The price of heat stress: functional and resource constraints to thermal tolerance in arid zone plants

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Certificate of original authorship

I, Kirsty Milner declare that this thesis, is submitted in fulfilment of the requirements for the award of, in the School of Life Sciences/Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Statement of thesis format

This thesis is submitted as a thesis by compilation. Chapter 1 provides a general introduction to the background literature, the gaps in the field and my research questions. The following three data chapters have been prepared as standalone manuscripts for publication. Chapter 5 provides a synthesis of my research and highlights the contribution this thesis makes to our knowledge on the thermal tolerance of desert plants, as well as identifying future directions.

Declaration of contribution to each publication

1. Chapter 2. Milner KM, French K, Ashcroft MB, Valenzuela SM, Leigh A (In preparation for submission). Capacity to tolerate, acclimate and recuperate. In preparation for submission to *Plant, Cell and Environment*.

I designed the experiment in consultation with my supervisors, Leigh and French. I conducted the field and laboratory work, collected and analysed the data. Ashcroft wrote the R scripts for Bayesian analysis of data. Valenzuela aided with laboratory work. I led the writing, with all authors contributing to the text.

2. Chapter 3. Milner KM, French K, Krix DW, Valenzuela SM, Leigh A (Submitted for revision). Plant stress under spring or summer extreme heat events**.** For resubmission to *New Phytologist.*

I designed the experiment in consultation with Leigh and French. I conducted the field work, including the development of the open-topped heat-stress chambers, collected and analysed the data. The Australian Arid Lands Botanic Garden nursery staff provided help with growing experimental plants in Port Augusta and watered them in my absence. Krix wrote the R scripst, which contributed to the data analysis. Valenzuela aided with laboratory work. I led the writing, with all authors contributing to the text.

3. Chapter 4. Milner KM, French K, Van Sluyter S, Leigh A (In preparation). Seasonal changes in functional protein groups, in contrasting desert plants

I designed the experiment in consultation with Leigh and French. I conducted the field and nearly all of the laboratory work, collected and analysed the data. Van Sluyter had previously developed the leaf proteomics methodology and aided with protein extraction and analysis, including running the samples and protein calculations. I led the writing, with all authors contributing to the text.

Preface

Plants! Why study them?

The more we learn about the way plants sense and respond to the environment the more interesting they become to me. Plants do not exist in isolation and despite appearing unchangeable are highly responsive to the conditions in which they grow. The close interactions between fungi and bacteria mean that plants are inextricably linked to one another. The sharing of nutrients and carbon between plants through mycorrhizal fungi moves resources from high to low gradients benefiting individuals, and not always of the same species (Arnebrant et al. 1993, Simard et al. 1997). And while the continued movement of water to a leafless-stump of *Agathis australis* to keep it alive does not appear advantageous, it is likely beneficial for its congeneric neighbours during water limitation (Bader and Leuzinger 2019). Plants sense their environment; detect emissions from other plants and prime themselves against herbivore attack (Frost et al. 2008). They share among themselves but exploit animals, including attracting parasitoid wasps to fend off herbivores (van Poecke and Dicke 2002), tricking animals into pollination (Jersáková et al. 2006) and in the extreme cases, eating them, a trait so good it evolved multiple times (Albert et al. 1992).

Despite all the interesting adaptations plants use to survive, plus making up ~ 80% of the 550 Gt of carbon of biomass on Earth (Bar-On et al. 2018) and providing vast ecosystem services (Costanza et al. 1997), plants suffer from being overlooked. The term ‘plant blindness’ was coined because plants go unnoticed, are not recognised as important, or less important than animals (Wandersee and Schussler 1999). The concern is that conservation funding for plants is lower than for animals (Balding and Williams 2016). All the while natural ecosystems face enormous pressure due to human activity; thus far resulting in 600 seed plant species having gone extinct (Humphreys et al. 2019). Threats to plants are mounting under climate pressure. Yet land plants currently draw down 30% of carbon emissions per year (Ciais et al. 2013) and re-forestation projects have the potential to further drawdown CO_2 emissions (Bastin et al. 2019). If re-vegetation is to work, the identification of appropriate species, ones that can cope with temperature change under climate change, is paramount.

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Abstract

Understanding how plants cope with extreme temperatures is key to determining species distribution under climate change. Plants possess an inherent ability to withstand high temperatures and acquire greater thermal tolerance seasonally. The membranes and photosynthetic apparatus in leaves are particularly susceptible to heat damage and likely to respond to different environmental cues. The question arises as to how these two systems differ in acquiring thermal tolerance and what roles proteins have in raising thresholds. As part of the stress response and to aid in thermal tolerance, heat shock proteins (HSP) are upregulated, but there are associated resource costs, of particular concern for natural populations. In extreme environments, like deserts, the additional stressors of water and nutrient limitation may affect how plants allocate resources to growth, reproduction and survival. My thesis is important in linking ecology, plant physiology and molecular biology over seasonal time scales in wild Australian desert plant species *in situ* in desert conditions. I estimated temperature thresholds of photosystem II (PSII, using chlorophyll *a* fluorescence) membrane stability (via electrolyte leakage) and fitness (via reproductive output) in response to heat stress across seasons. To determine how relative protein expression changes with conditions, I also quantified the complete proteome using shotgun proteomics with tandem mass spectrometry. Overall, species acquired higher thresholds of PSII and membranes and HSP expression was dependent upon season, with little sHSP detected in winter. Cost of three-hour heat stress was reduced in plants with access to additional nutrients, but unexpectedly, heat stress in spring was found to be less costly than in summer, likely due to more severe summer conditions making recovery hard. I show that changes to the proteome are complex, but consistent patterns emerged, with lipid metabolism, ROS homeostasis and HSPs meeting expectations of higher expression during summer. Also, regardless of species or heat-stress treatment, small HSPs were detected in greatest amounts in summer, emphasising the importance of small-HSPs for acquired thermal tolerance in desert species. Importantly, species differences were highlighted throughout the research. Across broad climatic zones, species have many modes for achieving the same outcome and microhabitat likely has an effect on driving adaptation. My work underscores the temporal dynamics of plant thermal tolerance in non-crop species in the environment and how this is achieved through proteome changes. However, my findings suggest that for species from harsh microhabitats, increasing heat stress in summer may have particularly severe consequences.

CHAPTER 1: INTRODUCTION

1.1 The environment is stressful

1.1.1 Species are constrained by stress

Species are adapted to the abiotic conditions they experience regularly and function efficiently within a restricted range. Therefore, it is physiological processes that limit the distribution of organisms (Addo-Bediako et al. 2000, Gaston and Spicer 2001, Chown et al. 2004). If we consider an increasing gradient of an environmental factor/abiotic condition, species niches can be described as a performance curve constrained by upper and lower critical limits, beyond which the organism cannot survive (example for plants, Figure 1.1a). At a point along this performance gradient, the physiological optimum (peak of the curve) is reached, where organismal systems function best (Bozinovic et al. 2011) and fitness is greatest. When conditions exceed optimal, a threshold is crossed, homeostasis—the ability of an organism to maintain normal physiological rates—is lost and the organism becomes stressed. When exposed to stressors, the inducible stress response is elicited (Laksanalamai and Robb 2004) (Figure 1.1a and see 1.2.3). There are costs associated with undertaking processes to reinstate balance (Miller and Stillman 2012).Stress can be any factor that decreases growth and reproduction below the potential of the genotype (Osmond et al. 1987). Since critical limits and thresholds are constrained by physiology, stress is defined based on the organism experiencing it (Bijlsma and Loeschcke 2005). The severity of stress depends upon intensity, duration and rate of change, as well as the developmental stage of the organism and the conditions prior to exposure (Bijlsma and Loeschcke 2005). At the individual level, exceeding critical limits will ultimately cause death. At the population or species level, evolutionary stress reduces fitness (Bijlsma and Loeschcke 2005) to the point of local or global extinction (Bozinovic et al. 2011).

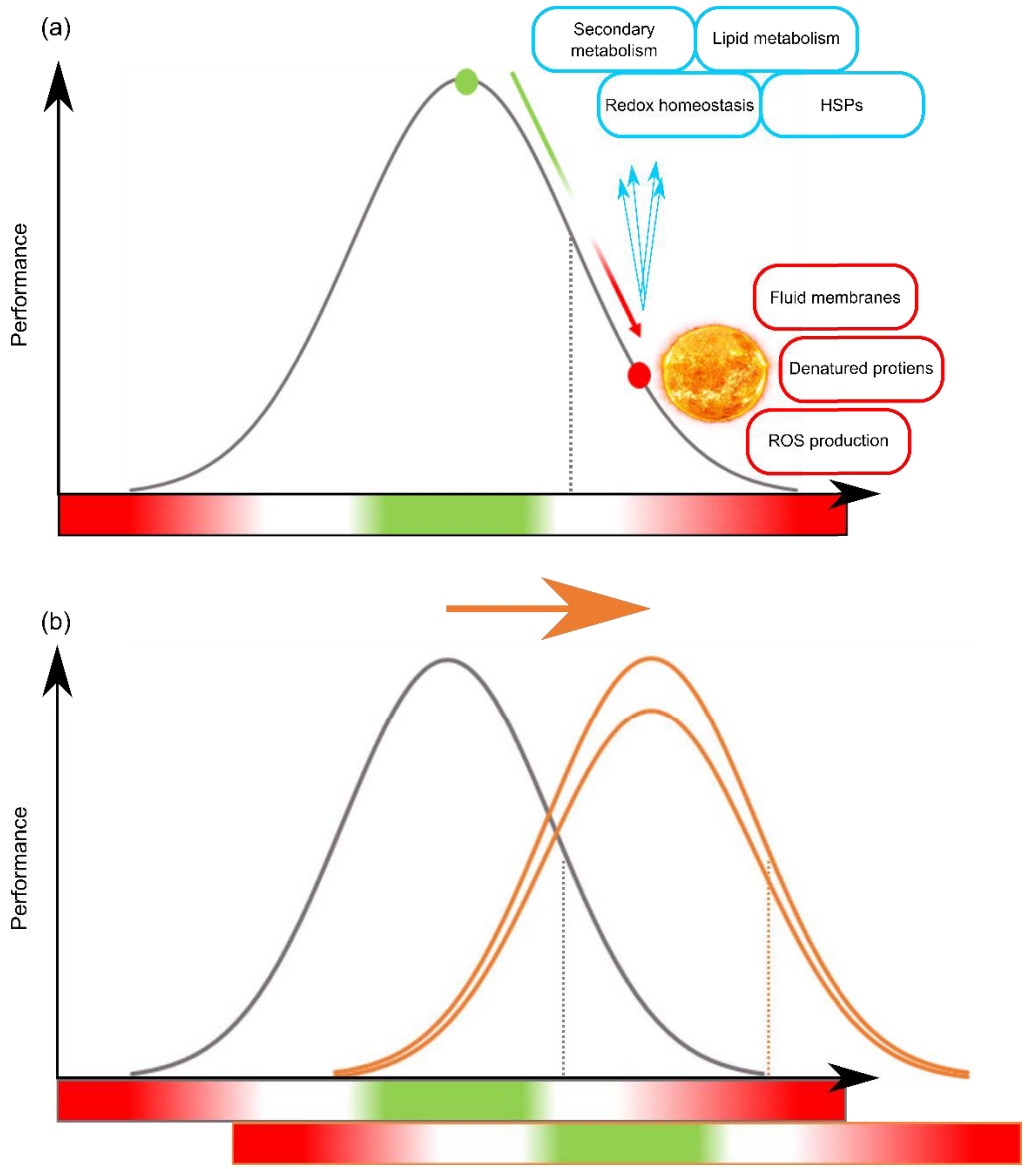

Figure 1.1. Plant performance curves defined by temperature. Along a gradient of increasing temperature, performance rises to optimal (green area), then declines as temperature becomes too hot. When tolerance thresholds (grey dashed line) are crossed (e.g. during a heatwave), the plant experiences stress. Performance falls due to loss of membrane stability, denatured proteins, and reactive oxygen species (ROS) production (red boxes). The stress response (blue boxes) is elicited as the plant attempts to return to homeostasis (a). Following priming and/or recovery from stress, the plant will shift from basal tolerance (grey curve) to acquired thermal tolerance (orange curves), a shift that includes higher thresholds. Ideally, acclimation would mean equal or enhanced performance at new temperatures, but often results in reduced performance (b).

1.1.2 Temperature as a significant abiotic factor

Temperature explains connections among energy, climate and biogeography (Bozinovic et al. 2011). Physiological rates are temperature dependent (Mitra and Bhatia 2008), controlled by maximum enzyme activity at low temperature and by limited substrates at high temperature (Atkin and Tjoelker 2003). Gaining an understanding of temperature-dependent processes not only helps explain species distributions today, but can inform future distributions under climate change (Somero 2010). Temperature is one of the key environmental drivers influencing plant traits (Moles et al. 2014), growth and physiology (Ghannoum and Way 2011), and photosynthetic function (Zhu et al. 2018). As sessile organisms responsible for most terrestrial productivity, understanding the influence of temperature on plants is vital.

