1990). Serine and putrescine on the other hand are not primary metabolites of respiration. As discussed below, there seems to be a tight coordination between amino acid synthesis and respiration which may account for this close association between serine, putrescine, and the respiratory flux at a common 30°C. Additionally, serine is a product of photorespiration, which may explain its variability across growth temperatures and the day and night periods (Figure 3.4). Photorespiration would not occur in the dark and would be stimulated by heating (Walker et al., 2016). Levels of serine were lower at night in agreement with reduced photorespiration. However, serine levels were often lower at 40°C than 25°C in the day (Figure 3.4), at odds with photorespiratory expectations. This highlights the difficulty in characterizing the relationships between metabolite pools and environmental perturbations. Many metabolites will have interconnected pathways that are influenced separately by temperature and light, such as the involvement of serine in photorespiration and protein synthesis.

3.5.3 Assimilate utilisation

Starch accumulated during the day in leaves, and was depleted during the night, reaching a minimum in the early morning (Figure 3.2); a pattern that is well documented (Graf et al., 2010; Graf & Smith, 2011). Interestingly, starch concentration followed a similar pattern irrespective of growth temperature, with no significant interaction between temperature and the starch diurnal cycle (Table 3.2). $^{14}CO_2$ pulse-chase experiments demonstrate that faster rates of R_{dark} occurs through faster depletion of starch (Hüve et al., 2012). However, transport of sucrose (as a result from starch conversion) out of leaves also has a major impact on leaf starch content (Stitt & Zeeman, 2012). The similar pattern

of starch drawdown despite differences in R_{dark} is therefore likely due to temperature mediated shifts in starch transport (as sucrose) out of leaves to other organs.

3.5.4 Amino acids respond strongly to the diurnal cycle and growth temperature

Long chained and aromatic amino acids such as the shikimate pathway derived aromatics phenylalanine, tyrosine and tryptophan; the oxaloacetate derived aspartic acid and lysine; and the α-ketoglutarate derived proline and GABA, all significantly increased at night or with warmer growth temperature (Figure 3.5, 3.6). Exceptions were amino acids with short catabolic pathways: alanine, glutamine and asparagine; as well as serine and glycine, amino acids with simple interconversion pathways (Hildebrandt et al., 2015). Such fluctuations among amino acid pools between the light and dark have previously been observed. For example, in Arabidopsis shoots, amino acid pools are greater in the day, such as glutamine, serine and threonine, while others such as glutamic acid, aspartic acid and asparagine were in greater abundance at night (Watanabe et al., 2014). Similarly, in tobacco leaves glutamic acid, aspartic acid, and asparagine are maintained at higher levels throughout the night relative to the day, while glycine, serine, and alanine are severely depleted at night (Fritz et al., 2006). The increase in abundance of certain amino acid pools in the dark was evident even over hours, with a nine-fold increase in isoleucine and greater than a five-fold increase in proline, lysine, and tyrosine levels within three hours of dark exposure (Figure 3.7). Of note, the relative abundance of metabolites from the short-term dark exposure (Figure 3.7) was much greater than the following growth temperature/diel metabolite analysis (Figure 3.5 and Figure 3.6). This is likely linked to the former being sampled from detached leaf sections rather than harvested from *in situ* leaves, and the associated changes this would have on metabolite transport and utilisation.

The synthesis of amino acids in the dark seems counterintuitive, as N incorporation into amino acids predominantly occurs in the light (Canvin & Atkins, 1974; Fritz et al., 2006; Nelson et al., 2014). Toxic ammonia is initially incorporated into glutamine and glutamate by glutamine synthetase and glutamate 2-oxoglutarate aminotransferase, an energy expensive process likely supplemented by energy generation by photosynthesis (Plaxton & Podestá, 2006; Tcherkez et al., 2017). However, I saw a greater accumulation of certain amino acids during the night which suggests that N was likely fixed into amino acids during the light, but at night catabolised through the respiratory pathways to synthesise a wider array of other common amino acids. The accumulation of these amino acids at night could also be due to an increase in protein degradation or a decrease in respiratory consumption. Alanine is the prime candidate for N recycling at night, as it was more abundant than other amino acids in the day, significantly depleted at night, and has the shortest anabolic/catabolic pathway, being converted between alanine and pyruvate by alanine aminotransferases (Hildebrandt et al., 2015). Furthermore, the *de novo* synthesis of alanine in the light is more rapid than other amino acids (Ishihara et al., 2015; Nelson et al., 2014; Szecowka et al., 2013), indicative of its role as an initial N storage point and mediator of carbon skeleton synthesis. Unsurprisingly, short-chained amino acids associated with photorespiration, glycine and serine (Dellero et al., 2016; Novitskaya et al., 2002; Timm et al., 2013) are highly abundant, accumulated during the day and are depleted at night (Fritz et al., 2006) likely playing a similar role to alanine in N and carbon recycling at night. Additionally, the strong positive correlation I observed between serine and R_{dark} at 30°C (Figure 3.4 and Table 3.4) may be an indicative of a coordination between amino acid recycling and respiratory acclimation.

The recycling of organic carbon skeletons through the TCA cycle can act as a mechanism to coordinate substrate supply with environmental dependent shifts in energy requirement, maintaining balanced redox poise (Igamberdiev & Eprintsev, 2016). An example of such coordination is evident in a study of tobacco plants with antisense enolase and reduced phosphoenolpyruvate (PEP) pools (Voll et al., 2009). With a decrease in PEP, respiration rates were constant despite a reduction in glycolytic substrate, due to downregulation of PEP entering the shikimate pathway, leading to a decline in aromatic amino acids (Voll et al., 2009). Thus, the reduced aromatic amino acid level I observed during the day and in cold-grown rice suggests less glycolytic metabolite partitioning to the shikimate pathway and a greater utilization of sugar substrates and organic acids for energy production at colder temperatures and during the day. This may be related to significantly reduced soluble sugar substrate concentrations noted in the 25/20°C grown rice (Figure 3.2). Together with higher levels of fructose-6-phosphate and glucose-6-phosphate at colder temperature (Figure 3.6) substrate flux through glycolysis was likely reduced at cold temperatures, which may have supressed the shikimate shunt. In the above analysis I am assuming changes in pools of amino acids are indicative of changes in biosynthesis rather than reduced incorporation of amino acids into proteins. On this point, in developed non-senescing tissue, the amino acid pools are not more than 1% of total amino acids incorporated into proteins, with the bulk of protein synthesis occurring through protein degradation and recycling rather than reliance on *de novo* amino acids synthesis (Hildebrandt et al., 2015).

If indeed my postulation that amino acid recycling was a mechanism to coordinate respiratory flux and redox poise with energy requirements, then TCA dependent complex-chain amino acids would likely increase when respiratory substrate supply in the form of organic acids and carbohydrates are unbalanced in relation to energy requirements. In

support of this, I did note a strong negative correlation between many long chained and aromatic amino acids and carbohydrate/organic acid pools (Figure 3.3). Furthermore, the general increase in the level of many long chained and aromatic chain amino acids occurred in warmer grown plants during the night/early-morning, coinciding with the fastest absolute R_{dark}. Significant catabolism of fructose-6-phosphate and glucose-6-phosphate pools also occurred during the dark and particularly at higher temperatures. These primary starting metabolites of glycolysis are control points for respiratory flux regulation (Plaxton & Podestá, 2006; Stitt, 1990). Thus, fluxes through the respiratory pathways were likely stimulated by transition from light to dark and from cold to warm environments. I therefore postulate that formation of long chained and aromatic amino acids in warmer grown rice at night consumed excessive reducing equivalents, balancing redox poise. The strong positive correlation between R_{dark} at 30°C and abundance of the amino acid breakdown product putrescine further supports amino acid recycling as a mechanism to balance respiration with the redox state of the mitochondria. Alternatively, a general increase in amino acid abundance in warmer grown rice at night, possibly through protein degradation, could indicate a greater reliance on amino acids as a substrate for greater energy demands. It is well established that protein degradation can be utilised as TCA cycle substrates (Araújo et al., 2011). In fact, R_{dark} can be directly stimulated by application of exogenous glycine, alanine and aspartic acid in Arabidopsis (O'Leary et al., 2017). However, the significantly faster leaf elongation rates I have previously observed in the hotter grown 40/35°C rice (Rashid et al., 2020) and the maintained soluble carbohydrate pools, is inconsistent with protein catabolism as a source of R_{dark} substrates at warmer growth temperatures.

3.5.5 TCA cycle organic acid intermediates

Irradiance or temperature did not lead to a uniform shift in TCA cycle intermediates. Succinate dehydrogenase and fumarase are downregulated in the light via phytochrome A (Eprintsev et al., 2013; Eprintsev et al., 2016) and also deactivated by thioredoxin enzyme (Daloso et al., 2015). This clearly leads to the high levels of succinate, fumarate and malate during the day (Figure 3.5). This tightly controlled light-dependent regulation of succinate dehydrogenase is the likely reason for succinate being the metabolite that correlated the strongest between R_{dark} at 30°C and the day/night cycle (Figure 3.4 and Table 3.4). In addition, the anaplerotic pathway involving the phosphorylation by PEPC (O'Leary et al., 2011; Tcherkez et al., 2009; Werner et al., 2011) could also contribute to the accumulation of malate and fumarate. Aconitate isomerase activity is greater in the day than at night which may explain a decrease in citrate levels during the day (Igamberdiev & Eprintsev, 2016). I can therefore attribute the significant shifts in TCA intermediates like malate, citrate, fumarate and succinate between day and night to shifts in enzyme velocities regulated by the day night cycle. Interestingly, the limited effect of growth temperature on succinate, malate or citrate levels indicates that these day/night enzyme activity dependent fluctuations are not disrupted by longer-term acclimation to growth temperature.

The accumulation of malate during the day and its depletion at night is linked to its prominent role as the main substrate for respiration, driving the TCA cycle. A link between photosynthesis and malate supply for respiration is evident in Arabidopsis where reduced plastid NAD-malate dehydrogenase activity results in higher night-time malate and starch content and reduced R_{dark} (Beeler et al., 2014). Similarly, malate depletion due to overexpression of NADP-malate dehydrogenase in Arabidopsis chloroplasts leads to a suppression of R_{dark} and ultimately plant starvation (Zell et al., 2010). My observation of malate declining at night can thus be attributed to malate synthesis during the day as a

storage of photosynthesis reducing potential, followed by oxidation at night to facilitate respiration. The use of stored malate as a TCA substrate at night would likely be another contributor to the disparity between growth temperature dependent R_{dark} and starch drawdown I observed.

3.6 Conclusions

From this work I have shown that the TCA cycle is highly responsive to both the day/night cycle and temperature acclimation. However, there is little overlap with minimal influence of one on the other. Similarities do exist in relation to long chained and aromatic amino acids which increase in abundance in response to reduced irradiance and higher growth temperatures, and associated changes in R_{dark}. The tight coordination of the TCA cycle and its link to amino acid synthesis is evident in that not only did the glycolysis substrate fructose-6-phosphate correlate strongly with environmental driven acclimation in R_{dark}, but so did serine and putrescine, TCA cycle-derived metabolites. Interestingly, shikimate pathway-derived aromatic amino acids were the only metabolites to interact in response to both growth temperature and the day/night cycle. Tight regulation between balancing redox potential with ATP demand seems to be a key feature of thermal acclimation, utilising these metabolites, as a means to achieve this balance between supply and demand of respiratory substrate.

Chapter 4 – Understanding mechanisms of respiratory thermal acclimation in chickpea: scaling from isolated mitochondria to whole leaves

4.1 Abstract

The way in which temperature influences the mechanisms of respiration at the organelle level is unclear. To investigate this, I grew chickpea plants at 15/10°C (light/dark), 20/15°C, 25/20°C and 30/25°C. Measurements of O_2 consumption showed that similar to rice, leaf dark respiration (R_{dark}) did acclimate to temperature changes in chickpea. However, R_{dark} did not significantly change over the diurnal cycle, irrespective of growth temperature. Although a greater leaf mass per area (LMA) was observed in 15/10°C grown compared to warmer grown plants, fumarase activity assays from leaves and isolated mitochondria showed no difference in mitochondrial protein content of leaf tissues between growth treatments. The results suggest that changes in mitochondrial characteristics rather than changes in mitochondrial abundance underlie the difference in leaf R_{dark} between growth temperatures. Further, O_2 consumption measurements in isolated mitochondria showed that associated with reduction of R_{dark} in warmer grown plants was the decline in rates of respiration per unit mitochondrial protein, most probably because there are reductions in the capacity of COX pathway owing to a decrease in the abundance of COX protein. Overall, it may be said that the altered cytochrome capacity is most likely to be the central of respiratory acclimation to temperature changes.

4.2 Introduction

Most of the reactions involved in respiration occur in mitochondria. It is important to understand how this organelle, particularly the two key pathways in the plant mitochondrial electron transport chain (ETC): the cytochrome *c* oxidase (COX) pathway, and the alternative oxidase (AOX) pathway (Vishwakarma et al., 2015; Yoshida et al., 2011) responds to global warming. Reducing equivalents generated by the tricarboxylic acid (TCA) cycle are oxidized, with liberated electrons transferred along the electron transport chain (ETC), producing a protein motive force for the generation of ATP. In the COX pathway, electrons in the ubiquinone pool are transferred indirectly to COX (Complex IV) via the ubiquinol-cytochrome *c* oxidoreductase (Complex III), in which the flow is coupled to proton transport from the matrix to the inter membrane space of mitochondria (Mansilla et al., 2018; Millar et al., 2004). A proton gradient generated is subsequently dissipated by the production of ATP via ATP synthase (Complex V) (Mansilla et al., 2018; Millar et al., 2004). By contrast, electrons in the ubiquinone pool transferred directly to AOX are not coupled to proton transport; thus, ATP production is decreased (Vanlerberghe, 2013). In addition to ATP synthase, another means of dissipating the proton gradient is by using uncoupling proteins (UCP) (Krauss et al., 2005; Sweetlove et al., 2006). As with AOX, engagement of UCP is not coupled to ATP synthesis (Jastroch et al., 2010). Considering the importance of the ETC in plant energy production, it is important that we understand how environmental changes, such as low and high temperatures, affect these pathways.