1.2 Plant thermal tolerance; from basal, to stress and recovery then acquired

1.2.1 Basal tolerance depends on where plants are from

The inherent ability of a species to withstand high temperatures, known as basal thermal tolerance, comes from long-term adaptation to an environment at the species level, so that metabolic function is optimised to growth environment (Berry and Bjorkman 1980). Heat stress is species-specific; for example, the temperatures noted in 1.2.2 provide a general guide for when temperature becomes stressful to plants, but mostly apply to temperate species from benign climates. Generally, species from warmer climates possess higher optima or thresholds of photosynthesis than those from cool climates (Nievola et al. 2017, Zhu et al 2018). Globally, the average optimum photosynthetic temperature is 23 ± 6°C, but increases to 30°C in the tropics, savannahs and arid lands (Huang et al. 2019) and see Table 1.1 for more examples. Recently, however, research suggests that thermal tolerance varies at much finer, microclimatic scales (Austin and Van Niel 2011, Slavich et al. 2014, Gollan et al. 2015, Curtis et al. 2016). In field studies, true basal tolerance is not determined but sampling in the coolest part of the year provides an estimate of thermal tolerance in the absence of heat stress (Sastry and Barua 2017, Zhu et al. 2018). Comparisons of organisms from hotter *versus* cooler environments show that, similar to the stress response, thermal tolerance is provided by heat shock proteins (HSPs; see 1.2.4.2), anti-oxidants and accumulation of solutes (Allakhverdiev et al. 2008), as well as membrane lipid saturation and thermally stable gene expression, translation and proteins (Tomanek and Somero 1999, Xu and Huang 2010).

Table 1.1. Examples of temperatures for optimal growth, photosynthesis or leaf thermal tolerance of different plant species or ecosystems/habitat/climate zones types.

Species or habitat	Temperature (°C)	Measure of optima or tolerance	Reference
Global average	23 ± 6	optimal photosynthesis	(Huang et al. 2019)
Psychrophiles	0-15/20	optimal growth	(Nievola et al. 2017)
Antarctic grass	10	optimal photosynthesis	(Nievola et al. 2017)
Mesophiles	10-30	optimal growth	(Nievola et al. 2017)
C_3 plants	10-35	optimal photosynthesis	(Yamori et al. 2014)
Temperate trees	20-35	optimal leaf temp.	(Sharkey 2005)
Tropical trees	<35	optimal photosynthesis	(Doughty and Goulden 2008)
Thermophiles	>30		(Nievola et al. 2017)
Tidestromia oblongifolia	40-45	high photosynthesis	(Berry and Bjorkman 1980)
Temperate tree species	45-50	leaf tolerance (T_{50})	(Cunningham and Read 2006)
Tropical tree species	50-52	leaf tolerance (T_{50})	(Cunningham and Read 2006)
Temperate arid zone species	48-54	leaf tolerance (T_{50})	(Curtis et al. 2014)
Yellowstone NP grass	43-57	max. root temperature experienced	(Stout and Al-Niemi 2002)
Max. temperature for survival	65	proteins denature	(Levitt 1980, Nievola et al. 2017)
	55-70	complete cell rupture	(Ilík et al. 2018)

1.2.2 Plant dysfunction under high temperature stress. What goes wrong and at what point?

Given that for much of the year temperatures do not exceed 5°C for greater than 80% of the Earth's surface (Margesin et al. 2007) it is no wonder that, in terms of plant thermal tolerance research, studies on cold stress have outnumbered those of heat stress (Gainge et al, in prep). However, for the remaining ~20% of the globe and at certain times of the year plants may experience high temperatures—and in recent decades increasingly extreme high temperatures (see 1.4)—highlighting the nessitiy of researching plant response to heat stress.

In plants, stress and damage caused by temperature exceeding upper thresholds can significantly reduce the ecosystem services they provide including carbon sequestration. Heat stress has decreased gross primary productivity (GPP) by as much as 30% (Ciais et al. 2005, Bauweraerts et al. 2014b) and caused worldwide reductions in crops productivity (Mittler et al.

2012). A difficulty for plants in terms of high temperature is balancing the energy budget (carbon out *versus* carbon in) due to numerous factors. Firstly, the temperature optimum of respiration is higher than the optimum for photosynthesis (Dewar et al. 1999, Atkin et al. 2006, Atkin et al. 2007). Secondly, not only are photosynthetic rates affected, but incoming light may not be used efficiently due to non-functioning photosystems. There are a number of pathways that can be followed by light energy hitting a leaf. The most beneficial of these pathways is assimilatory photochemistry, where light energy is used to fix inorganic carbon via the electron transport chain and Calvin cycle. During temperature stress, if either of these processes is interrupted, harmful excess light energy must be dissipated as heat or fluorescence (Bilger et al. 1984, Baruffo and Tretiach 2007). While photoprotective, these mechanisms mean a lower quantum yield of photosynthesis, i.e., less light energy used to make sugars. Finally, if photosystems are damaged, carbon gained must be spent on repair. Ultimately, photosynthesis is reduced and respiration rates increase (Atkin et al. 2005a, O'Sullivan et al. 2013), leading to reduced energy for growth and reproduction.

Through the body of research into high temperature stress in plants a general sequence of events, with increasing temperature, can be determined. In most terrestrial plants, temperatures above 35°C elicit a stress response (Larcher 2003). One of the most critical aspects of plants experiencing high temperature is maintenance of photosynthetic protein function. Early work suggested that PSII was the most thermally sensitive component of photosynthesis (see Berry and Bjorkman 1980). Evidence now suggests that, moderate heat stress likely causes inhibition of Rubisco activase (Law and Crafts-Brandner 1999, Sharkey 2005, Tang et al. 2007, Allakhverdiev et al. 2008), while damage to PSII occurs at severely high temperatures, i.e., above 45°C (Havaux 1993a, 1996). Early pioneers in plant thermal tolerance proposed that damage to membranes occur at temperatures ~10°C higher than damage to photosynthetic apparatus (Berry and Bjorkman 1980). During heat stress, membranes become fluid and disorganised, resulting in membrane permeability and a change in pH or ionic composition (Daniell et al. 1969). As most photosynthetic processes occur within the thylakoid membranes of the chloroplasts, the path of electron flow can be affected; unstacking occurs around 35-45°C (Gounaris et al. 1984), with increasing permeability to substrates and protons at 40°C (Bukhov et al. 1999, Atkin and Tjoelker 2003). Unstacking of thylakoids can be reversible or irreversible, depending on the stress imposed (Yamamoto et al. 2008). At extreme temperatures, membranes rupture, leading to cellular death (Daniell et al. 1969). Further to protein and membrane damage, metabolic imbalances and damage to macromolecules lead to accumulation of toxic by-products like reactive oxygen species (ROS) (Mittler 2002, Wang et al. 2003a, Suzuki and Mittler 2006). At moderately high temperatures (35-40°C), there is increased production ROS in chloroplasts and mitochondria (Mittler 2002,

Nievola et al. 2017). ROS causes lipid peroxidation damage to thylakoid membranes (Daniell et al. 1969, Djanaguiraman et al. 2018) and forms cytotoxic products, both of which damage the DI protein of PSII (Halliwell 1987, Yamamoto et al. 2008). ROS also inhibit the recovery of PSII (Allakhverdiev et al. 2008) by stopping *de novo* synthesis of proteins (Yamamoto et al. 2008). There is also a point at which high temperature affects the stability of DNA (Kültz 2005), RNA species and the cytoskeleton (Mittler et al. 2012).

1.2.3 What do plants do during stress? Elicit the stress response

Plants, as sessile organisms must endure heat stress events because they cannot moderate behaviour to maintain homeostasis, but instead must alter their cellular processes to safeguard photosynthesis; this is known as the stress response (Figure 1.1a). At first, heat-induced signalling results in the expression of genes coding for proteins with diverse functions in the heat stress response (Lindquist 1986, Al-Whaibi 2011), replacing normal cellular processes (Bijlsma and Loeschcke 2005, Biamonti and Caceres 2009, Bita and Gerats 2013). There is also the induction of a number of solutes and sugars to maintain osmotic pressure, increase protein stability and stabilise the membrane (Sung et al. 2003). Antioxidants are synthesised to scavenge damaging ROS (Mittler 2002, Wang et al. 2003b). Secondary metabolites are produced; for example, isoprene, which is suggested to have roles as ROS scavengers or in stabilising membranes (Velikova and Loreto 2005, Velikova et al. 2011), but is more likely protective during heat flecks (Sharkey et al. 2008). These aspects of stress response are touched on here only briefly (proteins involved in these responses are discussed in more detail in Chapter 4) as my focus of the stress response is HSPs.

1.2.4 The many roles of HSP

1.2.4.1 HSPs in the stress response

Initially identified in *Drosophila* in response to heat, HSPs have since been detected in all domains of life (Laksanalamai and Robb 2004), under normal cellular functioning with roles as chaperones (Boston et al. 1996) and in response to any type of stress that denatures or unfolds proteins (Feder and Hofmann 1999, Barua and Heckathorn 2004). During heat stress, constitively expressed HSPs, e.g. Hsp70s and Hsp90s, are upregulated (Tomanek and Somero 1999, Preczewski et al. 2000). While small HSPs (sHSPs) , not usually detected under non-stressful conditions, are upregulated later, or at high temperatures (Howarth and Ougham 1993). As chaperones, sHSPs prevent protein aggregation (Laksanalamai and Robb 2004), holding denatured proteins for ATP-dependent refolding (Żwirowski et al. 2017). Plants have a particularly high diversity of HSPs (Vierling 1991), especially sHSPs (Eisenhardt 2013) , likely due to their somewhat limited ability to moderate leaf temperature (especially under water stress). sHSPs localised in the chloroplast (chl-sHSPs) are known to stabilise the

thylakoid membranes (Downs et al. 1999a, Allakhverdiev et al. 2008, Bernfur et al. 2017) and protect protein components of PSII (Heckathorn et al. 1998, Chen et al. 2017). During the stress response, upregulation of HSPs is rapid, occurring within minutes (10-60 min) (Havaux 1993b, Knight and Ackerly 2003b, Zhang et al. 2014a), peaking between 60 and 90 minutes (Al-Niemi and Stout 2002) and continuing for 180 minutes or even up to 72 hours after heat stress (Charng et al. 2006). These proteins therefore act in cellular protection and repair as well as in acclimation.

1.2.4.2 HSPs and basal thermal tolerance

There is a well-established positive relationship between plant thermal tolerance and HSP levels (Vierling 1991, Downs et al. 1999a, Preczewski et al. 2000, Knight and Ackerly 2001, Knight and Ackerly 2003a, Bita and Gerats 2013). Although HSPs are highly conserved, the timing of production and amount produced is species-dependent (Feder and Hofmann 1999); however, clear patterns of association are yet to be determined. Plants from warmer environments are generally thought to express HSPs at higher temperatures than those from cooler environments (Shakeel et al. 2012). But there are conflicting results in plants and animals as to whether organisms with higher thermal tolerance express higher (Krebs and Feder 1997, Knight and Ackerly 2001, Dong et al. 2010, Shakeel et al. 2012) or lower levels of HSPs (Sørensen et al. 2005, Reusch and Wood 2007) than organisms with lower thermal tolerance. These opposing outcomes raise various questions. If organisms with high tolerance express low HSPs, what other mechanisms are used to tolerate heat stress? If organisms with high thermal tolerance produce large amounts of HSPs, what are the costs associated with this? With the cost of overexpression of HSPs to consider (discussed in 1.3) will organisms from stressful environments produce more or less HSPs? Most HSP studies are conducted under controlled conditions over short time periods (Preczewski et al. 2000, Baniwal et al. 2004, Wang et al. 2014a). The limited work under field conditions tends to be based on a small number of economically important crop varieties or plants from fairly benign climates (Barua and Heckathorn 2006). Few studies have considered plants from extreme climates, living at or close to thermal limits (Al-Niemi and Stout 2002, Stout and Al-Niemi 2002, Knight and Ackerly 2003b). Work on desert plants under natural conditions provides the opportunity to examine plants with inherently high basal thermal tolerance and how protein expression contributes to this.

1.2.5 Recovery following heat stress

After heat stress, there is a return to normal function, at which time repair can commence, with the time taken dependent upon the intensity of the heat stress (Howarth and Ougham 1993). Following moderate stress, net photosynthesis can return to similar pre-stress rates

(Haldimann and Feller 2004, Ameye et al. 2012), suggesting that not all damage to photosynthetic mechanisms is permanent. For example, the drop in photosynthetic rate is mainly explained by a reversible reduction in Rubisco activation (Haldimann and Feller 2004, Sharkey 2005). Also, photosynthetic activity can be sustained after heat stress if protein damage and repair are balanced (Takahashi et al. 2004, Allakhverdiev et al. 2008), such that there is a strong correlation between damage and recovery. Plants that are able to maintain a higher maximum quantum yield (measure of PSII health) also show a greater ability to recover photosynthetic efficiency (Krause et al. 2010, Curtis et al. 2014, Halter et al. 2016). On the other hand, if repair processes themselves are susceptible to elevated temperatures, recovery may be hampered (Takahashi et al. 2004, Takahashi and Murata 2008). Therefore, it has been suggested that, rather than the point of heat stress, it is the recovery stage – when conditions and physiological function return to pre-stress levels – that defines plant thermal tolerance (Sharkey 2005).

1.2.6 After recovery comes acquired thermal tolerance

Acquired thermal tolerance is a phenotypic response to change within an organism's lifespan and is important for improving functioning under new thermal conditions (Figure 1.1b). To acquire higher thermal tolerance, priming is required, whereby the stress response is elicited under sub-lethal stress temperatures and recovery occurs. Priming causes change to physiological processes because repair of heat-sensitive components is required for prevention of further heat injury and survival at normally lethal temperatures (Nievola et al. 2017). For example, some desert species that have been exposed to increasingly warmer conditions during spring can withstand summer temperatures up to 50°C with minimal photoinhibition and almost complete recovery (Curtis et al. 2014). Acquired thermal tolerance acts on different timescales, yet there is scant research on temporal changes in tolerance (but see Zhang et al. 2007, Aspinwall et al. 2017, Zhu et al. 2018). Elucidating the temporal response of thermal tolerance is critical to understanding how plants will be able to cope under a changing climate, where increased long-term average temperatures are overlaid with the short-term impacts of more frequent, extreme and unseasonal heatwaves.

In short-term acquired thermal tolerance, for example over the course of the diurnal cycle, cellular machinery is not altered, only the rates of processes (Berry and Bjorkman 1980, Atkin et al. 2006). Short-term adjustment involves the production of osmolytes to maintain protein stability other products to maintain faster metabolic rates (Hüve et al. 2006). Short-term acquired thermal tolerance is often linked to the expression of HSPs for protection and recovery after short-term stresses, such as those occurring during a heatwave. The acquired thermal tolerance returns to pre-heat tolerance within a few hours (Bahrndorff et al. 2009) to

days, shown to be associated with decay of HSPs (Charng et al. 2006, Charng et al. 2007, Lin et al. 2014). Over longer timeframes, such as days or months across seasons, acquired thermal tolerance raises temperature optima (Slatyer and Ferrar 1977, Cunningham and Read 2003) or thermal damage thresholds (Zhu et al. 2018, Curtis et al. In review), requiring relatively greater alteration of cellular machinery. As with basal thermal tolerance, the ability to acquire thermal tolerance, also known as plasticity, may be related to where plants live (Janzen 1967), but the evidence is not so straightforward. Plasticity in species from thermally stable regions, for example, in tropical plants has been found to be both limited (for photosynthesis Doughty and Goulden 2008, Drake et al. 2015) and plastic (for respiration Cheesman and Winter 2013, Slot and Kitajima 2015, Tan et al. 2017). While temperate deserts are highly seasonal, the fact that desert plants may already be at thermal limits raises the question as to whether their ability for acquired thermal tolerance is limited.

1.2.6.1 Changes required for long-term acquired thermal tolerance

Plants have two main options for improved thermal tolerance with season. They can produce new leaves with physiology to better suit conditions (Mulkey et al. 1992), most likely in nutrient-rich environments. Or remodel existing leaves, by altering structural and metabolic characteristics through major changes in transcriptome, proteome and metabolome (Bijlsma and Loeschcke 2005, Ahuja et al. 2010, Eisenhardt 2013). In the latter case, enhanced thermal tolerance changes the amount and type of enzymes (Al-Whaibi 2011) and substrates, as well as rearranging organelles (Davison et al. 1991, Stylinski et al. 2002). The expression of heat-stable Rubisco activase is one example (Atwell et al. 2014, Scafaro et al. 2016). Another is the reduction of cellular respiration to alleviate imbalances between carbon uptake and use (Atkin and Tjoelker 2003, Atkin et al. 2005b, Atkin et al. 2006). Membrane fluidity is maintained during high temperature by changes in composition of the phospholipid bilayer and the fatty acid saturation levels (Berry and Raison 1981, Murakami et al. 2000, Burgos et al. 2011, Miller and Stillman 2012, Zhu et al. 2018). Reorganisation of the thylakoid membrane takes days or more (Berry and Bjorkman 1980), whereas at longer timescales, both remodelling and degree of saturation are altered (Narayanan et al. 2015). Plants can swap phospholipids for other lipid types; galactolipids are such an inclusion for thermal tolerance and also convey the advantage of reallocating phosphate to other physiological processes (Härtel et al. 2000, Lambers et al. 2012). This mechanism is of particular relevance for plants in low phosphorous soils, such as in Australia, where they often occur in extreme high temperature environments (Lambers et al. 2012).

1.3 Cost of thermal tolerance

Thermal tolerance changes throughout the year and it is not only how this change occurs physiologically and morphologically that is of interest, but also why this change should occur at all. If plants are likely to experience high temperatures in their native environment, why are high thresholds not maintained at all times in preparation for such an occurrence? Organisms acclimate to have improved performance under new conditions (Huey et al. 1999, Wilson and Franklin 2002, Bozinovic et al. 2011), but adapting to new thermal regimes is costly. Acquired thermal tolerance in plants may result in negative net photosynthesis (Berry and Bjorkman 1980), carbon loss at higher temperatures (Barron-Gafford et al. 2013) or increased survival at high temperature with reduced carbon assimilation at low temperature (Bauweraerts et al. 2014a) (Figure 1.1b). Changes to saturation of membranes requires energy (Zheng et al. 2011) and synthesis and degradation of HSPs hijacks substrates, energy and transcription pathways (Feder and Hofmann 1999). Overexpression of HSPs conveys high thermal tolerance in the short term (Zhang et al. 2014b), but comes with the cost of lowered growth, development and survival (in animals; Krebs and Feder 1997, Bahrndorff et al. 2009). Increased temperature of initiation of HSP expression can occur with acquired thermal tolerance (Stillman and Somero 2000, Barua and Heckathorn 2004, Bozinovic et al. 2011), suggesting that enhanced thermal tolerance of the entire cell is a better strategy than inducing HSPs. In many studies, when cost is mentioned, it is used as a theoretical explanation for changes to physiological traits. Few studies follow organisms through to reproduction to measure the cost in terms of fitness (but see Krebs and Loeschcke 1994, re Drosophila). Examining trade-offs is a way to assess the cost of acquired thermal tolerance as there is a gain in one trait at the expense of another. Particularly in low nutrient environments, work on heat stress and reproductive fitness of plants provides the opportunity to examine the trade-offs associated with HSP expression.

1.4 The environment is becoming more stressful

1.4.1 Climate change and warming temperatures

Climate change has driven average global surface temperatures up by 0.85°C since 1880 (Hartmann et al. 2013) and will continue to warm by 2-4°C this century (IPCC 2014). The impact of climate change is already apparent. Phenological timing (Root et al. 2003) and shifts in species distributions (Walther et al. 2002, Kelly and Goulden 2008, Beever et al. 2015) have been recorded but what will happen next is more difficult to predict. Some species are expected to do better, some worse, depending on their current habitat conditions. Certain cold-adapted species will likely do well due to removal of low temperature limitations and extended growing time (Gunderson et al. 2000, Penuelas et al. 2004, Jentsch et al. 2007, Hansen et al.

2012). Organisms adapted to warmer areas may not fare as well. Rising temperature jeopardises those on the edge of their thermal maxima (e.g. tropical species or desert species), with performance optima already close to contemporary mean temperatures and thresholds close to current extremes (Stillman 2003, Pörtner et al. 2006, Somero 2010, Somero 2011). For tropical plants, the close proximity of thresholds to habitat temperatures means they have a limited ability to increase thresholds through acclimatisation (Doughty and Goulden 2008, Drake et al. 2015). Many climate change studies apply predicted conditions to organisms of today to see how they will respond in the future (Jentsch et al. 2007), treating species with enhanced *versus* ambient CO_2 concentrations often with a temperature increase (Stirling et al. 1997, Ameye et al. 2012, Gauthier et al. 2014). Others model predicted species distribution based on current climate and landscape variables (Austin and Van Niel 2011) or physiological constraints (Kearney and Porter 2009). The current research considers a more immediate future, under current CO_2 concentrations but where more extreme events are already occurring.

1.4.2 Extreme events under climate change

It is important to understand species responses to long-term average changes, but there are a range of circumstances for which increased annual average temperature may not be as harmful as extreme events. Impacts of extreme events are disproportionate to their short duration (Jentsch et al. 2007) and key determinants of adaptation and speciation (Gaines and Denny 1993). Extreme events are damaging because organisms are adapted to conditions they regularly experience, and may not tolerate the extreme conditions that occur sporadically (Bijlsma and Loeschcke 2005). In biological terms, the severity of an extreme event relates to the stressor exceeding the acclamatory capacity of an organism (Gutschick and BassiriRad 2003).

1.4.2.1 Heatwaves

The extreme event of interest in this research is heatwaves. While the formal definition for a heatwave is five consecutive days at least 5°C higher than the norm (IPCC 2007), other definitions are expanded to include a measurable response in the study organism to a high temperature extreme (Reichstein et al. 2013, Bauweraerts et al. 2014b). Here I refer to heatwaves in the broader sense, as being one or more days where high temperatures at least 5°C higher than the norm occur for several hours. Climate change has altered the likelihood of experiencing extreme events (Hansen et al. 2012). Heatwaves have become and will continue to become more frequent, intense and the duration has increased (IPCC 2007, Cowan et al. 2014, Perkins-Kirkpatrick and Gibson 2017). Increased frequency of extreme events may see an organism recover from a single event but be affected by several events in

succession. Heatwaves are also more likely to occur out-of-season (Steffen et al. 2014) with severity of impact dependent on season (De Boeck et al. 2011). An out-of-season event may be more damaging than one at a time when an organism has experienced preparatory priming events, a proposition that has yet to be fully investigated.

A heatwave affects plant functioning differently than a steady, constant temperature increase of equal heat sum (Bauweraerts et al. 2014b). Sudden heat stress results in reduced photosynthesis and carbon assimilation (Ciais et al. 2005, Ameye et al. 2012, van Gorsel et al. 2016) and even death (Harris et al. 2018, Ruthrof et al. 2018, Hoffmann et al. 2019). Further, heatwaves are often coupled with low rainfall (De Boeck et al. 2010) and the interaction of extreme heat plus drought results in greater plant stress or death (Hamerlynck et al. 2000, Matusick et al. 2018). Like increased annual average temperatures, heatwaves have forced shifts in plant species distributions; for example, woody plant encroachment of savannahs and grasslands in the US (Volder et al. 2013) and declines in European montane species (Abeli et al. 2012, Abeli et al. 2014). The thermal sensitivity of flower production has led to the proposal that extreme weather events could change plant reproductive strategies, e.g. alteration of the ratio between sexual and asexual reproduction (Abeli et al. 2012). In the long term, will reduction in genetic diversity halt the ability of plants to adapt to climate change?

My research is focused on Australia, where heatwaves have increased in frequency (Hartmann et al. 2013), are more intense and are occurring earlier (Steffen et al. 2014); for example, November 2012 was record breaking for maximum temperatures so early in spring (BoM 2012). In light of the predicted expansion of deserts in Australia with models predicting increased dryness (Hartmann et al. 2013) and global drought index over Australia (CMIP5 model, Lau and Kim 2015), the impact of heatwaves in these conditions requires urgent attention. While some coastal Australian species have been identified as resilient to repeated heatwaves (French et al. 2019), there is the danger that others will be outcompeted by stress-tolerant introduced species (French et al. 2017). Continued research into the impact of heatwaves on native species is required as extreme environments expand, in Australia and globally.

1.5 A challenging environment

1.5.1 Australian semi-arid and arid zone is grand (on two levels: large and impressive)

Globally, ~41% of the world's land is desert: arid, semi-arid or sub-humid (Safriel et al. 2005, Reynolds et al. 2007). In Australia, the percentage of arid and semi-arid land is far greater, with ~70% of the land mass being arid or semi-arid. The Australian outback is recognised across the world for its outstanding beauty and unique flora and fauna. In spite of supporting a strong rangeland grazing industry (Wilson 1994, Huang et al. 2016), it is one of the most

intact areas of desert remaining on Earth (Sanderson et al. 2002), supporting a diversity of life in often hostile conditions. Flora in Australian deserts provides food and habitat for native fauna (Bennison et al. 2013), is able to draw-down carbon (Cleverly et al. 2013, Poulter et al. 2014, van Gorsel et al. 2016) and holds the potential genes for greater thermal tolerance of crop species as many cultivated crops have wild Australian relatives (Jarvis et al. 2008, Scafaro et al. 2012, Atwell et al. 2014). We currently have little understanding of how Australian desert flora respond to high temperature extremes. While some researchers suggest that desert plants will do well under elevated CO_2 due to increased water use efficiency (Hamerlynck et al. 2000), others assert that, living near their thermal optima, desert plants will do poorly (O'Sullivan et al. 2013). Research to determine how desert flora will cope with extreme high temperature is urgently required.