When subjected to short-term changes in temperature, mitochondrial O_2 uptake typically doubles or halves for each 10°C increase or decrease in temperature, respectively (i.e. Q_{10} = 2.0), with the AOX and COX exhibiting relatively similar Q_{10} values (Atkin et al., 2002). However, with extended exposure to sustained changes in

growth temperature, respiration often acclimates, with cold-grown plants exhibiting higher rates of temperature normalized respiratory O_2 uptake than their warm-grown counterparts when measured at a common temperature (Armstrong et al., 2006; Atkinson et al., 2007; Covey-Crump et al., 2002). What is less clear, however, is whether changes in mitochondrial density and/or rates of O_2 uptake per unit mitochondrial protein contribute to the acclimation-based changes in temperature normalized rates of O_2 uptake in whole leaf tissues. It is also unclear whether acclimation is more strongly associated with changes in capacity of the AOX versus that of the COX. Studies on mitochondria isolated from leaves is required to tease apart the importance of individual ETC pathways to the acclimation response of respiration. Mitochondria are provided with exogenous substrates and maximum rates of O_2 uptake by the two terminal oxidases are assessed using inhibitors. I aimed to explore the extent to which acclimation was associated with changes in O_2 uptake per unit mitochondrial protein, whether acclimation was more strongly linked to changes in capacity of one pathway more than the other, and whether mitochondrial abundance per unit leaf mass was affected by growth temperature.

While photosynthesis in chickpea has been the subject of many studies (Bhasker et al., 2018; Chakrabarti et al., 2013; Hosseinzadeh et al., 2018), knowledge on respiration is more limited. This, despite chickpea being one of the most widely consumed legumes in the world (Esfahani et al., 2014; Merga & Haji, 2019). As a leguminous crop, chickpea is important not only in providing nitrogen to soil to enhance soil fertility, but also as an excellent source of protein for human consumption (Hiremath et al., 2011; Kaloki et al., 2019; Rasool et al., 2015). Because of the high content of protein, chickpea plays a significant role in food security, especially for people in poor developing countries with otherwise limited access to protein in their daily diet (Merga & Haji, 2019). Chickpea is a cool season crop with optimum temperature between 21-29°C during the day and

around 21°C at night (Kulkarni & Chimmad, 2014). However, similar to rice, climate change is significantly impacting on chickpea production. According to Devasirvatham and Tan (2018), high temperature is one of the most crucial stresses that has hampered chickpea production, with approximately 10-15% of yield losses for every 1°C increase beyond the optimum temperature (Upadhyaya et al., 2011). Thus, understanding how high temperature influences processes that control yield is needed if chickpea productivity (just like with rice) is to be maintained in warmer environments.

The aims of this chapter were to: characterize the extent of thermal acclimation of R_{dark} in chickpea to contrasting growth temperature regimes, and to investigate mechanistic changes in the mitochondrial ETC associated with leaf respiratory acclimation. I tested whether acclimation is associated with changes in: (1) mitochondrial protein per unit leaf mass; (2) maximal rates of O_2 consumption per unit mitochondrial protein; and, (3) O_2 uptake capacity via AOX vs COX in isolated mitochondria. The chapter also explores how growth temperature influences transcript abundance in leaves of chickpea. I hypothesised that thermal acclimation of respiration is associated with changes where cold grown plants (compared to hot grown plants) exhibit: (1) greater mitochondrial protein per unit leaf mass; (2) higher maximal rate of O_2 uptake per unit mitochondrial protein, when measured at a common temperature; and (3) lower O_2 uptake capacity via AOX in isolated mitochondria. Research at this level has the potential to explain how the whole plants respond to temperature changes.

4.3 Methodology

4.3.1 Plant materials and temperature treatments

Commercial kabuli type chickpea (*Cicer arietinum*; Katoomba, NSW, AU) plants were used in this study. Although most of chickpea production in Australia is desi (often for

exporting), kabuli is the most common chickpea grown for Australian local markets (Heard, 2017). The chickpea plants were grown in temperature-controlled growth cabinets at the Research School of Biology, Australian National University. Seeds were sown in seedling trays (30 x 37 x 7.5 cm) filled with seed raising mix (Debco Pty Ltd, Bella Vista, NSW, AU) and placed in two identical growth cabinets with eight trays per cabinet. The temperature was set to 15/10°C (light/dark) with a 16 h photoperiod and photosynthetic active radiation of 300 µmol m^{-2} s^{-1}, similar to previously reported growth conditions for chickpea (Sandhu & Hodges, 1971). Once all plants under 15/10°C were harvested, the entire set of experiments were repeated a further three times with growth temperature regimes set to 20/15°C, 25/20°C and 30/25°C (light/dark). Measurements were made on fully developed leaves at the 8- to 9-leaf stage, 2 h either side of lights turning on and off. For O_2 consumption measurements, leaves were placed in zip-lock bags containing moist paper towels and brought back to the lab to be processed. For fumarase activity and determination of transcript abundance, leaves were immediately frozen in liquid N_2 and stored in -80°C freezer until further analyses.

4.3.2 Leaf mass, area and O_2 consumption measurements

Three to four biological replicates were used to measure O_2 consumption in chickpea leaves. Fresh mass of each leaf was weighed, and leaf area was determined using a scanner (CanoScan LiDE110; Canon Australia, Macquarie Park, NSW, Australia) with analysis of scanned images using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). Thereafter, leaf dark respiration were calculated from O_2 consumption rates using a gas-phase fluorophore O_2-sensor system, utilising 2 mL O_2-sensor tubes as previously reported (Scafaro et al., 2017a). Rates were measured at a common temperature of 25°C at four different time points: two hours before (-2 h) and after (+2 h) lights were turned on/off. At the cessation of measurements, leaf sections

were collected, stored in paper bags, oven-dried at 70°C for 2 d and weighed to obtain dry mass. Q_{10} values were determined on leaves collected 2 h into the light by adjusting the temperature of four different bays in the O_2-sensor system (Figure S3.1) to 15, 20, 25 and 30°C, respectively. The measurements were carried out for 3 h and the Q_{10} values (i.e. proportional change in R_{dark} per 10°C rise in temperature) were calculated by using the equation:

$$Q_{10} = 10^{(10)(k)} \qquad \text{Equation 1}$$

where k is the regression slope of the $\log_{10}$ transformed R_{dark} plotted against measuring temperature (Atkin et al., 2000a).

4.3.3 Isolation of mitochondria

Isolation of intact mitochondria from chickpea shoots was conducted as described by Day et al. (1985) at the 8- to 9-leaf stage. Fifty grams of shoots were harvested, placed in a zip-lock bag and transported to the lab on ice. The shoots were washed with cold water and all subsequent steps conducted at 4°C. Shoots were disrupted with a stand homogenizer (Brinkmann PT-10-35 Polytron Homogenizer; Kinematica AG, Luzern, Switzerland)) in 200 ml of grinding medium containing 0.3 M sucrose, 25 mM tetrasodium pyrophosphate, 2 mM EDTA (disodium salt), 10 mM KH_2PO_4, 1% (w/v) PVP-40, 1% (w/v) BSA and 20 mM ascorbic acid, pH 7.5. The homogenate was filtered through 4 layers of damp, chilled Miracloth (Merck Millipore, Kilsyth, VIC, AU), then centrifuged for 5 min at 3000 RPM. The pellet was discarded and the supernatant was transferred to new tubes and centrifuged for 20 min at 12,300 RPM. The supernatant was discarded and the pellet was dispersed in residual supernatant using a small paint brush. 100 μL of wash medium consisting of 0.3 M sucrose, 10 mM TES and 0.1% (w/v) BSA, pH 7.5 was added to resuspend pellets, then the two centrifuge steps were repeated. The

final pellet was resuspended in 10 mL of wash medium, then layered over 35 mL of PVP gradient solution consisting of 0.6 M sucrose, 20 mM TES, 0.2% (w/v) BSA, 28% (v/v) Percoll and a linear gradient of 0-4.4% (w/v) PVP-40, pH 7.5. After centrifugation at 18,300 RPM for 45 min using a fixed angle-rotor Sorvall SS-34, the mitochondria were found as a tight light-yellow band near the bottom of the tube. The upper layer above the mitochondrial fraction was gently siphoned and the mitochondrial fraction was transferred to new centrifuge tubes. Approximately 30-40 ml of wash medium was added and centrifuged for 15 min at 15,000 RPM. The supernatant was discarded and the pellet was washed and centrifuged again. The final mitochondrial pellet was resuspended in approximately 1 mL of wash medium and used for O_2 consumption measurements, fumarase activity assays and protein abundance determination. Six to eight biological replicates (50 g of shoots for each mitochondrial isolation) were used for these measurements.

4.3.4 O_2 consumption measurements in isolated mitochondria

Following isolation, an Oxytherm Clark-type O_2 electrode (Hansatech Instruments, Pentney, UK) was used to measure respiratory O_2 consumption rates of: (1) uninhibited mitochondrial respiration, (2) cytochrome pathway capacity, and; (3) alternative pathway capacity for each sample at a common temperature of 25°C. Electrodes were calibrated with deionised water equilibrated to 25°C, and saturated with air. For each measurement, between 50 and 200 µL of mitochondrial suspension were mixed with 1 mL reaction medium (pH 7.2) containing 0.3 M sucrose, 10 mM KCl, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$,0.1% (w/v) BSA, and 10 mM TES. Substrates, effectors and inhibitors were added throughout each run (Table 4.1). Consumption of O_2 by the mitochondria was supported by the addition of external NADH and succinate as substrates (Millar et al.,

2001). Full activation of succinate dehydrogenase (for succinate oxidation) was ensured by adding ATP (Hong et al., 2005). ADP was supplied to ensure adenylate was not restricted (Atkin et al., 2002) in uninhibited mitochondrial respiration and cytochrome pathway respiration. The cytochrome pathway was measured in the presence of the AOX inhibitor, n-propyl gallate. The alternative pathway was measured by the addition of potassium cyanide (KCN), a COX inhibitor (Millar et al., 2001), as well as AOX activators, DTT and pyruvate (Atkin et al., 2002; Sweetman et al., 2019). Four to eight biological replicates were used to measure consumption of O_2, which was recorded using Oxygraph Plus v1.02 software (Hansatech Instruments, Pentney, UK). Protein concentrations were determined by Bradford method using Bovine Serum Albumin (BSA) as a standard (Bradford, 1976).

Table 4.1. Substrates, effectors and inhibitors used in determination of uninhibited mitochondrial respiration, cytochrome pathway capacity and alternative pathway capacity.

Uninhibited mitochondrial respiration	Cytochrome pathway	Alternative pathway
NADH (1 mM)	NADH (1 mM)	NADH (1 mM)
ATP (1 mM)	ATP (1 mM)	ATP (1 mM)
Succinate (0.5 mM)	Succinate (0.5 mM)	Succinate (0.5 mM)
ADP (1 mM)	ADP (1 mM)	KCN (0.1 mM)
	n-propyl gallate (30 μM)	DTT (1.5 mM)
		Pyruvate (5 mM)

4.3.5 Determination of fumarase activity in whole leaf extracts and isolated mitochondria

The activity of fumarase in whole leaf extracts and isolated mitochondria was determined spectrophotometrically as described by Noguchi et al. (1996); Noguchi et al. (2005), respectively. For whole leaf extracts, a sub-sample of frozen leaves (six to eight biological replicates) were crushed to powder in liquid N_2 using a mortar and pestle, and extracted

in an extraction buffer with a ratio of 1:7 leaf to buffer volume. The extraction buffer contained 100 mM HEPES-KOH (pH 7.5), 10 mM KH_2PO_4, 0.5 mM EDTA, 10% (v/v) glycerol and 10 mM DTT. The homogenate was centrifuged for 5 min at 12,000 RPM and 4°C. Supernatant (10 µL) was pipetted into a quartz cuvette (Hellma GmbH & Co. KG, Müllheim, Germany) and mixed with 990 µL of reaction mixture consisting of 70 mM KH_2PO_4-NaOH (pH 7.5), 50 mM malic acid, 4 mM DTT and 0.05% (v/v) Triton X-100. Fumarase activity was determined by measuring the conversion of malate to fumarate over time at 240 nm using a spectrophotometer (Cary 50 Bio UV-Visible; Varian Australia Pty. Ltd., VIC, AU). A molar extinction coefficient of 2.53 mM^{-1} cm^{-1} was used (Tong et al., 1991). The same procedure was used for isolated mitochondria, except 10 µL of mitochondria solution was mixed with 990 µL of reaction mixture with no DTT added.

4.3.6 Determination of protein abundance in isolated mitochondria

The abundance of cytochrome *c* oxidase (COX) and alternative oxidase (AOX) were determined using electrophoresis and Western blotting. Total protein concentrations of the isolated mitochondria extracted from leaves of plants grown at 15/10°C and 30/25°C were determined by Bradford method using Bovine Serum Albumin (BSA) as standard (Bradford, 1976). The total protein concentrations of each sample were standardized and solubilized in a NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) with 10% (v/v) DTT, then heated for 10 min at 95°C, and centrifuged for 30 sec at 12,000 RPM. Thereafter, supernatant was collected and 7 µL of each sample were loaded per lane and separated on 4-12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) using the MOPS-based buffer system. To blot, proteins from the gel were transferred to Immobilon-P Polyvinylidene Fluoride (PVDF) membranes (Merck Millipore, Kilsyth, VIC, AU) using an XCell II Blot module (Invitrogen, Carlsbad, CA, USA). Membranes were then

blocked for 2 h with 5% (w/v) skim-milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST). To probe, membranes were incubated for 2 h in primary antibody solution (5% w/v skim milk powder in TBST) containing commercially available antibodies (Agrisera, Vännäs, Västerbotten, Sweeden) at a dilution of 1:5000. Following washing with TBST, the membranes were incubated for 1 h in goat anti-rabbit antibody solution (5% w/v skim milk powder in TBST) at a dilution of 1:8000. Proteins were then visualized using the AttoPhos AP fluorescent substrate system (Promega, Madison, WI, USA), imaged using a Versa-Doc (Bio-Rad, Hercules, CA, USA) imaging system and quantified using Image Lab software (Bio-Rad, Hercules, CA, USA).