1.5.2 Difficulties for plants in the desert

Deserts are harsh environments in which temperature is high (often in excess of 45°C), rainfall is low (arid; <250 mm $year^{-1}$ and semi-arid; 250-500 mm $year^{-1}$) and light levels are high. Temperatures in the southern Australian arid zone have a large range (e.g. 4.7 to 34.3°C mean winter min and summer max in Port Augusta, South Australia (BoM 2016), with heatwaves exceeding 48°C (National Climate Centre 2009). With access to water, desert plants can tolerate high air temperatures by maintaining leaf temperatures below ambient air temperature through transpirational cooling and may decouple photosynthesis from stomatal conductance to do so, as identified in other ecosystems (Krause et al. 2010, Drake et al. 2018). However, the combination of high air temperature and water limitation restricts transpirational cooling. It is only when wind drops (Roden and Pearcy 1993) or transpiration is limited (Leigh et al. 2012) that leaf temperatures can rapidly exceed air temperature by 10°C (Vogel 2005) and stress thresholds may be crossed, even if for only minutes at a time.

Another stressor in desert systems is the lack of access to soil nutrients. In Australia, the age and isolation of the continent means that desert soils are extremely nutrient poor, especially in phosphorous (Stafford Smith and Morton 1990) and nitrogen, compared with other arid zones. Also, access to nutrients is restricted by water limitation, with the irregularity of the rainfall a more important stressor than the absolute amount (Orians and Milewski 2007). Nutrients are patchy in the landscape (Facelli and Brock 2000, Eldridge and Koen 2008, James et al. 2009) and water is in gradients perpendicular to creek lines, flood plains or dunes (Grigg et al. 2008). The desert flora are highly diverse due to functional diversity of nutritional strategies (Lambers et al. 2010) and predominantly evergreen to retain nutrients within long-lived leaves (Orians and Milewski 2007). Nutrient limitation, particularly N and P, necessitates careful resource partitioning by plants into cellular maintenance, growth and reproduction (see

1.3 for costs). The long-term growth and fitness costs following stress, when limited resources have been dedicated to survival and recovery are not well understood in general, with little known for Australian desert systems.

1.6 Research objectives

My research is concerned with species persistence in a changing environment, such that species composition will result from differences in the ability of species to cope with high temperature, particularly extreme high temperature. The overarching objective of the research described in my thesis is to provide insight into the mechanisms of thermal tolerance and identify the potential costs or trade-offs that species in hot, dry and low nutrient environments must employ in order to survive. To do so I addressed the following eight questions:

1. How do temperature thresholds of PSII efficiency and membrane stability relate to one another and to HSP expression?
2. What differences do we see in thresholds, protection and recovery in relation the seasons?
3. Are temperature thresholds and protein expression species-specific?

I address these questions in Chapter 2, where I used two Australian *Solanum* species native to differing microhabitats grown in a common garden. The temperature thresholds of membranes and PSII and the temperature of peak HSP expression were determined following a short (15 min) temperature stress. Sampling in winter and summer allowed for me to assess acclimation of thermal tolerances.

4. Is extreme heat stress in spring or summer more detrimental to plant fitness?
5. How does HSP expression differ after exposure to heat stress events at different times of the year?
6. How does nutrient availability influence downstream effects of heat stress?

In light of the increased likelihood of heatwaves occurring out-of-season due to climate change, in Chapter 3, I examined the cost incurred due to a heat stress imposed in spring (in the absence of significant temperature priming) compared to a heat stress in summer (after spring and early summer priming). I used the same two *Solanum* species as in Chapter 2 to address the above questions. In this instance, I grew potted plants under two nutrient regimes in ambient desert conditions. I exposed them to a long (3 h) heat stress and assessed short-term and long-term effects of timing and nutrient access. Importantly, I measured reproductive outcomes to assess the true fitness cost of heat stress.

7. Are there proteins that can be linked to physiological thresholds changes?
8. Did proteins of interest follow predicted expression paths with warming of the seasons?

In Chapter 4, I addressed questions seven and eight regarding seasonal change in protein expression and threshold temperatures by expanding the number of species, seasons (winter, spring and summer) and proteins examined. I used three desert species representing different functional types and from different families to increase coverage of proteins. With the main aim of investigating proteins responsible for seasonal change in thresholds, I *a priori* chose functional groups of proteins involved in photosynthesis, lipids and stress response.

CHAPTER 2: CAPACITY TO TOLERATE, ACCLIMATE AND RECUPERATE

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2.0 Abstract

An understanding of the upper limits of plant thermal tolerance will help elicidate which species may tolerate thermal stress under climate change. A plant's inherent ability to tolerate high temperatures often is driven by its climate of origin. With many physiological processes in leaves having their own temperature limits that affect the overall tolerance and recovery of the plant. How thermal limits of membranes and photosystem II (PSII) and the upregulation of protective heat shock proteins (HSPs) relate and change with warming of the seasons is not well understood, especially in wild species, particularly in arid systems. Two Australian arid *Solanum* species, *S. oligacanthum* and *S. orbiculatum*, were grown under ambient desert conditions and sampled in winter and summer to assess their seasonal plasticity. Seasonal temperature thresholds of membranes—via electrolyte leakage—and PSII function and recovery—using chlorophyll fluorescence parameters F_0' and F_v'/F_m'—were determined using Bayesian modelling. The temperature onset and peak of Hsp70 and a chloroplastic small HSP (sHSP) were also measured. I found distinct patterns in how thermal tolerance was acquired under heat stress. *Solanum oligacanthum* possessed high PSII thermal tolerance and *S. orbiculatum* high membrane thresholds. Both species acquired higher tolerances in summer and the temperature at which recovery was possible increased from winter to summer. The detection of sHSP only in summer suggests its importance in acquired thermal tolerance. That these species tolerate high temperatures in different ways highlights that there are a number of solutions for acclimation and persistence in harsh environments.

2.1 Introduction

As high temperature extremes become the new normal, uncovering how plants tolerate heat stress is critical for understanding species persistence in a warming world (for example, see Anderson 2016). Deliterous effects of stressfully high temperatures can include reduced photosynthesis (Teskey et al. 2015), biomass accumulation (Ameye et al. 2012) and even death (Harris et al. 2018). Identifying high temperature thresholds of plants, how plastic these thresholds are and exploring the mechanisms which have a role in thermal tolerance is crucial for estimating species vulerability to temperature extremes.

Most plants have some capacity to cope with high temperatures. They have an inherent or basal thermal tolerance due to long-term adaptation to their climate of origin. However, high temperature tolerance is not uniform across the globe, such that, species from warmer climates have greater ability to withstand high temperatures than their cooler climate counterparts (Cunningham and Read 2002, Knight and Ackerly 2002, Amano et al. 2012, Yamori et al. 2014) (Figure 2.1a). Even within a broad climatic zone species can differ based on their microhabitat preferences; for example, where wetter *versus* dryer habitats might

influence whether a given temperature is stressful (Curtis et al. 2016). Additionally, differences in plant traits reflect differential thermal tolerances amongst species. For example, species with high leaf mass per area (LMA) have been found to also have high thermal tolerance (Knight and Ackerly 2003a, Sastry and Barua 2017).

Further, thermal tolerance is not static through a plant's lifetime, as temperatures across much of the globe change with the seasons so too do thermal thresholds (Sastry and Barua 2017, Kumarathunge et al. 2019, Curtis et al. In review). Acquired thermal tolerance or acclimation is a physiologically induced multi-gene response (Halter et al. 2016) to recent climactic conditions. Metabolic and biochemical alterations occur and new thresholds are established to survive otherwise lethal stress (Sung et al. 2003). Changes include production of thermally stable proteins (Crafts-Brandner et al. 1997), saturation of membranes (Murakami et al. 2000, Feller 2016), accumulation of antioxidant enzymes and volatile organic compounds, and increases in osmolytes and soluble sugars (Hanson and Sharkey 2001, Sharkey 2005, Bita and Gerats 2013) (Figure 2.1c). Acclimation maintains a safe margin between stress thresholds and new habitat temperatures (O'Sullivan et al. 2017, Drake et al. 2018).

Acclimation, plasticity or potential—the amount by which species can adjust thresholds—differs among species and potentially ecosystems. Little is known about the extent to which acquired tolerance might buffer thermal vulnerability under climate change. As with basal thresholds, there are broad habitat differences in acclimation potential, whereby plants from thermally stable environments may be less able to shift than those from habitats with large seasonal differences (Cunningham and Read 2003, Atkin et al. 2006, Zhu et al. 2018) (Figure 2.1b). Additionally, plants from warm climates may have reached thermal limits and already be experiencing temperatures beyond their thermal thresholds (Doughty and Goulden 2008, O'Sullivan et al. 2017). Therefore, evidence suggests certain plant groups could be susceptible to increasing temperatures due to their climate of origin, for example tropical species (Krause et al. 2010, Sastry and Barua 2017, Sastry et al. 2018), but knowledge of species in other warm biomes is limited. Given that deserts are highly seasonal, one might expect plants in these environments to be able to acclimate by shifting their thresholds up substantially in summer. However, desert plants are at risk due to extreme temperatures potentially exceeding their thermal thresholds (O'Sullivan et al. 2017). Within such harsh ecosystems, the importance of refugia for species survival (Ashcroft et al. 2009, Graham et al. 2019) highlights the necessity of understanding species responses at fine scales.

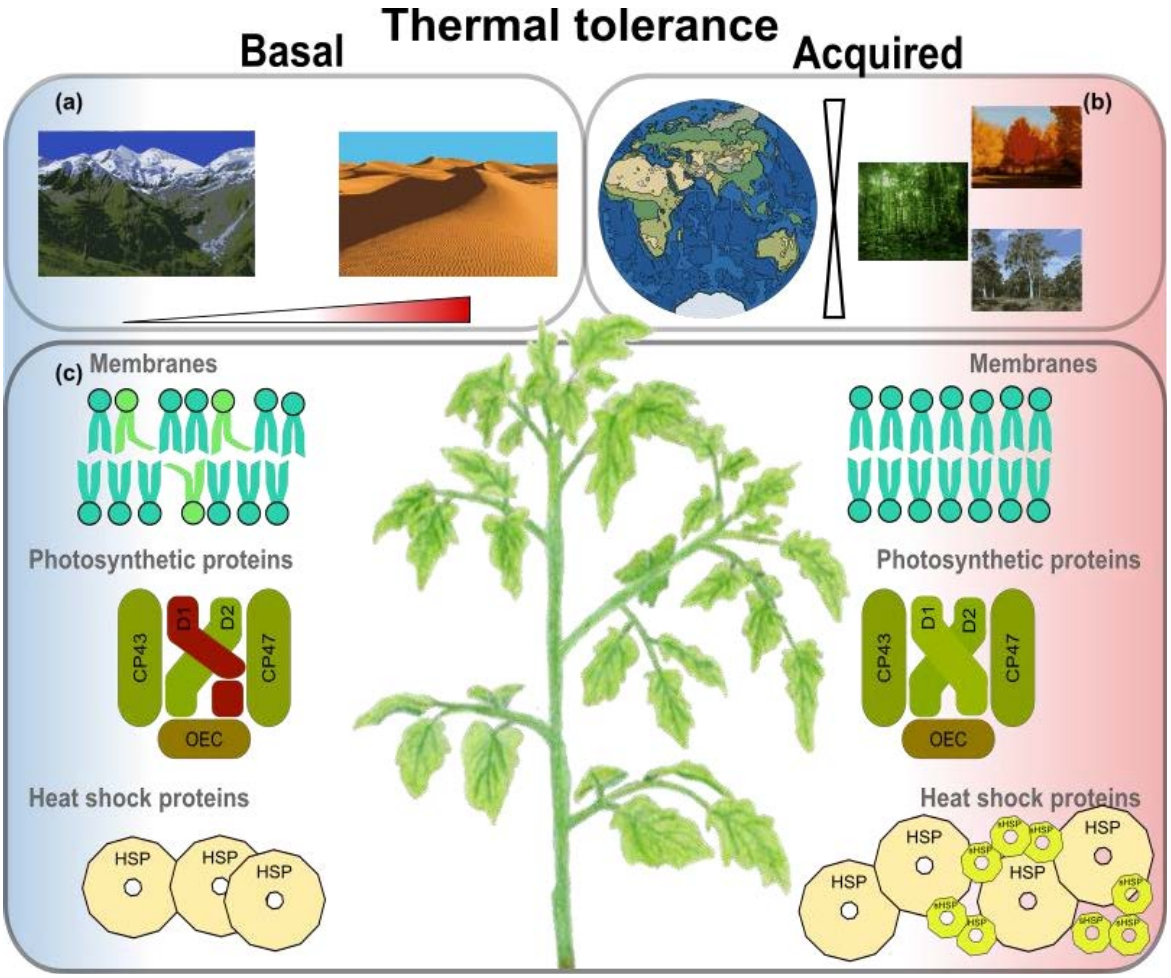

Figure 2.1. Habitat and physiological patterns of thermal tolerance in plants. Basal thermal tolerance (a) is a plants inherent ability to withstand temperatures and often relates to habitat of origin. Basal thresholds are generally higher in warmer than cooler habitats. Acquired thermal tolerance (b) requires physiological changes within the lifetime of a plant and the acclimation ability of plants to acquire higher thresholds may be greater at high latitudes. Physiological changes (c) to acquire thermal tolerance include more saturated than unsaturated fatty acids in membranes, thermally tolerant proteins (e.g., the D1 protein in Photosystem II) and high expression of heat shock proteins. Image of "Healthy tomato plant" by Davis & Mitra (2019), figshare, https://doi.org/10.6084/m9.figshare.8049962.v1