4.3.7 Quantitative PCR analysis in whole leaf extract

A sub-sample of frozen chickpea leaves (six to eight biological replicates) was sent to Flinders University (Professor David Day) for the determination of genetic response of selected respiratory genes. For the purposes of the current experiment, new primers were designed to quantify transcript levels of genes encoding a putative NADH: ubiquinone oxidoreductase (Complex I) subunit, six cytochrome *c* oxidase (Complex IV) subunits and five ATP synthase subunits (Supplementary Table S4.1). Previously published qRT-PCR assays were used to analyse alternative oxidase (AOX) gene expression (Sweetman et al. 2019). Primers targeting putative uncoupling proteins (UCP) were also previously developed (Booth, 2018).

RNA was extracted from ~75 mg of frozen tissue powder in a TRIzol-like extraction buffer (Shavrukov et al., 2013). The clarified extract was purified using chloroform phase-separation and overnight precipitation in 35% (v/v) isopropanol, then the pellet was washed with ethanol and resuspended in RNase-free water (Sweetman et al., 2019). RNA quality was checked in a 1% agarose gel and quantified using a Nanodrop ND1000 (Thermo Scientific, Australia). Genomic DNA contamination was removed

using DNase I (NEB, VIC, Australia) and approximately 1 µg of RNA was used for cDNA synthesis by Protoscript II reverse transcriptase (NEB, VIC, AU).

Transcripts were analysed using KAPA SYBR-Fast qPCR Universal ReadyMix (Roche, NSW, Australia) as previously reported (Sweetman et al., 2019). Standard curves were generated using a dilution series of purified PCR products at known concentrations (1, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} fmol/µl) for each gene. Transcript levels for each gene of interest were calculated relative to the geometric mean of three reference genes, which were analysed using previously-optimised primers targeting Elongation Factor 4 (ElF4; AJ004960), Heat Shock Protein 90 (Hsp90; GR406804) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AJ010224) (Garg et al., 2010).

4.3.8 Statistical analyses

Data were analysed using one- or two-way analyses of variance (ANOVA) in SigmaPlot v11.0 software (Systat Software Inc, London. UK). Following ANOVA, multiple comparison tests were performed using SigmaPlot v11.0 software. six to eight biological replicates were used for measurements of fumarase activity, uninhibited isolated mitochondrial respiration, capacities of cytochrome and alternative pathways as well as abundance of transcript.

4.4 Results

4.4.1 Effect of growth temperature and the diurnal cycle on leaf dark respiration

Table 4.2 shows that thermal acclimation of leaf respiration in the dark (R_{dark}) does occur in chickpea, as growth temperature significantly influenced R_{dark} when measured at a common temperature of 25°C (P<0.001). However, this thermal acclimation phenotype was evident only for the coldest 15/10°C grown chickpeas, which had noticeably faster R_{dark} at 25°C than all other growth temperatures (Figure 4.1). Plants grown at the warmer

20/15°C, 25/20°C and 30/25°C did not differ from one another in R_{dark} at 25°C. Irrespective of temperature, R_{dark} did not significantly change over the diurnal cycle (P>0.05; Table 4.2) and thus no interaction was found between the growth treatments and time of sampling. Although 15/10°C grown plants had significantly greater leaf mass per area (LMA) compared to warmer grown plants (Table 4.2), analysing on a fresh or dry mass basis (Figure 4.1B,C) rather than by leaf area did not change the significant differences in R_{dark} between growth treatments, indicating that thicker leaves for the cold grown plants while contributing to, did not fully explain the faster R_{dark}. Furthermore, as presented in greater detail below, fumarase activity assays from leaves and isolated mitochondria allowed for the determination of the mitochondrial content in leaf tissue, and no significant difference was found (Table 4.3).

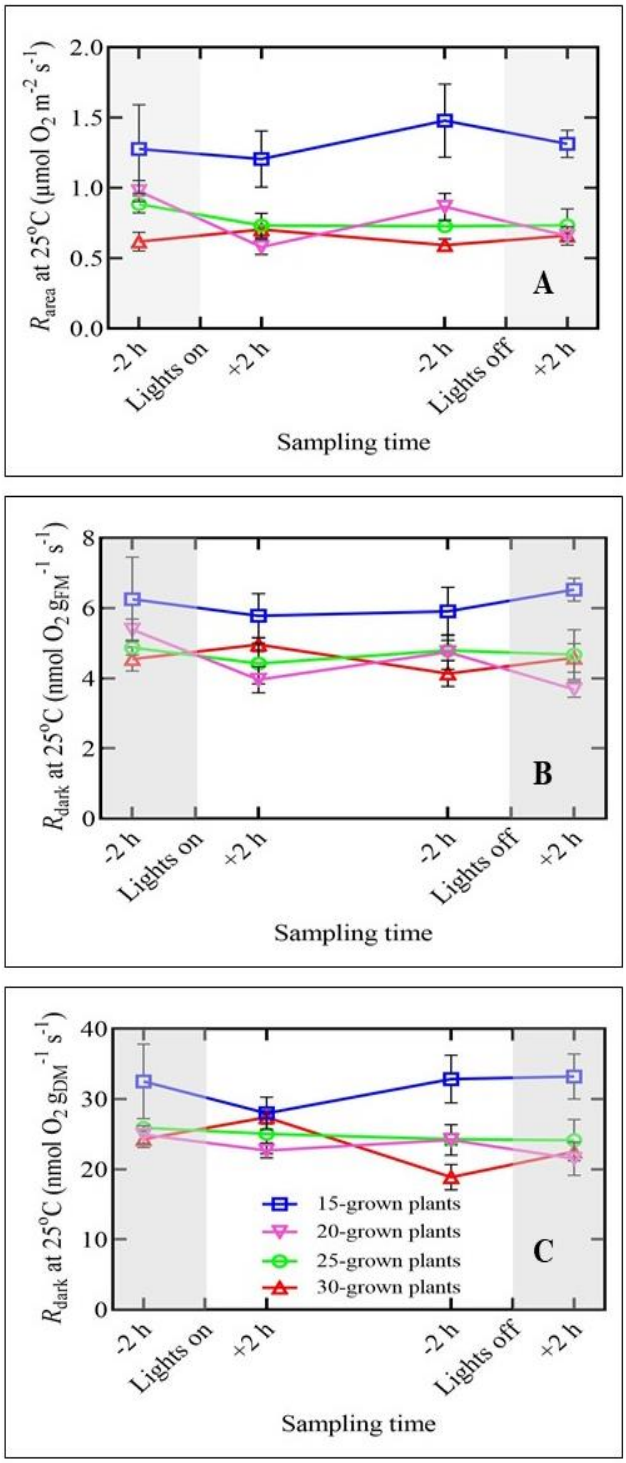

Figure 4.1. Dark respiration (R_{dark}) of 15/10°C, 20/15°C, 25/20°C and 30/25°C grown plants measured at a common temperature of 25°C at 2 h before lights on/off (-2 h) and 2 h after lights on/off (+2 h). Measurements are presented on a (A) leaf area, (B) fresh mass (FM); and (C) dry mass (DM) bases. Grey area represents the dark period. Points are means of three to four biological replicates ± SE.

Table 4.2. Two-way ANOVA of temperature and time effects on R_{dark} of 15/10°C, 20/15°C, 25/20°C and 30/25°C grown plants measured at a common temperature of 25°C. Measurements are presented on a leaf area, fresh mass (FM) and dry mass (DM) bases. Asterisks (*) denote statistically significant difference (P<0.05) among the levels of temperature treatments and time points.

R_{dark} measure	Temperature		Time		Temperature x Time	
	F	*P*	*F*	*P*	*F*	*P*
R_{area} (μmol O_2 m^{-2} s^{-1})	26.636	<0.001*	1.604	0.201	0.633	0.763
R_{FM} (nmol O_2 g^{-1} s^{-1})	12.505	<0.001*	1.369	0.263	1.213	0.310
R_{DM} (nmol O_2 g^{-1} s^{-1})	12.598	<0.001*	1.471	0.234	1.218	0.306

Table 4.3. Leaf mass per unit area (LMA) of 15/10°C, 20/15°C, 25/20 and 30/25°C grown chickpea leaves. Values are means of three to four biological replicates ±SE. One-way ANOVA of growth temperature effect is reported with asterisks (*) indicating significance at P<0.05. Superscript letters show significant differences between growth temperature treatments according to a Tukey test.

Growth temperature	LMA (g m^{-2})
15/10°C grown	42 ± 0.0004[a]
20/15°C grown	25 ± 0.0001[b]
25/20°C grown	29 ± 0.0001[b]
30/25°C grown	26 ± 0.0001[b]
F	15.754
P	<0.001*

4.4.2 Temperature sensitivity of respiration

To determine the short-term sensitivity of R_{dark} in chickpea leaves to temperature, I calculated Q_{10} values by adjusting the short-term measurement temperatures during O_2-consumption runs (Figure 4.2). Contrary to expectations, R_{dark} when grown at the colder temperatures of 15/10°C and 20/15°C were considerably less temperature sensitive with the Q_{10} values for both were 1.7 ± 0.1. The warmer grown 25/20°C and 30/25°C plants had greater Q_{10} values of 2.1 ± 0.1 and 2.2 ± 0.1, respectively.

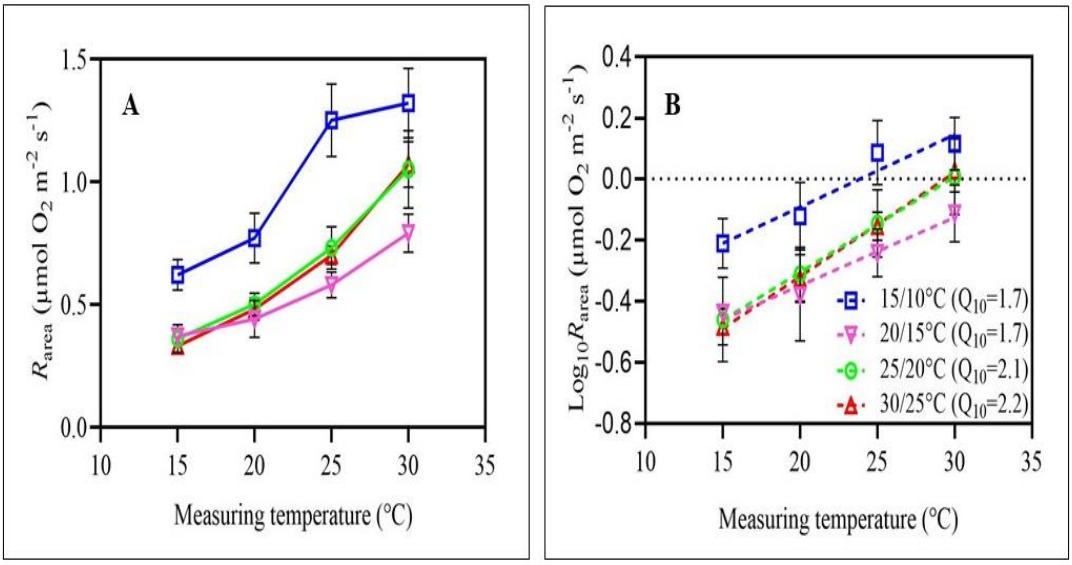

Figure 4.2. Dark respiration (R_{dark}) and log_{10} transformed R_{dark} on a leaf area basis of 15/10°C, 20/15°C, 25/20°C and 30/25°C grown plants. Dashed lines are regression lines of R_{dark} versus measuring temperature and points are means of three to four biological replicates.

4.4.3 Tissue mitochondria content

Fumarase, one of the enzymes in TCA cycle was used as a marker for mitochondrial protein in leaves (Nast & Müller-Röber, 1996; Noguchi et al., 2005; Noguchi & Terashima, 2006). No statistically significant difference was found in fumarase activities in either leaf extracts or isolated mitochondria of 15/10°C and 30/25°C grown plants (Table 4.4). As a result, when dividing activity in leaf extracts over that in isolated mitochondria to provide the estimate of mitochondrial protein content (Noguchi et al., 2005), no difference was observed between the two growth treatments. This denotes that growth temperature doesn't affect mitochondrial protein content in chickpea leaves.

Table 4.4. Fumarase activities in 15/10°C and 30/25°C grown plants. Activity was measured in (A) leaf extract, and; (B) isolated mitochondria. (C) Tissue mitochondria content was calculated using both (A) and (B). Values are means of six biological replicates ±SE and statistical data (*F*-value and *P*-value) is based on one-way ANOVA of temperature treatment effect.

Fumarase activities	15/10°C	30/25°C	*F*	*P*

Leaf extract (μmol g_{DM}^{-1} min^{-1})	1.1±0.1	0.8±0.1	3.01	0.11
Isolated mitochondria (nmol $mg_{protein}^{-1}$ min^{-1})	48.1±11.7	40.2±7.8	0.32	0.58
Tissue mitochondria content ($mg_{protein}$ g_{DM}^{-1})	30.3±7.6	28.80±11.6	0.01	0.92

4.4.4 Effect of growth temperature on respiration of isolated mitochondria

When isolated mitochondria were uninhibited (with the presence of NADH, ATP, succinate and ADP), a faster rate of R_{dark} were observed for 15/10°C grown plants compared to 30/25°C grown plants when measured at 25°C (P=0.013; Figure 4.3A). This faster rate from mitochondria of plants grown at colder temperatures seems associated with a significantly greater COX capacity from the mitochondria of cold-grown plants (P=0.028; Figure 4.3B). Interestingly, unlike the dramatic divergence in COX capacity, alternative oxidase (AOX) capacity was not altered by growth temperature (P=0.954; Figure 4.3B). The acclimation of R_{dark} observed in leaves (Figure 4.1) may therefore be related to changes in capacity of the cytochrome pathway (Figure 4.3B).

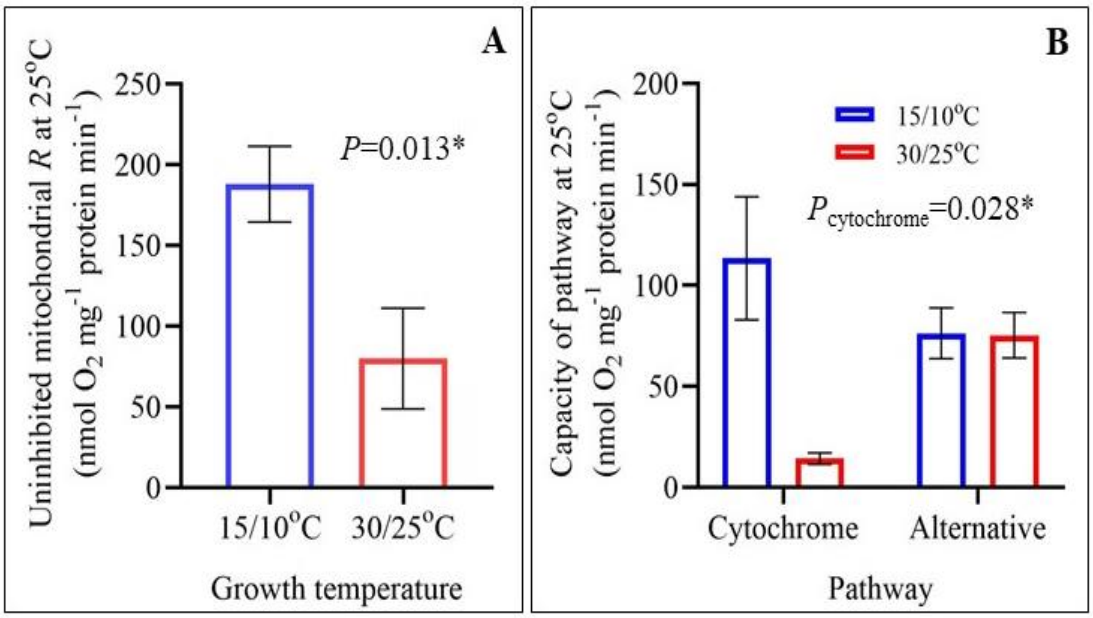

Figure 4.3. Effect of growth temperature on (A) uninhibited mitochondrial respiration, and (B) cytochrome and alternative pathways of 15/10°C and 30/25°C grown plants measured at a common temperature of 25°C. Values are means of four to eight biological replicates ±SE. Statistical data (P-value) is based on a one-way ANOVA of

growth temperature effect and reported in each graph with asterisks (*) indicating significance at $P<0.05$.

4.4.5 Molecular responses of leaves to growth temperature

Western blot analysis showed a statistically significant decrease in COX abundance when grown at 30/25°C compared to 15/10°C (Figure 4.4, Supplementary Figure S4.1). Contrastingly, no difference was observed between temperature treatments for the abundance of AOX. Quantitative PCR on specific transcripts of interest (Figure 4.5, Supplementary Table S4.2) showed no statistically significant difference in any of the COX-encoding transcripts between 15/10°C and 30/25°C grown plants. Contrastingly, a transcript encoding Complex I exhibited a decline (P=0.016) in abundance in leaves grown at 30/25°C. Similarly, two out of four AOX-encoding transcripts (*aox2a* and *aox2b*; with P-values of 0.028 and 0.009, respectively), and four out of five ATP synthase-encoding transcripts (*orf25*, *orfb*, *mgp1*, and *atp3*; with P-values of 0.002, 0.005, 0.026, and 0.026, respectively) showed a decrease in abundance when grown at 30/25°C.

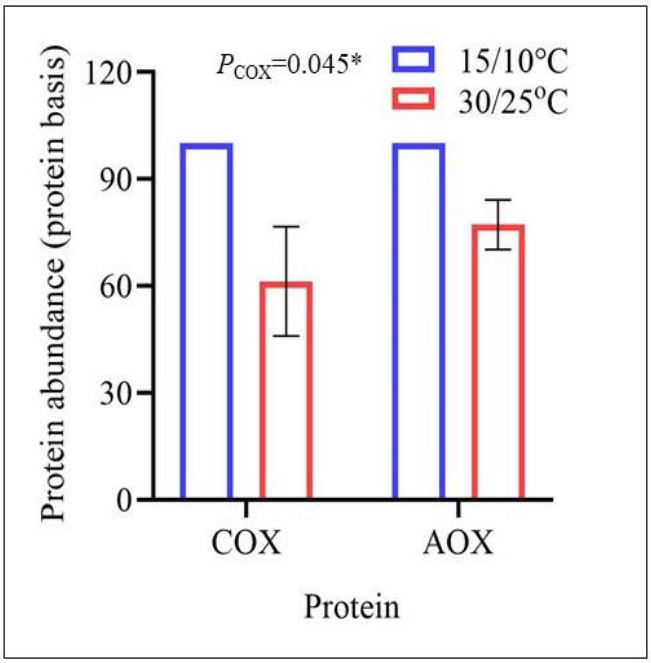

Figure 4.4. Abundance of mitochondrial electron transport chain proteins determined by Western blot analysis for mitochondria isolated from chickpea leaves grown at 15/10°C and 30/25°C. Data were normalised by adjusting the value from 15/10°C grown plants to 100. Data represent mean ±SE of four to five independent western blots, with each blot representing mitochondria isolated from a separate plant. Statistical data based on one-way ANOVA of growth temperature effect is reported (*) with asterisks indicating significance at $P<0.05$. Representative blots are presented in Supplementary Figure S4.1.

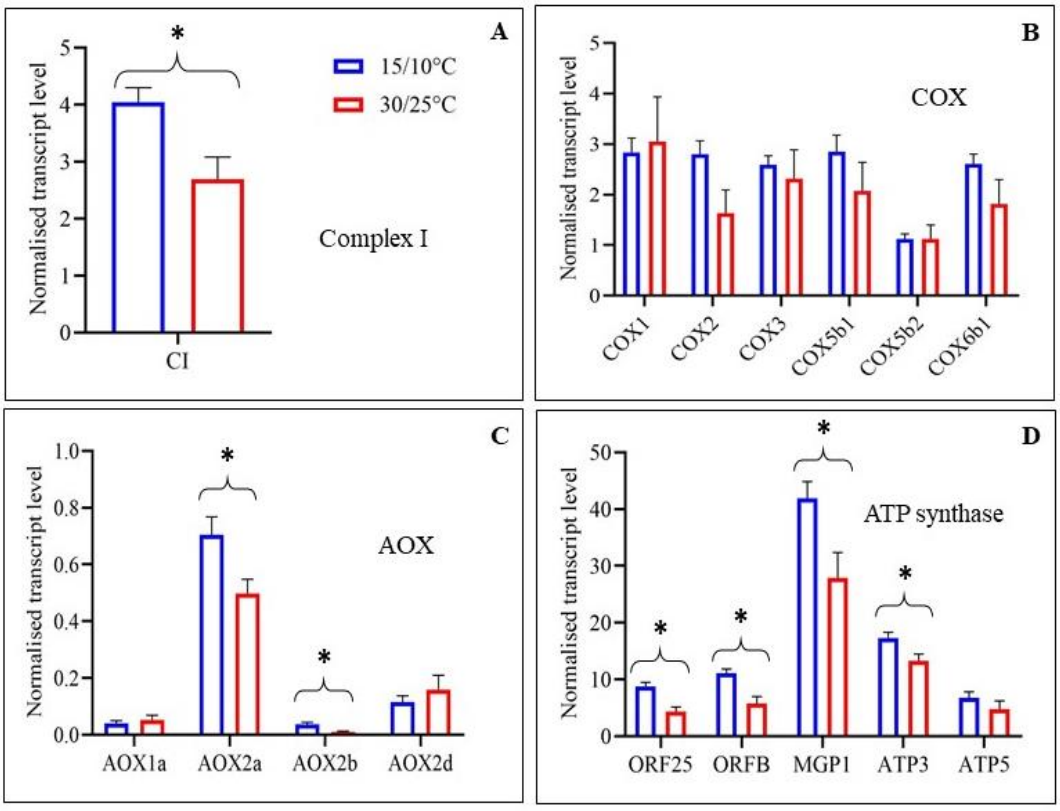

Figure 4.5. Quantitative PCR analysis of mitochondrial transcript abundance in 15/10°C and 30/25°C grown plants. Genes were analysed encoding isoforms of (A) Complex I, (B) cytochrome *c* oxidase (COX), (C) alternative oxidase, and (D) ATP synthesis. Values are means of six biological replicates ±SE and statistical data based on one-way ANOVA of growth temperature effect is reported (*) with asterisks indicating significance at $P<0.05$.

4.5 Discussion

4.5.1 Acclimation of leaf dark respiration to temperature changes

At the measurement temperature of 25°C, the rates of R_{dark} in chickpea leaves were evidently faster in 15/10°C when compared with their warmer grown counterparts (Figure 4.1). Thus, chickpea demonstrates a strong ability to thermally acclimate to the low

growth temperature treatment, an observation that may relate to the fact that that chickpea is a cool season crop (Croser et al., 2003; Kulkarni & Chimmad, 2014). Low temperature sensitivity (i.e. low Q_{10}) exhibited by chickpea plants grown at cold environment (Figure 4.2) may be another factor contributing to the ability of chickpea to grow in cold conditions, through low Q_{10} values enabling rates of O_2 uptake to be maintained in leaves first exposed to cold temperature. Interestingly, previous studies have reported that to achieve high yield of chickpea, higher temperature between 25-30°C is needed (Kulkarni & Chimmad, 2014; Patra et al., 2011), suggesting that while chickpea respiration acclimates well to lower temperatures, warmer environments are needed to maximise growth and yield. This sensitivity of yield to growth temperature – being optimal at 25-30°C – highlights the need to determine the factors controlling growth and ultimately yield of this protein-rich crop as daily mean temperatures increase in response to global warming (IPCC, 2013).

4.5.2 Lower capacity of cytochrome pathway in warmer grown plants

It seems that biochemical changes in the characteristics of mitochondria, rather than changes in their absolute abundance, are what leads to the observed temperature-dependent difference in leaf R_{dark}. Associated with slower temperature-normalized rates of leaf R_{dark} exhibited by chickpea grown under 30/25°C (compared to 15°C grown plants) was the reduction in the rates of R_{dark} per unit mitochondrial protein when isolated mitochondria were uninhibited. It seems likely that the reduction was caused by the significantly lower capacity of the cytochrome pathway due to the drop in COX protein abundance. This result is certainly consistent with the findings in rice when the COX protein abundance was lower in 40/35°C grown than 25/20°C grown plants (Chapter 2). Although only *cox2* and *cox3* transcripts showed the potential toward significant decreases (Supplementary Table S4.2), substantial reduction in transcripts for most

ATPase genes tested might suggests a drop in ATP production. On the other hand, less transcripts for most ATPase genes does not necessarily mean a decrease in ATP production, instead, an appropriate level of ATP is actually produced to maximise the growth and eventually yield of chickpea, which usually grows in a temperature range of 25-30°C (Kulkarni & Chimmad, 2014; Patra et al., 2011). However, suitable measurements are still needed to quantify the production and concentration of ATP. Other than Arabidopsis leaves (Armstrong et al., 2008) and wheat roots (Kurimoto et al., 2004b) as well as rice leaves in this research, not many studies have reported the alterations of COX in plants in response to growth temperature.

Alternatively, AOX has been noted to be commonly involved in plant responses to stresses such as low and high temperature (Searle et al., 2011; Wagner et al., 2008). However, in this study, no significant difference was observed in the capacity of alternative pathway via AOX between plants grown under cold and hot, although the expressions of *aox2a* and *aox2b* were enhanced under cold treatment (Figure 4.5). This finding was consistent with a previous study by Sweetman et al. (2019) where mitochondria isolated from chickpea seedlings exhibited AOX capacity constitutively even when grown under normal conditions (i.e. 25-27°C day/12-14°C night). Similar result was also found in the cotyledon, leaf and root of soybean (*Glycine max* L.) and shoot and root of siratro (*Macroptilium atropurpureum*) (Kearns et al., 1992). Here, the same capacity of AOX with reduced capacity of cytochrome in hot conditions suggests the involvement of AOX in stabilizing the respiratory redox poise by directly reducing oxygen to water (Saha et al., 2016; Vanlerberghe, 2013). This supports the general role of AOX in controlling the operation of TCA cycle by liberating the energy as heat (Pu et al., 2015; Schertl & Braun, 2014).

4.6 Conclusion

Overall, results in this chapter indicate that akin to rice, R_{dark} in chickpea can also acclimate to temperature changes, particularly to 15/10°C where the R_{dark} rates were higher compared to warmer grown counterpart. Associated with a decrease in the rates of R_{dark} per unit mitochondrial protein at high temperature were the decline in LMA, along with the reductions in the capacity and abundance of COX. However, this change did not align with changes in transcript abundances. Though, further work is needed to understand why reduced cytochrome capacity would occur, adjustment in the capacity of cytochrome pathway could probably the causative of the changes in R_{dark}. In general, this chapter again demonstrates that COX and AOX could be the target in regulating the mechanism of leaf R_{dark} in order to achieve a better crop yield in fluctuating global temperature.

Chapter 5 – General discussion

5.1 Principal findings

5.1.1 Acclimation of respiration and photosynthesis in rice is asynchronous in response to contrasting growth temperatures

Rice is a staple food for nearly two-third of the world population (Khush, 2005), and thus more research is needed to better understand the biological characteristics of this important crop, including how rice responds to sustained changes in the temperature. Physiological acclimation to long-term changes in temperature have important consequences for a plants carbon balance (Atkin et al., 2015; Gifford, 2003; Heskel et al., 2016). A critical area of research is therefore to understand how, and to what extent, the acclimation process alters carbon budgets in rice.