The overall thermal tolerance of a plant, at any given time, is comprised of temperature thresholds of various metabolic processes and protective mechanisms. Many studies base critical thermal thresholds on the health of the photosynthetic apparatus, particularly photosystem II (PSII) but we know that other systems, such as membranes are susceptible to heat. Heat stress impairs protein function within thylakoid membranes (Ilík et al. 2000, Krause et al. 2010, O'Sullivan et al. 2017, Ilík et al. 2018) and instability in the membranes themselves (Schreiber and Berry 1977, Havaux 1992). Early research suggests that membrane damage

occurs 10°C above the temperature for damage to PSII (Berry and Bjorkman 1980), but this has not been formally tested. In terms of acclimation potential of thresholds questions remain as to whether all thresholds have the same plasticity. Further, it is not clear how thresholds for membrane stability and PSII function relate to mechanisms that protect against and repair protein damage, such as upregulation HSPs.

The role of heat shock proteins (HSPs) in thermal tolerance is well established. However, comparative studies have provided contradictory results as to whether plants with higher basal thermal tolerance express more (Preczewski et al. 2000, Srikanthbabu et al. 2002) or fewer (Barua et al. 2008, Garavaglia et al. 2009, Aspinwall et al. 2019) HSPs than species with lower thermal tolerance. That HSP expression is not uniformly higher or lower in heat tolerant species suggests that these plants may utilise either HSPs or other mechanisms to withstand high temperatures. How short-term HSPs expression alters with the season and acquired thermal tolerance, particularly in wild plants, is not so well understood. There are four possible scenarios: 1) plants do not change their HSP expression; 2) plants primed for warm weather produce more HSPs because they are ready to do so, or have a stock of HSPs ready; 3) primed plants produce fewer HSPs as the process of priming has generated more thermally tolerant cellular machinery, enabling them to withstand higher temperatures; or 4) different kinds of HSPs are expressed after plants are primed.Generally, increased HSP expression is related to acquired thermal tolerance (Heckathorn et al. 1999, McLoughlin et al. 2016), but considering the potential cost of HSP production (Heckathorn et al. 1996a, b, Feder 1999), there should be a change in seasonal HSP expression to optimise resource allocation.

I investigated two *Solanum* species, adapted to differing microhabitats in Australian deserts. In winter and summer, I measured PSII health through chlorophyll fluoresence and membrane thresholds along with a cytosolic Hsp70 and a chloroplastic small HSP (chl-sHsp24) . My experiment was designed to address the following questions: 1) How do temperature thresholds of PSII efficiency and membrane stability relate to one another and to HSP expression? My expectation was that temperature thresholds of membranes would be greater than PSII thresholds, with the extent of the difference being ~10°C (Berry and Bjorkman 1980); 2) What differences do we see in thresholds, protection and recovery in relation the seasons? I predicted a change in all three parameters, with the simplest outcome being an increase in thresholds, protection and therefore recovery from winter to summer; 3) Are temperature thresholds and protein expression species-specific? Given previous work has identified that thermal tolerance is related to microhabitat and plant traits (Knight and Ackerly 2003a, Curtis et al. 2016), I expected to see species differences in thermal tolerance.

2.2 Methods

Site and growth conditions

This research was conducted *in situ* at the Australian Arid Lands Botanic Garden (AALBG), Port Augusta, which lies within the Australian southern arid region in South Australia (SA; 32° 27´42´´ S, 137° 44´36´´E). The desert location of the AALBG allows for the comparison of desert plant species grown under comparable conditions (mean annual rainfall 220 mm, mean maximum temperature 34.2°C; BoM 2018) and in naturally occurring sandy soil. This congeneric study compared two species of *Solanum* that occur in similar broad scale climatic conditions, but differ in their microhabitat preference; of either more favourable (e.g., high water and nutrient runoff) or harsher (hardpan) habitats. *Solanum oligacanthum* F. Muell is found in seasonally flooded watercourses across arid New South Wales, SA and Queensland (Figure 2a,b). It is a perennial herb or subshrub to 1 m with small, tomentose leaves (8-30 mm long) (PlantNET 2004). *Solanum orbiculatum* subsp. *orbiculatum* Dunal ex Poiret (herewith, *S. orbiculatum*) grows on desert dunes and sandy plains in central Western Australia, southern Northern Territory and western SA. It is a shrub to 1.5 m with orbicular leaves (30-60 mm long) covered with stellate hairs (eFloraSA 2013) (Figure 2a,c). Leaf samples for this study were taken from established, mature plants grown at the AALBG in non-light limiting conditions with supplemental irrigation sufficient to keep plants alive during severe drought. Samples of *S. oligacanthum* were collected from five individuals at a single location, while *S. orbiculatum* was sampled from five individuals at three locations within the AALBG.

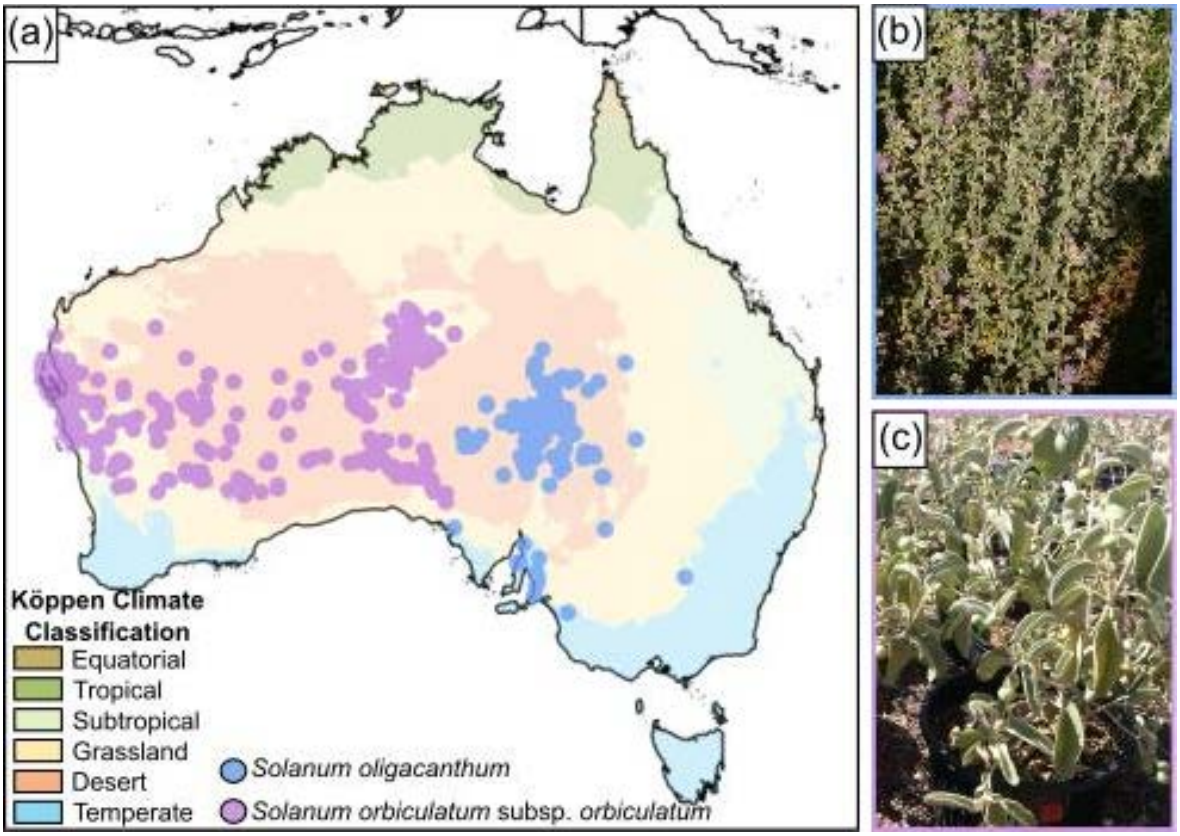

Figure 2.2. Arid Australian *Solanum* species and their distribution (a). *Solanum oligacanthum* (b) is restricted in distribution and grows in relatively wetter microhabitats while *Solanum orbiculatum* (c) has a broad distribution and grows in relatively drier microhabitats. Species distribution map was produced using the Atlas of Living Australia (ALA 2018).

Thermal tolerance assays for photosystem health and membranes

Sampling was conducted twice; first in the austral winter (June 2016) in the absence of heat stress and; second in summer (January 2017) after plants had acclimatised to warmer temperatures. Fully expanded leaves were harvested pre-dawn and used to measure PSII thermal tolerance, membrane stability assays and HSP expression (see below). Leaves were collected from at least five individuals per species, some of which may have been vegetative clones but were considered individuals for this study. For each species, all three assays were conducted on the same day and species were tested on consecutive days.

Photosynthetic thermal tolerance thresholds were measured using a chlorophyll fluorescence assay developed by Curtis et al. (2014). Leaves were kept in moist, dark conditions until ten leaves were randomly assigned to one of six treatment temperature baths (a 28°C control, plus 42, 44, 46, 48, 50°C in winter and 28, 44, 46, 48, 50, 52°C in summer). Maximum bath temperatures were set to ~4°C above expected threshold, to obtain a complete response

curve by which to ascertain thresholds. For each batch, the ten leaves were placed on moist paper towel and sealed into zip-lock plastic bags for dark adaptation prior to the start of the experiment. Dark-adapted leaves were removed from bags for measurement of chlorophyll *a* fluorescence using a MINI-PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany), then re-sealed and placed in the control bath for 15 min. Following the control bath, leaves were placed in their respective temperature treatment bath for 15 min, before re-placement in the control bath for a 90 min recovery. Leaves were then left to recover overnight. While in the baths, leaves were exposed to sub-saturating light (~280 µmol photons m^{-2} s^{-1}). Light-adapted chlorophyll *a* fluorescence was measured after 90 min recovery and dark-adapted following overnight recovery, in sealed, moist bags in the dark at room temperature.

Chlorophyll *a* fluorescence can identify whether light energy has been captured for carbon fixation or dissipated due to impairment along the photosynthetic pathway making it useful for temperature experiments. Three fluorometry parameters were used in this study: effective quantum yield of PSII (EQY; $F_v'/F_m'_{90}$) and minimal fluorescence ($F_0'_{90}$) 90 mins after heat treatment; and overnight values of F_v/F_m $_{ON}$. Minimal (F_0) and maximal fluorescence (F_m), are the levels of fluorescence when all PSII reaction centres are open or closed respectively (Baker 2008). These values are used to estimate F_v/F_m, a measure of PSII photochemical efficiency (Kitajima and Butler 1975, Lazár and Ilík 1997). A temperature related fall in F_v/F_m is related to increased photoinhibition through a decrease in F_m or rise in F_0 (French et al. 2019). During heat stress F_0 rises quickly indicating that PSII function has been impaired (Schreiber and Berry 1977, Havaux 1992). Light-adapted chlorophyll fluorescence provides an understanding of the operational efficiency of PSII following heat treatment (Murchie and Lawson 2013). To ascertain the extent to which quenching represented photoinhibition (short-term) or photoinactivation (permanent damage), recovery (R_{Fo}) was calculated. To find the temperature at which leaves are able to recover from heat stress, R_{Fo} was calculated using the ratio of starting F_0 values to overnight F_0 values.