The experiments reported in this thesis aimed to investigate the extent of thermal acclimation of respiration and photosynthesis in rice. In Chapter 2, the measurements of respiration and photosynthesis were carried out on leaves at different developmental stages. The responses of pre-existing leaves (PE) already developed at a control environment of 30/25°C, and leaves newly developed (ND) following temperature shifts to 40/35°C and 25/20°C were characterised. I hypothesised that ND leaves would acclimate to a greater extent than PE leaves, with subsequent acclimation in ND leaves associate with recovery of photosynthesis and reduced rates of respiration, resulting in a lowering of the respiration/photosynthesis ratio. The hypothesis supported by the data suggesting that acclimation occurred to a greater extent for ND leaves compared to PE leaves. Instantaneous temperature response curves (Chapter 2; Figure 2.7) showed that although respiratory homeostasis (i.e. identical respiration rates even when measured at contrasting growth temperatures) was not achieved, respiration rates were consistently faster in 25/20°C and slower in 40/35°C acclimated leaves at any given temperature when

compared to leaves grown at warm control temperatures. This demonstrates respiratory acclimation had occurred, albeit limited in extent considering there were differences in rates of leaf respiration at the prevailing growth temperature (R_{growth}) and respiratory homeostasis was not achieved (Chapter 2; Table 2.3). A stronger acclimation phenotype was observed for light-saturated net photosynthesis, with the optimum rates (A_{opt}) and rates measured at the prevailing day-time temperatures (A_{growth}) being stable across growth temperature treatments in ND leaves, even at 40/35°C (Chapter 2; Table 2.3). Taken together, these results pointed to acclimation of respiration and photosynthesis being asynchronous in ND rice leaves due to stronger photosynthetic than respiratory acclimation (i.e. homeostasis of photosynthesis but not respiration). A number of previous studies on acclimation of respiration and photosynthesis show asynchronous responses to temperatures. However, in most cases, respiration normally acclimated to a greater extent than photosynthesis, rather than the reverse (Way & Oren, 2010). Examples of stronger respiratory than photosynthetic acclimation include studies using spinach (*Spinacia oleracea*) (Yamori et al., 2005), black spruce (*Picea mariana*) (Way & Sage, 2008), white spruce (*Picea glauca*) (Benomar et al., 2017), and loblolly pine (*Pinus taeda*) (Wertin et al., 2012). The finding of asynchronous acclimation favouring photosynthesis rather than respiration in rice is indeed interesting, and might influence how this major crop responds to climate change. Further, the finding that photosynthesis acclimates to a higher degree than respiration has implications for how rice carbon fluxes of modelled in future warmer climates, with current models usually assuming that the relationship between respiration and photosynthesis at any given measuring temperature remains constant (Gifford, 2003). This might be true at moderate temperatures; however, at higher temperatures the ratio could be greater, as I have observed in rice.

The rise in respiration rates with increasing temperatures, and homeostasis of photosynthesis led to an increase in the ratio of respiration to photosynthesis with rising measurement temperatures. However, due to the small but significant suppression of respiration in 40/35°C acclimated plants, the respiration to photosynthesis ratio was not stimulated as much by heat in hot grown rice when compared to the colder grown rice (Chapter 2; Figure 2.8). Thus, although respiratory acclimation was not strong enough to maintain homeostasis, it did lead to reduced carbon loss as a percentage of assimilate gained in rice leaves acclimated and exposed to hot temperatures. These results provide key attributes of acclimation in rice leaves, providing targets for what attributes of rice acclimation can be improved in future breeding and genetic engineering projects. For example, I would suggest that minimising respiratory loss (which has less intrinsic acclimation) rather than promoting photosynthetic gain (which already has strong intrinsic acclimation) should be the main focus when attempting to improve the carbon balance and subsequent vegetative growth of rice in warmer growth environments. Indeed, the stimulation of respiration at higher temperatures likely explains the decline in rice yield associated with rising minimum night-time temperature in the tropics (Nagarajan et al., 2010) and the subtropics (Peng et al., 2004). Low yield with increased respiration is also reported in wheat (Johkan et al., 2011; You et al., 2009). Essentially, faster rates of respiration are linked to a high ATP production, which provides more energy for metabolism and growth. However, excessive and inefficient ATP production/use comes at the expense of carbon lost as respiratory substrate that otherwise could have been used for biomass accumulation (Keller, 2010), and hence a potential reduction in crop yield. There are studies that have identified ways to cut respiratory carbon loss. Amthor et al. (2019) reviewed some specific proteins, pathways and processes as candidates that can be engineered to reduce respiratory costs in an effort to

enhance crop productivity. Further understanding of the mechanisms that control plant respiration at high temperature, such as the molecular and biochemical results obtained in this thesis, is an important step in developing strategies to reduce unnecessary respiratory loss. As discussed below, capacity changes in the alternative and cytochrome pathways of respiration seem to play an important function in acclimation of rice to growth temperature, and manipulation of these pathways may be one key target for improving respiratory efficiency.

The lower respiratory acclimation ability in rice compared to many other species is interesting. It may be possibly related to the fact that tropical species are more vulnerable to warming than temperate species (Way & Oren, 2010). Rice being a tropical grass would have evolved in an environment where the climate is generally stable. A more stable environment is thought to reduce the acclimation potential of some species (Cunningham & Read, 2003). Why a stable climate would limit respiration but not photosynthesis acclimation potential is an interesting question that requires more study. As a starting point, further experiments are needed to assess whether tropical species in general have greater ability in acclimate photosynthesis rather than respiration, in contrast to temperate species. This is especially important to consider as this might effect global temperature-dependent changes of respiration to photosynthesis ratios, which could lead to an overestimation or underestimation of the future global carbon balance (Gifford, 2003).

5.1.2 Diurnal variations in respiration acclimation phenotypes

It is crucial to explore the extent of respiration acclimation throughout the day/night cycle, given that minimum night-time temperatures are rising more rapidly than maximum day-time temperatures in many rice-growing regions (Laborte et al., 2012; Nagarajan et al., 2010; Peng et al., 2004). In Chapter 3, I aimed to understand how the diurnal pattern in

respiration changes with growth temperature. I investigated if the acclimation pattern of Chapter 2 holds at other times of the day. The experiments of Chapter 3 involved five measurement-times of respiration in a single 24 h period on rice plants grown again at 25/20°C, 30/25°C and 40/35°C. Considering there are fluctuations in temperature even within a single day, I hypothesised that there are also diurnal variations in thermal acclimation of respiration in rice. Indeed, when measured at a common temperature of 30°C, the greatest divergence in respiration between the growth treatments was observed at the end of the day/light period. Differences were not as pronounced between treatments at night. This result showed that the thermal acclimation of respiration varied diurnally, although the interaction between time of day and growth temperature was not significant.

Many studies measure respiration at only one set time point during the day, usually between 09:00 am and 4:00 pm, regardless of the growth temperature. However, the present findings suggest that the time of day when the measurements are taken should be considered, as time of day does to some extent influence the thermal acclimation of respiration. It is understandable that measuring respiration at multiple time points throughout a day is not practical, especially when conducting large-scale studies in the field. However, it is still very important to consider the variations in respiration that occurs diurnally and factor this into my interpretation of respiratory thermal acclimation.

5.1.3 Genetic and biochemical responses underpin acclimation

While there are many possible mechanisms that underlie thermal acclimation, I hypothesised that acclimation of respiration is associated with changes in transcript and protein abundance in key pathways connected with energy capture and use. Indeed, the most striking finding in this study was the significant decline in COX protein abundance at 40/35°C, both in 24 h hot PE and ND leaves of rice. When normalised to porin, a similar trend was observed.

Given the focus in earlier chapters on rice, ideally, I would have isolated mitochondria from rice. However, it was not possible to isolate rice mitochondria due to the toughness of rice leaves and the mechanical damage sustained to mitochondria during the extraction process. Considering this limitation in methodology, I isolated leaf mitochondria from another important crop, chickpea (*Cicer arietinum*) to gain a better understanding of changes in mitochondrial characteristics at the organelle level (Chapter 4). With the isolation of chickpea mitochondria, direct measurements of the cytochrome and alternative oxidase pathways of respiration were obtained in order to examine which pathway shows changes associate with respiratory acclimation to temperature. Consistent with the changes in COX protein abundance in rice, the capacity of the cytochrome pathway along with the abundance of COX protein was lower in 30/25°C compared to 15/10°C in chickpea mitochondria. This decline in COX capacity and abundance were not due to a concurrent decline in mitochondrial abundance, which remained consistent based on the activity of fumarase, a marker enzyme of mitochondria abundance. Thus, in chickpea, acclimation to high temperature conditions is associated not only with a decline in rates of leaf *R* at a given temperature, but also with a decreased investment in the capacity and abundance of COX protein, and decline in the rates of respiration per unit mitochondrial protein – a result that is similar to that in rice. It could be argued that reduced mitochondrial capacity through reduced COX abundance is a mechanism to counteract faster enzyme velocities at hotter growth temperatures, or that reduced COX capacity indicates a reduction in ATP demand (Noguchi & Terashima, 2006). However, it is unlikely COX capacity is the limiting factor on overall respiratory flux, as this would imply movement towards a fully reduced ubiquinone pool. A highly reduced ubiquinone pool is unlikely to occur as it would lead to excessive production of reactive oxygen species and lead to reduced plant fitness. Further, maintenance of AOX and UCP capacity

whilst COX declined indicates a greater proportion of electrons may have passed through the alternative pathways at warmer growth temperatures. This proportional increase in the alternative relative to cytochrome pathways may be a mechanism to dissipate excessive respiratory redox poise and proton motive force under hot growing conditions.

Evident from the transcript profiling presented in Chapter 2 that the hot treatment promoted a greater gene expression response than the cold treatment. However, the influence of temperature on particular genes did not necessarily correspond to changes in protein abundance (Das & Strasser, 2013; Jorrín-Novo et al., 2018). For example, *cox* gene expression did not align with COX protein abundance. The ambiguity between transcript and protein abundance findings is not entirely unexpected and is likely related to a few major factors. Firstly, translational and post-translational modifications can account for a divergence between the two (Jorrín-Novo et al., 2018; Stermer, 1995). Secondly, isoforms of the same protein may be expressed differentially in response to temperature. This seemed to occur in relation to *aox* gene expression, where a more stress responsive *aox1a* isoform was expressed with heat, while the *aox1c* isoform declined. Thus, the general pattern of *aox* expression would be different, depending on which isoform was assessed. Thirdly, the mechanisms by which DNA is transcribed and translated is responsive to temperature in-of-themselves. For example, higher temperatures do alter transcription rates and mRNA decay rates in Arabidopsis (Sidaway-Lee et al., 2014). Whether such changes contribute significantly to variation between transcript and protein abundance is an interesting proposition that requires further study. A final point on the mismatch between protein abundance and transcript level relates to the reference genome used in RNA-seq analysis. I used the rice cultivar Nipponbare as the reference genome for expression analysis. Although Nipponbare and IR64 are closely related subspecies of rice, the subtle differences that do exist between their genomes can

lead to significant differences in transcript abundance calculations during RNA-seq analysis, related to single nucleotide polymorphisms and annotation difference (Slabaugh et al., 2019).

It is unlikely that substrate limitations account for the suppression of respiration at higher measuring temperatures exhibited by hot acclimated rice. No correlation was observed between respiration rates and concentration of soluble sugars at any temperature regimes. In fact, soluble sugar concentration was remarkably consistent, irrespective of growth temperature or time of day. The fact that Q_{10} values were largely unaffected by growth temperature, and that sugar concentrations were homeostatic, points to substrates being unlikely to be limiting. Alternative limiting factors are posited below.

5.1.4 Time of day and temperature drive variations in the levels of respiratory metabolites

The synthesis of ATP via oxidative phosphorylation requires a transfer of electrons from reducing equivalents generated by the TCA cycle, in the form of NADH and FADH (Millar et al., 2011). Prior to the TCA cycle, glycolysis takes place consuming photosynthates as substrate (Plaxton & Podestá, 2006). Interrelation between respiration and photosynthesis, especially in the light period, lead to variations in metabolic pathways (Hurry et al., 2005; Urbanczyk-Wochniak et al., 2005). I hypothesised that variations in respiratory metabolomic profile are influenced by day/night cycle and growth temperature. As known, carbon assimilated in photosynthesis during the day is stored as starch in leaves, which is then broken down at night to provide sugars in order to sustain metabolic processes (Smith & Stitt, 2007). Thus, unsurprisingly, starch was accumulated during the day and degraded at night, following the expected trend to a similar extent regardless of the growth temperature. Contrastingly, the concentration of soluble sugars

across the day/night cycle was largely unchanged at all temperature regimes. This result contrasts with that seen in other species. For example, in *Arabidopsis thaliana*, levels of soluble sugars in leaves were lower at night when compared to the levels in dark-adapted leaves measured during the day (Florez-Sarasa et al., 2012). Similar results have been observed in leaves of sugarcane (Du et al., 2000) and barley (Müller et al., 2018).

From the metabolite profiling analysis, approximately 60 metabolites with various responses were detected: some responded to growth temperature and some responded to the diurnal cycle. Only two metabolites (shikimate pathway derived aromatic amino acids tyrosine and tryptophan) showed interaction between growth temperature and diurnal cycle, suggesting that diurnal responses of metabolites were mostly not related to responses of metabolites to growth temperature. Overall, the most noticeable trend, evident from the principle component analysis (Figure 3.3), was a negative correlation between majority of the amino acids and carbohydrates as well as organic acid intermediates of the TCA cycle. Negative associations among substrates and primary metabolites of the TCA cycle might be indicative of shifts in TCA dependent pathways (e.g. changes in flux through the shikimate and GABA shunts). I postulate that this may be a mechanism to coordinate adenylate demand and the redox potential of the cell. In other words, cycling of intermediates to generate reducing equivalents in the form of NADH and FADH when adenylate is needed may coordinate the generation of primary carbon compounds in the form of amino acids when reducing equivalents and adenylates are in lower demand. Although sucrose levels were stable throughout the 24 h cycle, some key intermediates in carbohydrate synthesis and degradation at the top of glycolysis, namely glucose-6-phosphate and fructose-6-phosphate, were in significantly higher abundance during the day and lower at night. Additionally, increasing growth temperature markedly reduced glucose-6-phosphate and fructose-6-phosphate. This probably implies

higher activity in the cold and during the day of the enzymes hexokinase and phosphoglucose isomerase, involved in the formation of glucose-6-phosphate and fructose-6-phosphate, respectively (Berg et al., 2002a). A decline of glucose-6-phosphate at night is also seen in *Arabidopsis* (Bläsing et al., 2005), attributed to the export of carbohydrates from the leaf (Gibon et al., 2004); this might provide a potential explanation for what I observed in rice.