The high temperature tolerance of cell membranes (membrane stability) was determined by measuring the electrolyte leakage from leaves after exposure to treatment temperatures following the methods of French et al. (2019), modified from Rollins et al. (1962). To test the suggestion that membrane thermal tolerance is 10°C greater than tolerance of PSII, temperature treatments for membrane stability were extended to 56°C. To compare membrane stability with photosynthetic thermal tolerance, leaves were exposed to temperature baths in the sequence described above. Leaves were placed in five replicate tubes of deionised water (in a ratio of approx. 0.5 g leaf weight: 15 ml water) for 5 min before an initial electrical conductivity measurement was taken (EC_0) using a conductivity sensor (TetraCon925; WTW, Weilheim, Germany). Electrical conductivity was measured again 90

mins after treatment (EC_{90}). Tubes were placed in an oven (100°C for 2 h) and maximum solute leakage (EC_{max}) was measured the following morning. A relative membrane stability index (MSI) was calculated using the equation:

$$MSI = 1 - \left(\frac{EC_{90} - EC_0}{EC_{max} - EC_0}\right)$$

where values close to one indicate healthy membranes and values closer to zero indicate greater damage.

Measurement of HSP expression levels in leaves

Levels of HSP were measured firstly to investigate the relationship between HSP expression and protection of photosynthetic proteins and/or membranes; and secondly to test for any species- and/or season-specific variations. Detached leaves, collected from plants over both winter and summer, had been naturally exposed to a wide range of temperatures. To capture the onset of HSP expression in response to temperature stress, which is suggested to begin approximately 10°C above growing temperature (Vierling 1991), treatment bath temperatures were extended to include several degrees below those used for photosynthetic and membrane tolerance assays, i.e., 36-52°C, in 2°C increments. Leaves from three replicate plants were placed in moist bags and heat-treated as described above. Leaves were left to recover for 2 h following treatment (to maximise HSP expression (Al-Niemi and Stout 2002)) before being snap-frozen in liquid nitrogen and stored at -80°C, until needed for analysis back in the laboratory.

Leaf protein extraction

Proteins were extracted from approximately 100 mg of fresh leaf weight. Frozen leaf samples were placed in 2 ml tubes (Snap-Cap Microcentrifuge Safe-Lock™ Tubes, Eppendorf™, Hamburg, Germany) with a 3 mm glass bead and ground using a tissue homogeniser (MM300, Retsch GmbH, Haan, Germany). Grinding of samples required repeated rounds of bead beating (7 x 1 min at 100 Hz) with snap-freezing in liquid nitrogen between rounds, in order to keep samples cold. Once a fine leaf powder was achieved, proteins were extracted using an SDS extraction buffer (100 mM Tris, 2.5% w/v SDS, 5 mM EDTA a with protease inhibitor cocktail (cOmplete™ ULTRA tablets; Merck, KGaA, Darmstadt, Germany)), modified from Knight (2010). Samples in extraction buffer were heated to 95°C for 5 min then left to stand for 1 h at room temperature. SDS has a high binding capacity for proteins and when heated to 95°C improves the solubilisation of membrane proteins (Molloy 2000) and inhibits protease activity (Jefferies et al. 2000). It is important that membrane proteins are solubilised as chl sHSPs are known to associate with thylakoid membranes (Glaczinski and Kloppstech 1988,

Bernfur et al. 2017). Samples were centrifuged at 20 000 g for 10 min and supernatant collected for analysis. Total protein yield, in mg, was quantified in triplicate using a BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) and an 8-point standard curve using BSA. Samples were stored at -80°C when not in use.

SDS-PAGE and Immunoblotting

Detail on antibody validation and optimisation is provided in the supplementary material (**Methods S2.1**). All products used for SDS-Page and immunoblotting were sourced from Bio-rad (Hercules, CA, USA) unless otherwise stated. All gels (10-well, Mini-PROTEAN TGX Stain-free gels 4-15%) were loaded with a molecular mass standard (Precision Plus Kaleidoscope Standard) and three amounts of the relevant positive control protein (30, 20, 10 µg). Gels were also loaded with three replicates of each of two temperature treatments. This meant a total of 20 gels were run (two species x two seasons x ten temperature treatments). All test samples and positive controls loaded on the gels contained equal amounts of total protein (30 µg) and were mixed 1:1 with 2x sample Laemmeli buffer, heated for 5 min at 95°C before being loaded onto gels. Gels were subjected to electrophoresis using standard techniques (Laemmli 1970), run at 100 V for 70 min.

Protein samples were transferred from gel to PVDF membranes at 90 V for 90 min using the wet western transfer technique (Towbin et al. 1979). Membranes were blocked with blocking buffer (5% skim milk in TBST) for 1 h at room temperature. The membrane was incubated with primary antibodies in blocking buffer overnight at 4°C (dilution of 1:2000 mouse anti-Hsp70 monocolonal antibody (N27F34; ENZO Life Sciences Inc., Farmingdale, USA) and 1:5000 rabbit anti-chl sHSP. The polyclonal antibody was raised in rabbits, against a synthetic peptide based on the amino acid sequence found in the consensus region III of a ~21 kDa chl sHsp published by Downs et al. (1998; see Methods S2.1 and Table S2)). Membranes were washed, then incubated in horseradish peroxidase conjugated secondary antibodies at a dilution of either 1:2000 for Hsp70 (anti-mouse IgG, 9044) or 1:5000 for sHSP (anti-rabbit IgG, A9169; both from Merck KGaA, Darmstadt, Germany). Visualisation of protein bands on western blots was done using chemiluminescence (Clarity™ Western ECL substrate) and an Amersham Imager 600 (GE Healthcare, Little Chalfont, UK). Membranes were probed for Hsp70 first and then stripped using a mild stripping buffer (1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% (v/v) Tween20, pH 2.2), blocked and probed for chl sHSP.

Post-antibody staining for total proteins on the membrane was done using Amido black stain. Membranes were first placed in Amido black stain solution (0.1% (w/v) naphthol blue black; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, 10% (v/v) methanol, 2% (v/v) acetic acid) for 3 min and then de-stained (50% (v/v) methanol, 7% acetic acid) for 15 min until bands were

clear. As with detection of HSPs, total proteins were imaged using CCD camera. Densitometry methods were conducted to semi-quantify HSP expression using ImageJ (National Institutes of Health; Bethesda, MD). Proteins of interest were normalised to total protein of each sample and positive controls were used to standardise immunoblots so blots could be compared (Degasperi et al. 2014).

Data Analysis

Statistical analyses were carried out in the R environment for statistical computing v.3.5.1 (R Core Team 2018). Starting values of F_v/F_m and F_0 (taken on all leaves before the experiment began) were compared to test whether fluorescence values were similar in different seasons. There were significant season * species interactions for both F_v/F_m and F_0 (ANOVA; F_v/F_m: $F_{1,236}$ = 82.90, p <0.001 and F_0: $F_{1,236}$ = 82.90, p < 0.001, Figure 2.3) so correction was required. Corrections were made relative to the control (leaves exposed to 28°C) for chlorophyll fluorescence parameters and MSI. The mean of control leaves and mean starting F_m, F_v and F_0 were used in calculations because different batches of leaves were used at each temperature and the randomised order of leaf measurement meant starting values could not be matched to a particular leaf.

To test for differences between species within a season and between seasons within a species, temperature thresholds of photosynthesis ($F_v'/F_m'_{90}$ and $F_0'_{90}$) and membranes (MSI) 90 min after temperature treatment and the temperature at which recovery (R_{Fo}) was possible were determined using Bayesian models (Ashcroft et al. 2016, French et al. 2019). Previously thresholds have been found from the inflection point of the T-F_0 curve (Schreiber and Berry 1977, Ilík et al. 2000, Knight and Ackerly 2002, Kouřil et al. 2004, Zhu et al. 2018) or 50% decline in F_v/F_m (Curtis et al. 2014, Drake et al. 2018, French et al. 2019). However, unlike chlorophyll fluorescence responses, the high temperature asymptotes of MSI curves were not reached (see Figure S2.2c,d). To make comparisons among threshold temperatures, thresholds were found based on a 12% decline (or 12% rise for $F'_{0\,90}$) in health (from the low temperature asymptote; see Figure 2.4). A 12% threshold was selected as this point is close to the inflection point of the T-F_0 curve which marks the onset of photoinhibition (Schreiber and Berry 1977, Havaux 1992). Generalised logistic functions were fitted (using R2OpenBugs package for R) to temperature- $F_v'/F_m'_{90}$, -$F_0'_{90}$ -MSI and -R_{Fo} curves. Bayesian models iteratively and stochastically fit response curves and the 95% credible intervals (equivalent to confidence intervals) that best explain the data (Ashcroft et al. 2016). Rather than produce a p-value, for each of 20 000 iterations, thresholds of the two response curves were compared, if the threshold of a curve was higher in 95% of the iterations the result was substantive (equivalent to α = 0.05)(Ashcroft et al. 2016).

Two-way ANOVA, was used to compare the LMA of leaves by species and season. The leaf area (measured using ImageJ) and dry mass of leaves was used to calculate LMA (g m^{-2}) on a subset of 20 leaves from temperature assays.

To explore whether the potential to recover (R_{Fo}) was correlated with other chlorophyll fluorescence parameters ($F_v´/F_m´_{90}$ and $F_0´_{90}$, $F_v/F_{m\ ON}$), membrane stability (MSI) or HSP expression (Hsp70 and chl-sHsp24), Spearman´s rank coefficients and p-values were produced in Hmisc package (Harrell 2019) and visualised using Corrplot package (Wei and Simko 2017). Means of parameters at each temperature were used in correlations.

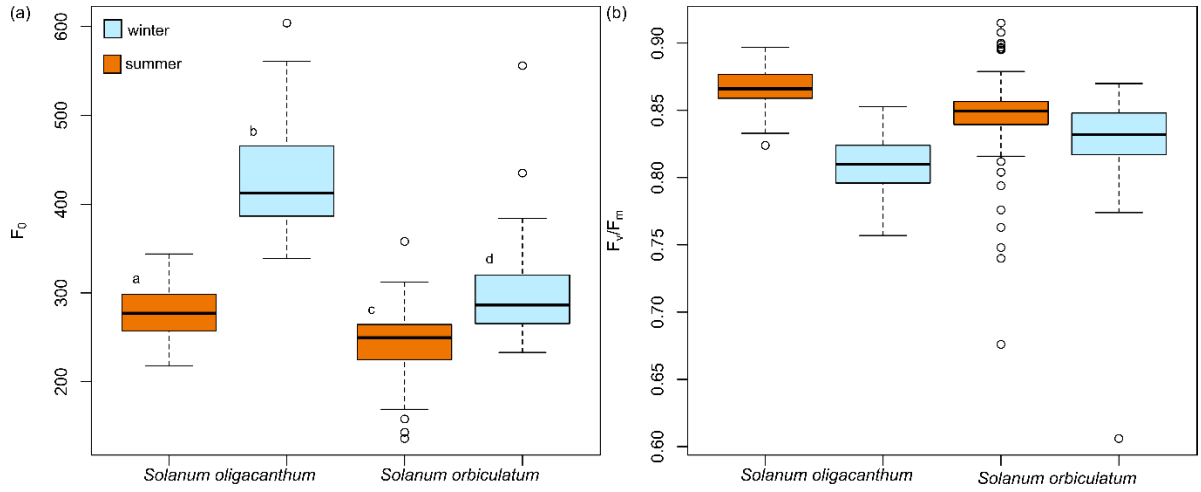

Figure 2.3. Comparison of starting values of F_0 (a) and F_v/F_m (b) of two desert Solanums in winter (blue) and summer (orange). There was significant species by season interaction for both F_v/F_m and F_0 (ANOVA; F_v/F_m: $F_{1,236} = 82.90$, $p < 0.001$ and F_0: $F_{1,236} = 82.90$, $p < 0.001$). Different letters signify significantly different means among groups ($p < 0.05$). Boxplots show mean and interquartiles with whiskers extending to 2.5% and 97.5% confidence intervals.

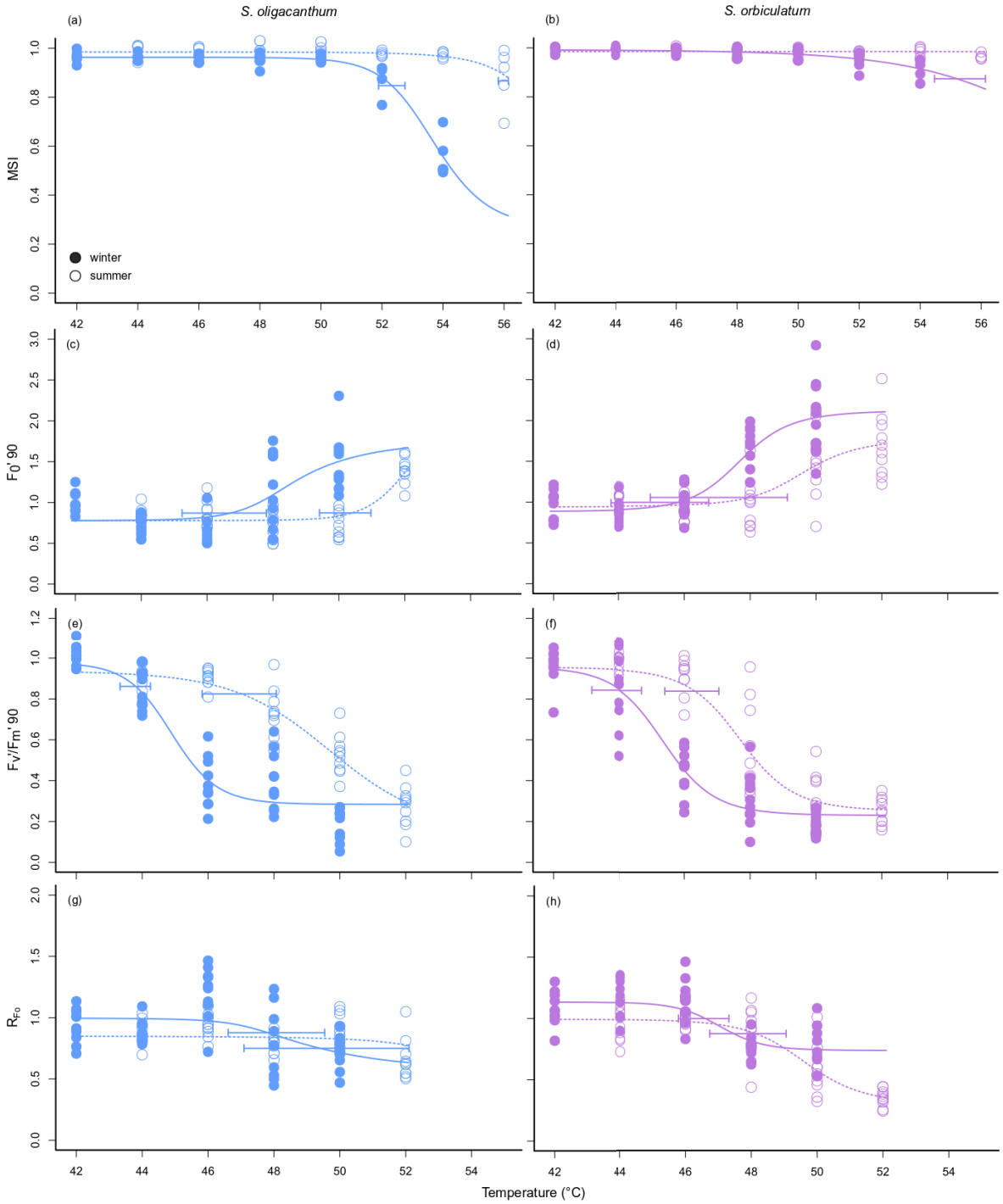

Figure 2.4. Seasonal differences in temperature decay curves of membrane stability (a,b), minimal fluorescence (F_0') (c,d), effective quantum yield (F_v'/F_m') (e,f) and recovery of F_0 (g,h) of two species of Australian *Solanums.* Values are relative to control. Thresholds (88% (12% for F_0') of low temperature asymptote) with 95% CI error bars were found using generalised logistic curves using Bayesian Monte Carlo iterative modelling for *Solanum oligacanthum* (blue) and *S. orbiculatum* (violet) in winter (full circles, solid line) and summer (empty circles, dashed line).

2.3 Results

The way in which thermal thresholds of MSI, PSII efficiency and recovery relate to one another was similar for each species and across seasons. The ranking of thresholds for the two Solanums was as follows: the temperature threshold of MSI was highest followed by $F_{0}'_{90}$ and $F_v'/F_m'_{90}$ (Figure 2.5; Table S2.1). The temperature at which recovery (R_{Fo}) was possible lay between thresholds of MSI and $F'_{0\,90}$. Overall, the difference between membrane stability and the lowest threshold of PSII health ($F_v'/F_m'_{90}$) was ~10°C; however, differences were smaller in *S. oligacanthum* (8.4 and 9.2°C in winter and summer) than *S. orbiculatum* (11.2 and 10.0°C in winter and summer).

Seasonal comparisons of thermal tolerances showed both species were able to upwardly adjust all temperature thresholds from winter to summer. All *S. oligacanthum* thermal thresholds were significantly higher in summer than winter (Figure 2.5; Table S2.1), while *S. orbiculatum* significantly shifted its thresholds of photosynthesis but not membrane stability. Species differed in their acclimation potential: *S. oligacanthum* thresholds were 3-4°C higher, whereas *S. orbiculatum* thresholds were 1-2°C higher in summer than winter.

When comparing thermal tolerance between the species within a season, generally *S. oligacanthum* had higher thresholds than *S. orbiculatum*, with the exception of MSI in both seasons, and $F_v'/F_m'_{90}$ in summer (Figure 2.5; Table S2.1). That said, there were only two instances in which thresholds were significantly different between species: *S. orbiculatum* had a significantly higher MSI threshold in winter and *S. oligacanthum* had significantly higher $F_{0}'_{90}$ threshold in summer.

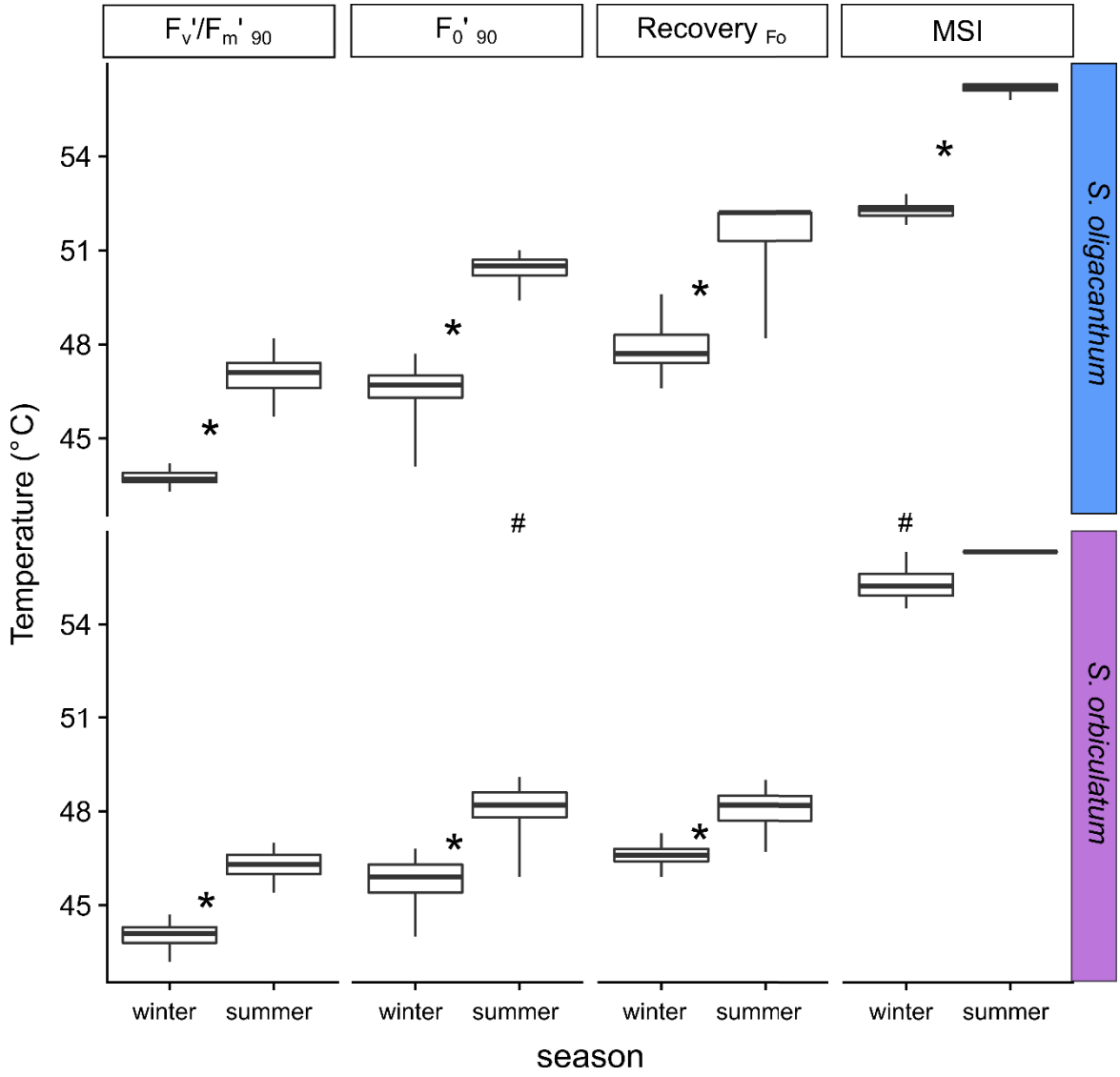

Figure 2.5. Upward adjustment in temperature thresholds of two Australian desert *Solanum* species from winter to spring. Thresholds of $F_v'/F_m'_{90}$, $F_0'_{90}$, Recovery of F_0 and membranes (MSI) are given for *S. oligacanthum* (top) and *S. orbiculatum* (bottom). For comparisons between seasons (within species), thresholds that were significantly higher in summer than winter are denoted with *. For comparisons between species (within seasons), significantly different thresholds are denoted with #. Thresholds were found using Bayesian modelling and differences were considered significant when curves (Figure 2.4) were different ≥95% of 20 0000 iterations. Boxplots are described in Figure 2.3.

Seasonal and species differences were apparent in the amounts of HSP produced and the temperature of peak HSP expression. Hsp70 expression was generally greater in *S. orbiculatum* than *S. oligacanthum* in both seasons (Figure 2.6a). For *S. oligacanthum*, Hsp70 expression peaked at 42°C in both seasons, but there was a greater amount of Hsp70 upregulated in summer than winter as seen in the immunoblot (Figure 2.6b). In contrast,

S. orbiculatum upwardly shifted the temperature at which Hsp70 expression peaked from winter to summer (from 40 to 44°C) and the relative amount of Hsp70 at the peak was higher in winter than summer (Figure 2.6a). Despite species differences in Hsp70 expression, in both species the temperature at which peak expression of Hsp70 occurred was lower than the temperature marking onset of damage to PSII (threshold of $F_v'/F_{m\ 90}$)(Figure 2.6a).

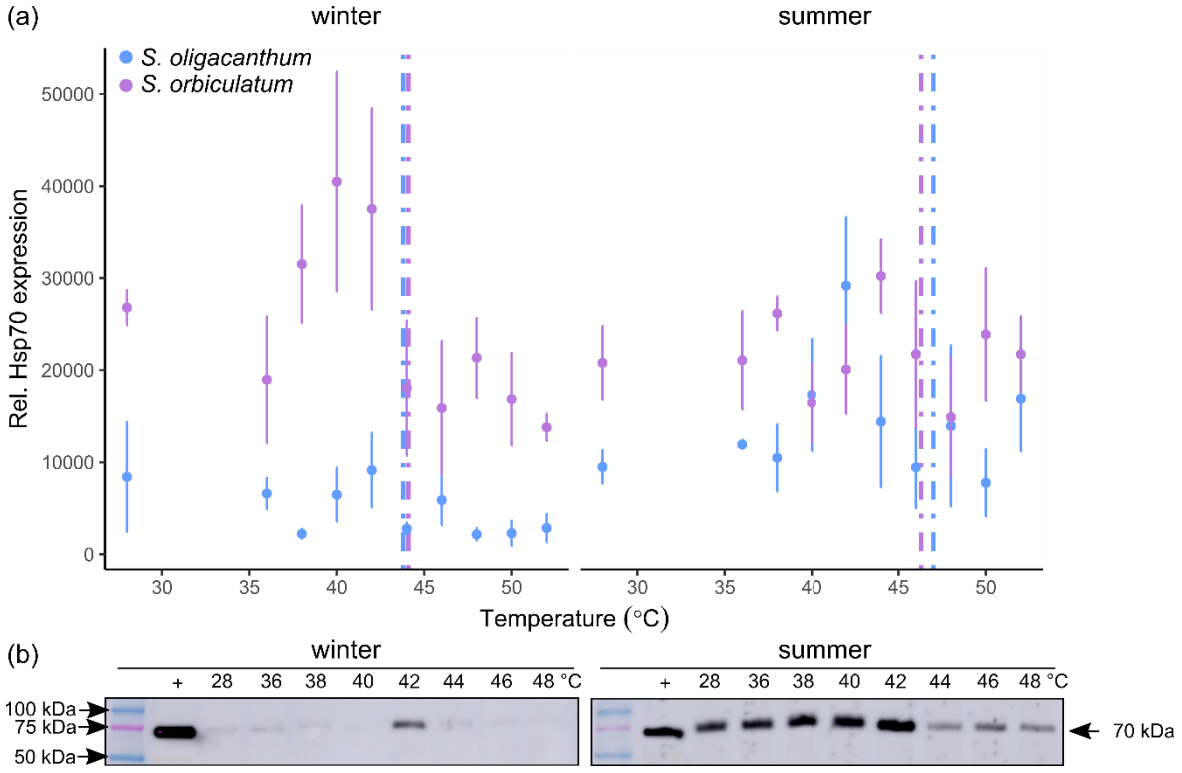

Figure 2.6. Seasonal relative expression of Hsp70 (Hsp70 relative to total protein; mean ± SE) from leaves of *Solanum oligacanthum* (blue) and *S. orbiculatum* (purple) following exposure to treatment temperature for 15 min plus 2 h recovery (a). Species were sampled in winter and summer. Dashed vertical lines show the temperature thresholds of $F_v'/F_{m'\ 90}$. Example immunoblots of Hsp70 expression in *S. oligacanthum* from winter and summer samples (b).