Previous studies showed that total leaf amino acids typically increased during the day and decreased at night (Matt et al., 2001; Sulpice et al., 2014; Watanabe et al., 2014). Urbanczyk-Wochniak et al. (2005) also found differences in amino acid levels between light and dark periods in potato leaves, in which most of the amino acids were increased in the presence of light, and decreased in the dark. In this study, amino acid alanine was significantly accumulated and depleted during the day and at night, respectively. Tryptophan, phenylalanine, tyrosine an aspartic acid responded oppositely, independent of growth temperature treatment. The increase in alanine level during the day might be a result of alanine aminotransferase upregulation (Miyashita et al., 2007; Watanabe et al., 2014).

Most of the TCA cycle organic acid intermediates were not altered with growth temperature. However, the diurnal cycle changed metabolites significantly. For example, citrate significantly accumulated at night and was depleted during the day. By contrast, there was low abundance of α-ketoglutarate, succinate, fumarate and malate at night. Enzymes are key regulators that determine the speed of each reaction in the TCA cycle. For example, light inhibition of pyruvate dehydrogenase complex (PDC), the enzyme that catalyses the formation acetyl-CoA from pyruvate (DeBrosse & Kerr, 2016; Tovar-Méndez et al., 2003) results in partial operation of TCA cycle during the day. This slows the flux through succinyl co-A and succinate dehydrogenase (Tcherkez et al., 2009),

likely causing the high levels of succinate during the day. Additionally, phosphorylation by PEPC (O'Leary et al., 2011; Tcherkez et al., 2009; Werner et al., 2011) and downregulation of fumarase in the presence of light (Eprintsev et al., 2016) also increase the levels of fumarate and malate during the day. Accumulation of fumarate and malate during the light period has been previously reported in many species (Brauner et al., 2018; Pracharoenwattana et al., 2010; Zell et al., 2010). Both organic acids can be used as respiratory substrates at night (Watanabe et al., 2014). I postulate that the significantly greater abundance of organic acids such as malate during the day, followed by depletion during the night, might be a mechanism by which rice can utilise substrates for respiration without relying on starch pools alone.

5.2 Limitations of the study

The first limitation of this study was the use of only single cultivar for either rice (IR64) and chickpea (Kabuli type). In relation to temperature sensitivity among rice, Glaubitz et al. (2014) classified IR64 as an 'intermediate' cultivar, particularly to high night temperature. This characteristic makes this cultivar an appropriate candidate for the study where three distinct temperature regimes (i.e. cold, warm and hot) were used. However, the results, for example the levels of primary and secondary metabolites in response to diurnal respiration, might vary if a contrasting set of cultivars were considered. Other rice cultivars that may have been suitable for comparative studies are N22 and IR72 (high temperature tolerant cultivars) as well as Azucena and DR2 (temperature sensitive cultivars) (Glaubitz et al., 2014; Jagadish et al., 2008). As for chickpea, more specific genotypes such as ICC 3362, ICC 6874 and ICC 12155 (heat tolerant genotypes) or ICC 16374, ICC 4567 and ICC 10685 (heat sensitive genotypes) are good candidates to investigate the physiological responses to growth temperature (Krishnamurthy et al., 2011).

Another limitation involved the application of hydroponics system, in rice experiments (refer to Chapter 2-3). This system was chosen for several reasons including that a large number of plants could be grown in a small area of glasshouse, hence increase the replicate numbers. More importantly, in contrast to soil based systems, hydroponics allows for complete control over water and nutrients availability (du Toit & Labuschagne, 2007), with the concentrations of all the macro- and micro-elements mixed appropriately [refer Section 2.3.1 (Hubbart et al., 2007)]. This difference, however might possibly affect the amounts or concentrations of nutrients received by plants and eventually influence the synthesis of primary and secondary metabolites (Ibrahim et al., 2011; Okazaki et al., 2008). In a study by Tamura et al. (2018) on leaf lettuce (*Lactula sativa*), the level of amino acids (e.g. lysine and phenylalanine) was higher in hydroponically grown plants than soil cultivated plants. On the other hand, soil cultivated leaves accumulated more sugars (e.g. arabinose and sucrose) and fatty acid-derived alcohols (e.g tetracosanol and hexacosanol) than leaves from plants that were grown hydroponically.

To have a study that included the flowering, ripening and harvesting phases of growth and development would have been desirable. Unfortunately, growing rice to these later stages of development would have substantially increased the time and complexity of my studies. Given the unavoidable time and resource constraints, I chose to focus on the vegetative stage of rice development. However, knowledge about the physiological responses of rice to growth temperature during the vegetative stage is valuable, given that increasing biomass production in the lead up to anthesis would rise crop yield (Hatfield & Prueger, 2015). The asynchronous acclimation of photosynthesis and respiration during the vegetative stage that I have identified in rice surely has an impact on the photosynthate production and usage, and ultimately plant growth. Theoretically, rapid respiration rates in plants grown at 40/35°C (when measured at prevailing temperatures), will contribute

to carbon loss, despite photosynthesis being homeostatic, especially if night temperatures rise faster than day temperatures. If night warming occured at the reproductive stage, the rice carbon budget will likely be negatively affected, and as a consequence grain filling. Ultimately, measuring photosynthesis and respiration rates during flowering and post flowering phases, would be required to answer how growth temperature impacts yield over the entire rice life-cycle.

One caveat to the work I present in Chapter 3 is that the measurements of respiration in the day were measured as dark respiration, by dark-adapting leaves for 30 minutes prior to measuring respiration under darkness. It is well established that respiration is light-sensitive, such that respiratory CO_2 released in the light is usually up to 80% lower than in darkness (Atkin et al., 2006b; Pärnik et al., 2007; Zaragoza-Castells et al., 2007). These light dependent changes in respiration might be a result of changes in substrates supplied by photosynthesis, given that photosynthesis occurs only during the day and respiration occurs round-the-clock. If *in situ* respiration was measured over the day/night cycle a different pattern of flux and acclimation may have been observed. However, I chose to focus on dark respiration as this is the most common form of respiration reported in the literature.

5.3 Conclusions and future directions

Overall, my PhD research aimed to understand the thermal acclimation of physiological processes in rice. The results I present provide important insights about the extent of thermal acclimation of respiration and photosynthesis in rice (Chapter 2). As far as I am aware, this is one of only a few studies to investigate both respiration and photosynthesis simultaneously, and the only study specific to rice, particularly on comparing the PE and ND leaves. Acclimation of respiration was indeed greater in rice ND leaves compared to PE leaves and resulted in homeostasis across the two lower growth temperatures.

However, homeostasis was not achieved when plants were grown at the hottest temperature. Contrastingly, the consistency in light-saturated photosynthesis at growth temperatures indicated that ND leaves strongly acclimated to temperature changes. Taken together, rice ND leaves showed asynchronous acclimation response favouring photosynthesis. Following this important finding, the study linked the physiological acclimation to the associated genes, proteins and metabolites at different time points following temperature shifts. Two main findings were: (1) within the first 24 h following temperature transfer, there was a transient adjustment in gene expression of PE leaves, which fits with the perturbations of respiration and photosynthesis in PE leaves; and (2) when exposed for a longer period, the abundance of COX declined in hot acclimated ND leaves. These results, especially the rapid responses in carbon fluxes and transcript abundance observed in PE leaves (which linked to the lower degree of acclimation compared to ND), indicate the sensitivity of abrupt temperature changes on crop functionality. Some of the molecular responses to short-term adjustments in temperature that I characterised, such as the variation in COX abundance, may be utilised by breeders to identify rice genotypes with greater thermal plasticity. In regards to the vegetative stage of development, the results suggest that rice plants have the potential to withstand more frequent heat waves, at least for temperatures equal to or below 40°C. Rice seems even more capable of acclimating to longer-term subtle increases in average global temperatures, considering the greater ability of ND leaves to acclimate to growth temperature, particularly in regards to photosynthesis acclimation.

Chapter 3 provides information on respiration acclimation throughout the day/night cycle for rice. In general, acclimation of respiration mostly holds diurnally. The strongest phenotype was observed at the end of the day. By contrast, differences in the acclimation phenotype were not seen at night or in the early morning. I investigated if the

diurnal changes in acclimation phenotype were related to changes in pool sizes of metabolites. Metabolite profiling revealed how respiratory metabolite levels were altered in response to contrasting growth temperatures and the diurnal cycle. In general, many amino acids were negatively correlated with carbohydrates and organic acids of the TCA cycle, particularly at night and at warmer growth temperatures.

The results presented in this thesis provide a strong foundation for future work probing the energy utilisation processes in plants. Given the fact that dark respiration acclimated differentially throughout the day, more detailed studies of respiration, including at least two (e.g. at the end of the day and night) if not more different time-points within a single 24 h period, should be considered. Further it would also be more informative of natural systems to assess light rather than dark respiration during the day. Although measuring light respiration is a complicated task – requiring respiratory, photosynthetic, and photorespiratory CO_2 fluxes be distinguished – a number of methods can be utilised. The three most common methods of determining light respiration are the Kok method (Kok, 1948), the Laisk method (Laisk, 1977), and the isotope-ratio mass spectrometry method. Future experiments building on the work I present here can use these methods to build a more comprehensive analysis of the respiratory day/night cycle. Indeed, variations in the temperature sensitivity of light and dark respiration, together with photosynthesis would be required for a more complete estimate of daily net carbon balance of plants, and to accurately predict the yield of crops exposed to global warming. Expanding the analysis from one focused on leaf tissue to a study of entire above and below ground biomass of rice would be another step in advancing the work I present here.

My results revealed that the capacity of COX declined in hot-acclimated plants (chickpea mitochondrial experiments in Chapter 4), likely as a result of reduced COX protein abundance (rice immunoblot experiments). The reason why there was a decline

in the abundance and capacity of COX at warmer growth temperatures is not clear and further experimentation is required. However, based on this work, COX emerges as a key target for the manipulation of the thermal acclimation potential of respiration. As such, it would be worth trying to understand the enzymatic capacity and abundance of COX in other crops when growing above their normal growth temperatures. It is understandable that results obtained from leaves may not fully reflect the overall impact on the entire plant level (Impa et al., 2019; Van Dingenen et al., 2016). Similar to this study, for example the results of transcript abundance in rice leaves which experienced major changes at 2 and 6 h, then acclimate within 24 h, may or may not capture the impact of high growth temperature on respiration at the whole rice plant (e.g. the whole plant would probably require more than 24 h to achieve homeostasis). This emphasizes the need to consider measuring the gas exchange together with the associated mechanistic traits at the whole plant level as well, and not only in a controlled growth facility, but also at the scale of paddy fields. The significant differentially expressed genes (Figure 2.1, Table 2.1) along with the results of COX protein abundance and capacity could be used as a starting point to develop markers to support rice breeding programs focusing on improving heat tolerance in rice.

Additionally, more studies need to be carried out aiming to identify valuable genetic variation in crops. The findings could be used by plant geneticists and breeders for improving the quality of crops. For example, breeding new rice cultivars that are not only heat tolerant, but also high in nutritional values [e.g. Frontière – a high-protein rice cultivar; Wenefrida et al. (2017)] and resistant to pests and diseases [e.g. Makassane – an IRRI-bred rice variety that resistant to bacterial leaf blight disease; Singh et al. (2013)] will ultimately ensure food security and reduce hunger especially in developing countries in the face of a changing climate and a rapidly rising world population.

The transient adjustment in metabolic transcript abundance of PE leaves of annual crops, particularly rice and chickpea, in response to growth temperature may not translate into perennial crops such as alfalfa (*Medicago sativa*) which needs to survive winter. Although the effect of temperature on photosynthesis and respiration on perennial crops has been studied previously (Yamori et al., 2014; Zaka et al., 2016), little has been done to compare the findings with annual crops. Carbon balance, which hinges on the prevailing climate and the crop developmental stages (Impa et al., 2019), may differ specifically in a seasonal context. In regards to respiration, it is a difficult task to interpret the relationship between temperature, respiration and crop yield, not only because it involves many other physiological processes (e.g. photorespiration), but also because the respiration alone can be divided into two components: growth and maintenance respiration. Maintenance respiration, compared to growth respiration is the most responsive to environmental changes (Caleb et al., 2016; Ryan, 1991), including temperature (Krishnan et al., 2011). The higher the rate of maintenance respiration, the lower the amount of assimilates available for growth (van Iersel & Seymour, 2000; Weraduwage et al., 2015) and eventually yield (Amthor et al., 2019). As stated in Eagles and Wilson (2018), maintenance respiration is more dominant towards the end of life cycle in annual crops, and after the initial establishment of a high leaf area index (LAI) in perennial crops. Understanding how changes in temperature, particularly in the short-term, affect physiological, metabolic, protein, and transcript processes in both annual and perennial crops, and across the level of whole tissues, would be of considerable value. This would provide a more holistic understanding on how temperature influences growth, development and ultimately yield for many crops with varying life strategies.

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Supplementary

Table S2.1. Concentrations of essential macro- and micro-nutrients in hydroponic solution for rice plants.

Element	Chemical formula	Concentration (mM)
Macronutrients		
Nitrogen (N)	NH_4NO_3	1.4
Phosphorus (P)	$NaH_2PO_4.2H_2O$	0.6
Potassium (K), Sulfur (S)	K_2SO_4	0.5
Calcium (Ca)	$CaCl_2.2H_2O$	0.2
Magnesium (Mg)	$MgSO_4.7H_2O$	0.8
Micronutrients		
Iron (Fe)	Fe-EDTA	0.07
Boron (B)	H_3BO_3	0.037
Manganese (Mn)	$MnCl_2.4H_2O$	0.009
Zinc (Zn)	$ZnSO_4.7H_2O$	0.00075
Copper (Cu)	$CuSO_4.5H_2O$	0.0003
Molybdenum (Mo)	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0001
Vanadium (V)	NH_4VO_3	0.000138
Silicon (Si)	Na_2SiO_3	0.0012963

Table S2.2. Primers used for qPCR gene expression analysis.

Protein	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Size	Location	mRNA
Ferredoxin NADP reductase	TCAGGCTCTACTCCATCGCC	20	487	506	58.9	60	152	exon 2	NM_001063105.1
	ACCAGGCTTCAAGTCACAGAGG	22	638	617	60	54.6		exon 3 or 4	
Phosphoribulokinase	TTTGATGCCTTCATTGATCCCC	22	865	886	56.8	45.5	80	exon 3 or 4	NM_001054360.1
	TCATCAGGAATAAGCTGGGTTGG	23	944	922	58.1	47.8		exon 4	
Uncoupling protein	CCTCTACGAGCCCGTGAAATCC	22	348	369	60.6	59.1	134	exon 2 or 3	NM_001075091.1
	TGACAAGGTCAGTGGGGTTGG	21	481	461	60	57.1		exon 4	
Cytochrome *c* oxidase (COX)	GCCATTGCAGGAGTGATGGG	20	73	92	59.2	60	85		LOC_Os12g33946.1
	TCCCACCAAGAATTTGATCGCC	22	157	136	59	50			
Alternative oxidase (AOX)	TGCTTTAGGCCATGGGAGACC	21	416	436	59.9	57.1	84	exon 1 or 2	NM_001060293.1
	CTTGTCGAGCAGCGTCTTGG	20	499	480	59.5	60		exon 1	
Sucrose-phosphate synthase (SPS)	ATGCGAAGCCTGAAACCCCC	20	1317	1336	60.9	60	124	exon 8 or 9	NM_001052401.1
	CACACGGAGGTGCAGAAAAGG	21	1440	1420	59.6	57.1		exon 9	
eIF4-gamma/eIF5/eIF2-epsilon domain containing protein (Eukaryotic initiation facctor 5C)	CTGATGGAGGCTGTTCAGTGG	21	1035	1055	58.5	57.1	81	exon 6 or 7	NM_001074292.1
	AGCCCATGCTTTCACCTGCC	20	1115	1096	61.2	60		exon 7	

Table S2.3. Differentially expressed genes. At each time point, differential expressed genes were assessed for the hot and cold treated plants relative to the warm grown control plants sampled at the same time (n = 4). Genes were considered differentially expressed an false discovery rate adjusted P (padj)<0.05.

Treatment	Time point	Upregulated	Downregulated
Cold	2 hr	4	0
Hot	2 hr	858	960
Cold	6 hr	2	0
Hot	6 hr	685	780
Cold	24 hr	0	0
Hot	24 hr	0	0

Table S2.4. Protein concentrations of 25/20°C and 40/35°C day/night grown leaves. Data represents mean of three to four biological replicates ± SE.

Time point	Protein concentration (mg ml^{-1})	
	25/20°C	40/35°C
6 hr [Pre-existing (PE) leaves]	7.7 ± 0.8	7.0 ± 1.0
Day 1 [Pre-existing (PE) leaves]	8.8 ± 1.9	6.9 ± 1.3
Newly-developed (ND) leaves	8.5 ± 0.7	7.8 ± 0.8

Table S2.5. Pearson's correlation coefficients between rates of dark respiration and concentration of soluble sugars for plants grown at different growth temperature regimes.

	Dark respiration x soluble sugars		
	25/20°C	30/25°C	40/35°C
Day 1			
Correlation coefficient	-0.999	-0.999	0.049
P-value	0.024*	0.032*	0.969
Day 7			
Correlation coefficient	-0.805	-0.990	-0.461
P-value	0.405	0.090	0.695
Newly developed (ND)			
Correlation coefficient	-0.420	-0.560	0.879
P-value	0.724	0.621	0.317

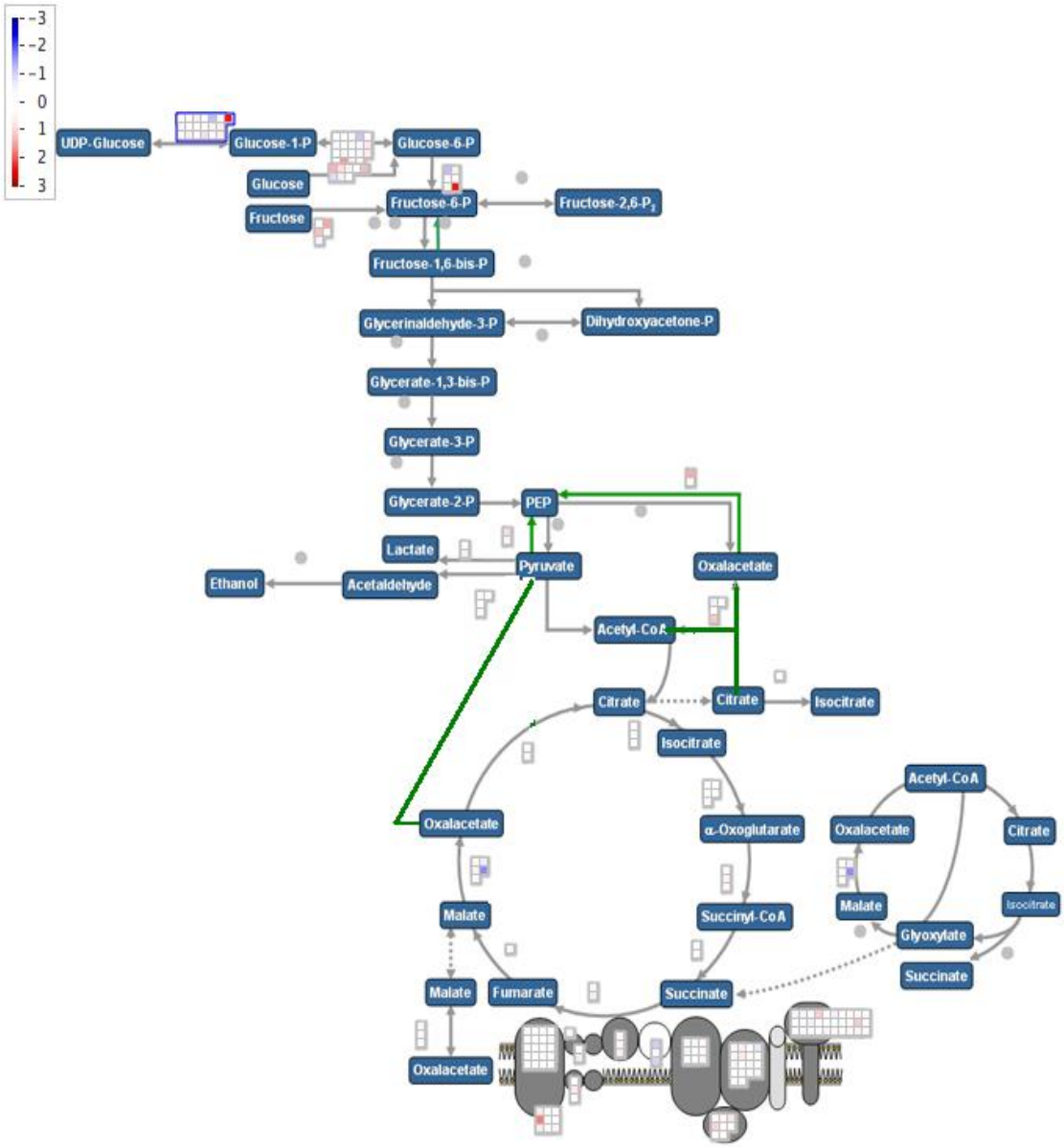

Figure S2.1. Differential gene expression in the Glycolysis and TCA under hot treatment. Gene expression is visualised in a MapMap pathway view of Glycolysis and the tricarboxylic acid (TCA) cycle. Each box represents a rice gene with homology to an Arabidpsis gene annotated in the MapMan pathway. Data show for the comparison of 2 hours hot treatment compared to 2 hours control treatment (warm); red shading represents up-regulation and blue shading down-regulation on a log2 fold-change scale.

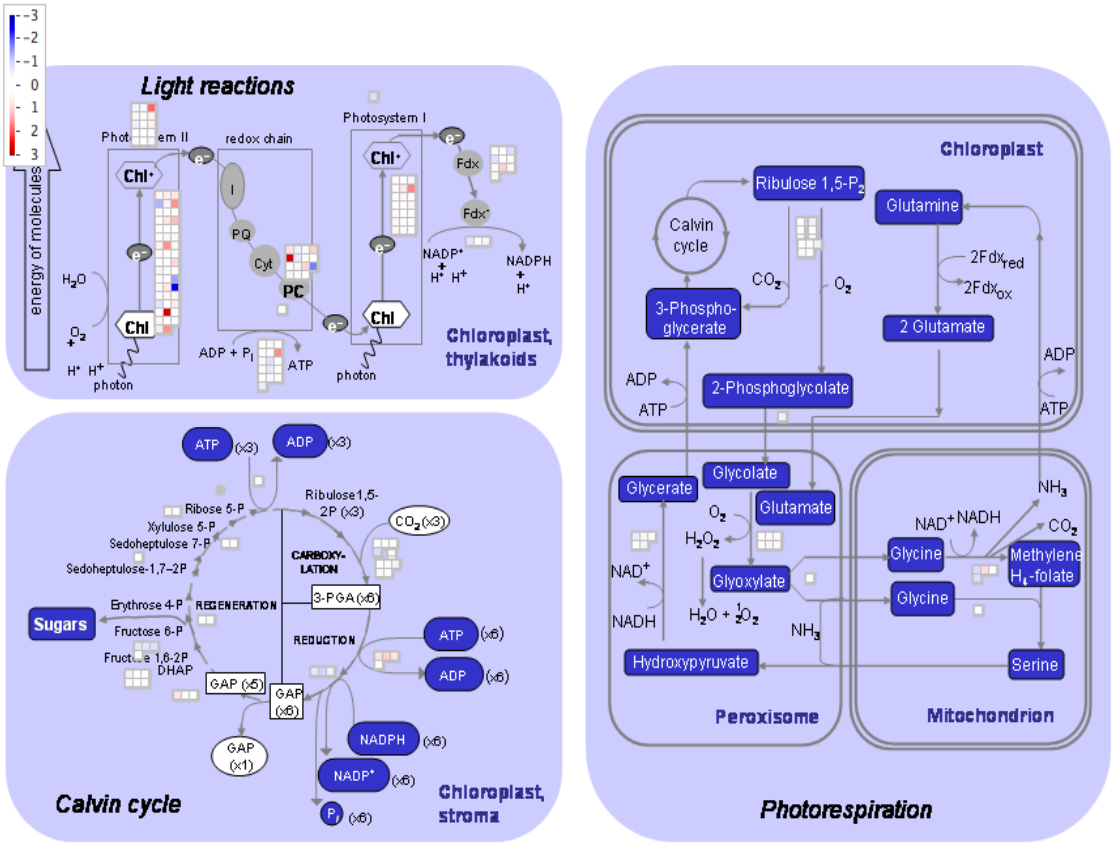

Figure S2.2. Differential gene expression of photosynthetic genes under hot (40/35°C) treatment. Gene expression is visualised in a MapMan pathway view of photosynthesis. Each box represents a rice gene with homology to an Arabidopsis gene annotated in the MapMan pathway. Data show for the comparison of two hours hot treatment compared to two hours control (30/25°C) treatment; red shading represents up-regulation and blue shading down-regulation on a log2 fold-change scale.

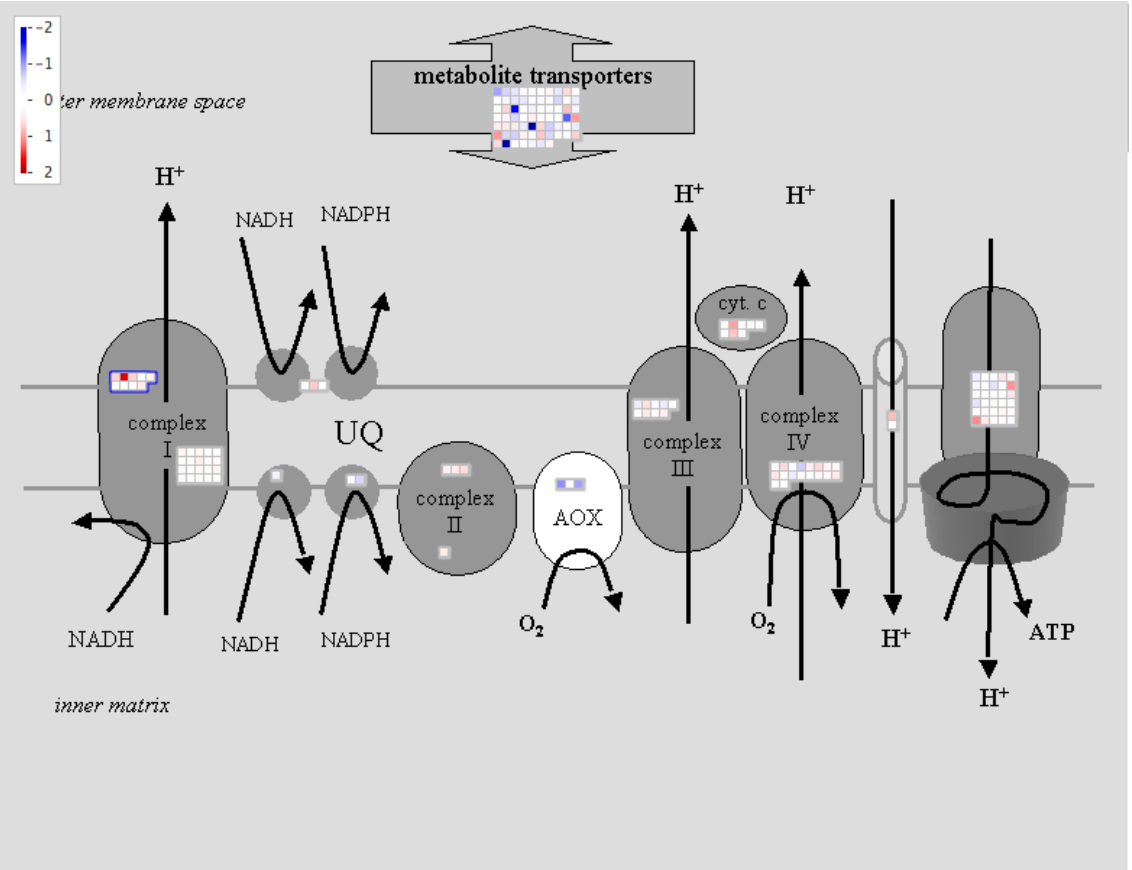

Figure S2.3. Differential gene expression in the mitochondrial electron transport chain under hot treatment. Gene expression is visualised in a MapMan pathway view of the mitochondrial electron transport. Each box represents a rice gene with homology to an Arabidopsis gene annotated in the MapMan pathway. Data show for the comparison of two hours hot (40/35°C) treatment compared to two hours control treatment (30/25°C); red shading represents up-regulation and blue shading down-regulation on a log2 fold-change scale.

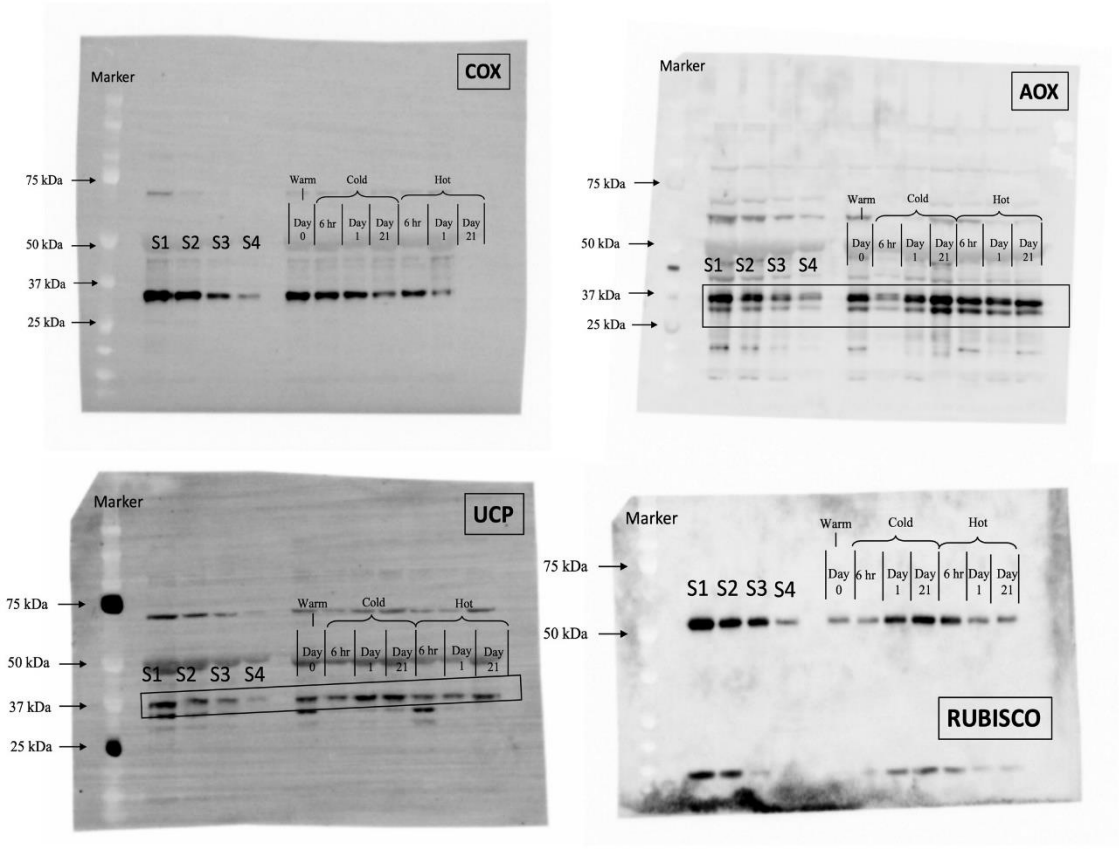

Figure S2.4. Representative western blots of cytochrome *c* oxidase (COX) subunit II, alternative oxidase (AOX), uncoupling protein (UCP), and Rubisco, used for determination of protein abundance of leaves. S1 to S4 is a dilution series of the warm/control (30/25°C) samples. This is followed by the initial day 0 warm leaves prior to temperature transfer, the 6-h and 1-day times after transfer of PE leaves, and ND leaves after 21 days of temperature transfer, for the cold (25/20°C) and hot (40/35°C) treatments as indicated on the images. For AOX and UCP, the bands within the box are what was classified and analysed as the proteins of interest based on the molecular weight marker markers.

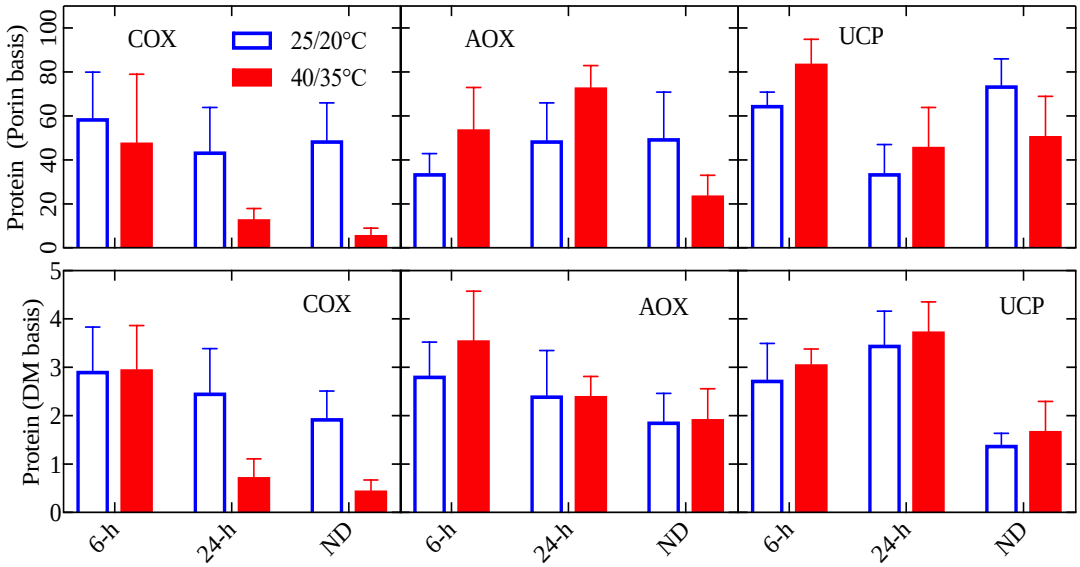

Figure S2.5. Abundance of mitochondrial electron transport chain proteins determined by Western blot analysis for pre-existing rice leaves sampled at; six and 24 h after *T* transfer to 25/20°C or 40/35°C, and leaves newly developed (ND) post *T*-transfer. The top three panels are protein abundance of cytochrome *c* oxidase (COX), alternative oxidase (AOX), and uncoupling protein (UCP) normalised to porin abundance and adjusting the largest value in each dataset to 100. The bottom three panels are leaf area values normalised to a dry mass (DM) basis by factoring in the leaf mass per area of pre-existing and newly developed leaves as presented in Table 2.2 of the text. Data represent mean ± SE of four independent western blots, with each blot representing leaf tissue from a separate plant.

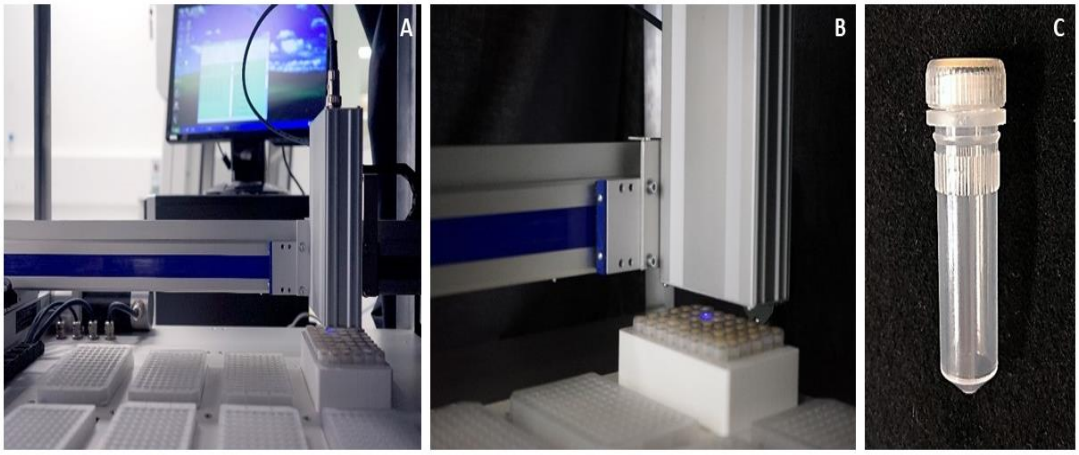

Figure S3.1. The high throughput fluorophore measurement system (Q2 O2-sensor) used in the measurements of dark respiration on the O_2 consumption basis (A-C). The leaf samples were placed in (A) the 2 ml capacity Q2 tubes and sealed with specialised caps containing a fluorescent metal organic dye that sensitive to O_2 quenching. The tubes were then placed in the racks of 48 tubes. During measurements, (B-C) a blue-spectrum LED excitation, followed by a red spectrum emission (approximately 480 nm and 580 nm, respectively) pulse onto the surface of caps. This O_2 quenching phenomenon allows the O_2 dependent decay in fluorescence signal to be quantified. The system was covered with black cloth during the measurements to maintain darkness.

A

```
##
## Call:
## adonis(formula = met_c ~ rel_temp, data = dat, method = "eu")
##
## Permutation: free
## Number of permutations: 999
##
## Terms added sequentially (first to last)
##
##           Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
## rel_temp   1    299.71 299.707  6.7082 0.10367  0.001 ***
## Residuals 58   2591.29  44.677         0.89633
## Total     59   2891.00                 1.00000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

B

```
##
## Call:
## adonis(formula = met_c ~ Treatment, data = dat, method = "eu")
##
## Permutation: free
## Number of permutations: 999
##
## Terms added sequentially (first to last)
##
##           Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
## Treatment  2    494.87 247.437  5.8861 0.17118  0.001 ***
## Residuals 57   2396.13  42.037         0.82882
## Total     59   2891.00                 1.00000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure S3.2. The R outputs of permutational multivariate analysis of variance (PERMANOVA) showing that (A) the day/night phase explained 10% variation in metabolite levels (P<0.001), and (B) the growth temperature explained 17% variation in metabolite levels (P<0.001).

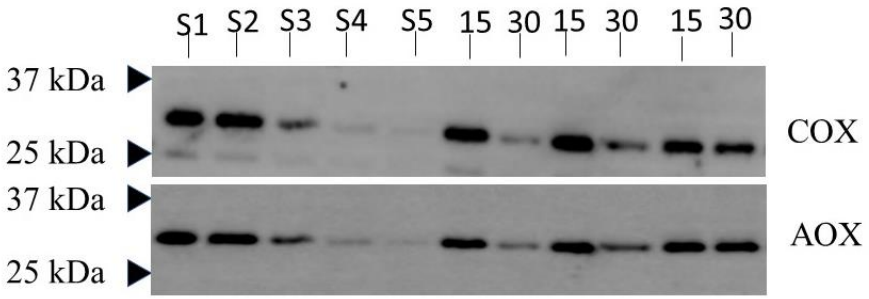

Figure S4.1. Representative western blots of cytochrome *c* oxidase (COX) subunit II and alternative oxidase (AOX) used for determination of protein abundance of leaves. S1 to S5 is a dilution series of the 15/10°C leaves. This is followed by the 15/10°C leaves and 30/25°C leaves from 3 biological replicates.

Table S4.1. Forward and reverse primer sequences and expected product size for the different genes studied.

Primer set	Accession	Forward primer	Reverse primer	Expected product size (bp)
CI(NDHF1)	XM_004497802	GATTGAAGCCCCCTTTCCCA	ATTCTGGCCCACGCCTTAAA	111
COX1	TC01181 RC	GCCATTCTGGAGGAGCAGTT	ATTCCAGGTCCACGCATGTT	118
COX2	TC13198	TGGGCTGTACCTTCCTTTGG	ACGACGATAGGCATAAAGGCA	146
COX3	TC20962	TTTGGCAACCACCGTAGGAG	CATCGCGCCACCATACAAAC	116
COX5b1.1	TC17999 RC	AGGGTTGAGGATGTTGTCCC	GCAGGTGCTTCCTTTGTGC	128
COX5b1.2	TC18790	GGATAGTTGGATGCCCAGGT	AGGTCCTACCACTTCGAGCA	128
COX6b1	TC18097 RC	ACGGAAATCTACTGGAGCGG	GAAAGCAGTGAAGTTGCCCC	144
ORF25	TC22885 RC	TGCGCCTAAGTGCGAAAAGA	CGGATGCGACGGGAAGAAAT	99
ORFB	TC13780	CAAAATCCGGAGCAACGACC	CGGCGTTACACCATTGGGAT	116
MGP1	XM_004503953	GGAGACTCAAAAGAAAAGGGAGG	AGGAAGTTCCGGATGTCCAA	81
ATP3	XM_004507724	TCCCCAGAGGTTGTTGAGAGA	GTCTCACCCCCTTCAATCTCA	83
ATP5	XM_004499817	ACTGTCTTTCCTCTTCCCCCT	TCCAGCACTAGACCACCAAG	131
UCP1-like	XM_004506600	CCCCGGTTGATGTGGTCAAG	CAAGATCCCAGCCGTCCAAA	147
UCP1L-like2	XM_004514430	CCCCGGTTGACGTGGTTAAA	TCCAAGATCCTAGCCGTCCA	147
UCP5-like	XM_004489704	AGGTGGAGGCAGGTAAGGAA	AGGACCCTGCCTCGAAATTG	125
UCP5-like2	XM_004509622	GTGACGAATCCGGTGGATGT	TAGGACCCTCACCACGAACA	124

Table S4.2. Statistical data (*F*-values and *P*-values) of quantitative PCR analysis of gene expression based on one-way ANOVA of temperature treatment effect. Asterisk (*) show significant differences between growth temperatures at $P<0.05$.

	F	*P*
Complex I (CI)	8.385	0.016*
COX		
COX1	0.053	0.823
COX2	4.724	0.055
COX3	0.213	0.054
COX5b1	1.423	0.260
COX5b2	0.000296	0.987
COX6b1	2.315	0.159
AOX		
AOX1a	0.298	0.597
AOX2a	6.609	0.028*
AOX2b	10.652	0.009*
AOX2d	0.631	0.446
ATP synthase		
ORF25	17.000	0.002*
ORFB	12.938	0.005*
MGP1	6.827	0.026*
ATP3	6.814	0.026*
ATP5	1.198	0.299