In contrast to Hsp70, the seasonal differences in chl-sHsp24 expression were more prounounced, but the species differences in amounts of chl-sHsp24 produced were less obvious. For both species, there was no chl-sHsp24 detected in winter, with high expression in summer (Figure 2.7a). In winter, there was a protein detected greater than 25 kDa but its size and constant expression with temperature suggests it is not my sHsp (Figure 2.7b). Species appeared to express similar amounts of chl-sHsp24 in summer, but peak expression occurred at a lower temperature in *S. orbiculatum* than in *S. oligacanthum* (44 *versus* 48°C). The lower temperature of peak expression in chl-sHsp24 by *S. orbiculatum* means that

expression occurred before the onset of PSII damage for that species; conversely, the peak occurred after photosynthetic damage had begun in *S. oligacanthum* (Figure 2.7a).

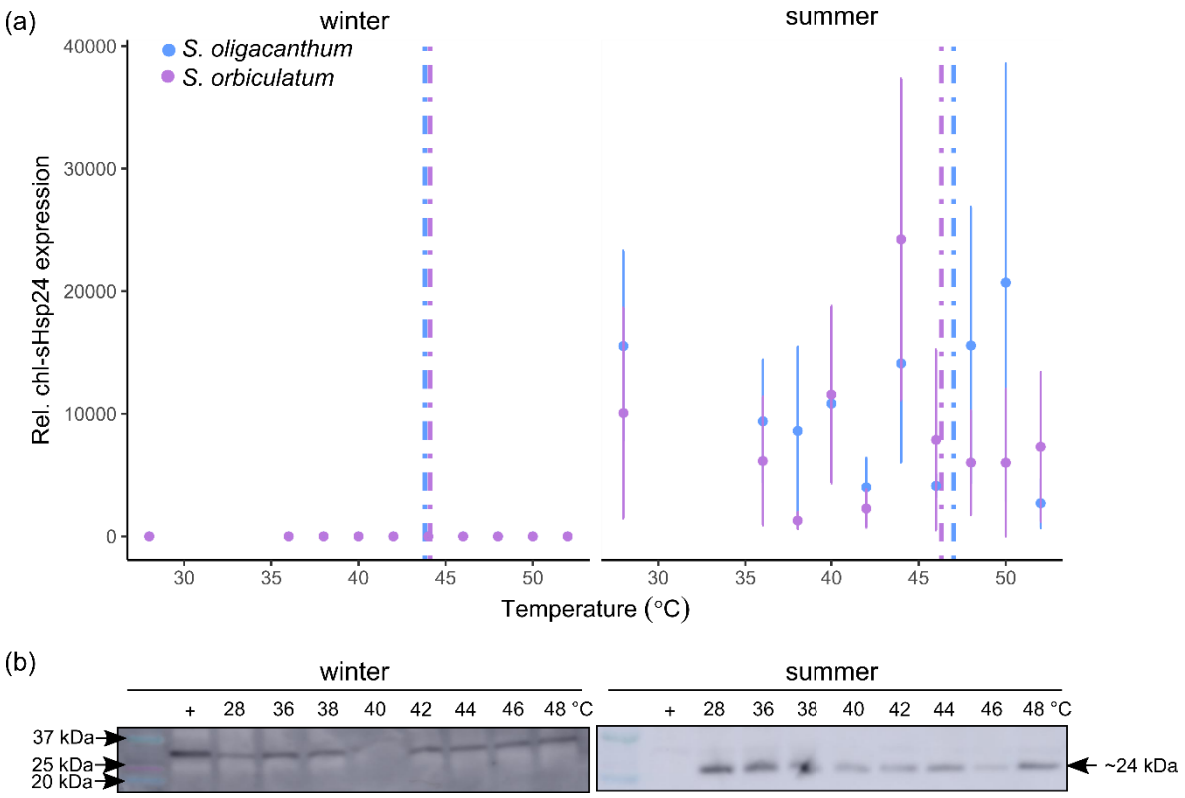

Figure 2.7. Seasonal relative expression of chloroplastic sHsp24 (chl-sHsp24 relative to total protein; mean ± SE) in detached leaves of *Solanum oligacanthum* (blue) and *S. orbiculatum* (purple) after 15 mins at treatment temperature plus 2 h recovery. Details same as for Figure 2.6.

Spearman's rank correlations were used to determine whether any parameters correlated with recovery or if the protective mechanisms helped enhance thermal tolerance. Given that HSPs are protective, it was my expectation that these parameters would be strongly correlated with recovery. Correlations between recovery and these parameters were generally in the direction expected and the same for both species in both seasons; however, the correlations were not strong (Figure 2.8, Table S2.2). R_{Fo} was positively correlated with chlp-sHsp24, when detected, with greater chlp-sHsp24 resulting in a higher temperature at which recovery was possible (Figure 2.8b,d). The direction of correlation between recovery and Hsp70 was inconsistent (Figure 2.8). Stronger correlations were found between recovery and thermal thresholds. R_{Fo} was positively related with membrane stability (with one exception; Figure 2.8c) and $F_v'/F_m'_{\ 90}$ and $F_v/F_{m\ ON}$, while being negatively correlated with $F_{0\,90}$ (with one exception; Figure 2.8a).

When considering how protective mechanisms of HSP expression and thresholds of photosynthesis or membranes, there were both species and seasonal differences. For *S. oligacanthum* during winter, Hsp70 positively correlated with $F_v'/F_m'_{90}$ and $F_v/F_{m\ ON}$ (Figure 2.8a); in summer, chl-sHsp24 was negatively correlated with $F_{0\ 90}$(Figure 2.6b). When *S. orbiculatum* was sampled in winter there were no strong correlations between protective mechanisms and thresholds (Figure 2.8c), whereas in summer, MSI was positively correlated with both $F_v'/F_m'_{90}$ and $F_v/F_{m\ ON}$ (Figure 2.8d).

Generally, *S. orbiculatum* had higher LMA than *S. oligacanthum* but there was a significant species * season interaction (ANOVA: $F_{1,76}$= 44.46, $p < 0.001$, n = 20, log transformed). *Solanum oligacanthum* had significantly higher LMA in winter than summer, whereas LMA of *S. orbiculatum* was significantly higher in summer than winter (Figure S2.1).

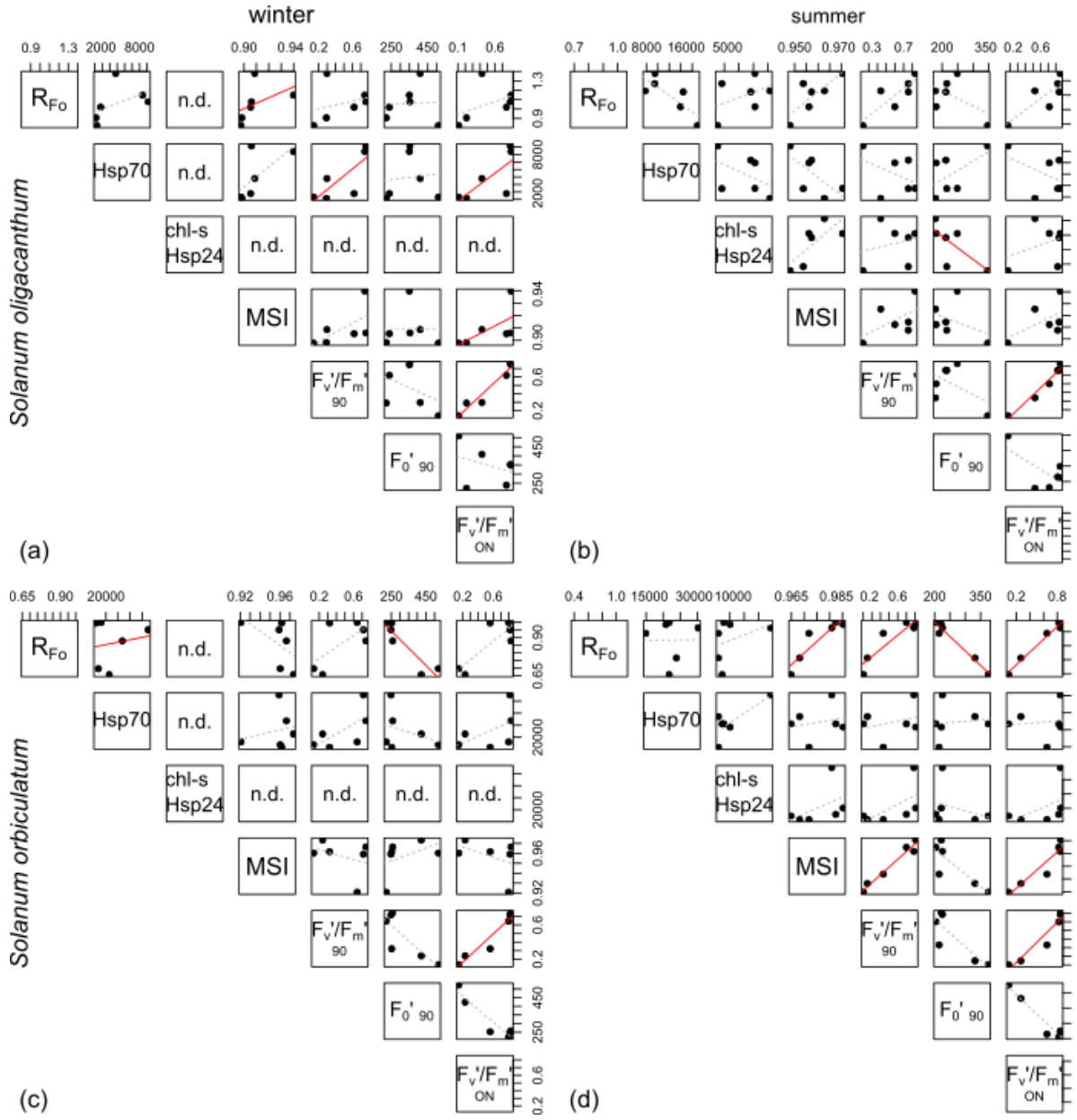

Figure 2.8. Relationships between chlorophyll *a* fluorescence and physiological parameters of *Solanum oligacanthum* (a, b) and *S. orbiculatum* (c, d) following 15 min heat treatment at six temperatures in winter (a, c) and summer (b, d). Significant Spearman's rank correlations are indicated by solid red lines. chl-sHsp24 was not detected (n.d.) in any samples in winter. For Spearman's rank correlation coefficients (R^2) and p values see Table S2.2.

2.4 Discussion

Current understanding of climatic or habitat driven patterns in basal and acquired thermal tolerance is still rudimentary, especially within arid systems, where high temperature tolerance is critical. In my study, *Solanum* species from contrasting microhabitats in the arid zone were found to have high basal thermal tolerance of photosynthetic function and membrane stability,

consistent with high growth temperatures. PSII thresholds were ~10°C lower than membrane thresholds and protective HSP expression usually peaked prior to the onset of PSII damage. These species were able to alter their tolerances to withstand, and also able to recover from, higher temperatures in summer. Nonetheless, the species differed in both thresholds and the degree to which they acquired thermal tolerance.

High winter PSII thresholds of my desert species follow observations that plants from high temperature biomes maintain relatively high basal thresholds (Cunningham and Read 2002, Knight and Ackerly 2002, Amano et al. 2012).Although sampling only once within in a season, my winter thresholds for *Solanum* were similiar to those found in previous research. For example, winter thresholds were comparable to the basal thresholds of rainforest trees in the cool season ($F_v´/F_m´_{90}$ ~44°C *versus* F_v/F_m T_{50}; 44-49°C respectively (Sastry and Barua 2017) and species in semi-arid woodland (46°C) and tropical savannah ($F_{0\ 90}$ ~46°C *versus* F_0 T_{crit} = 47°C respectively (Zhu et al. 2018). Noting that my use of a 12% threshold will estimate a lower temperature than the use of a 50% or inflection point (~20% known as T_{crit}) threshold (Knight and Ackerly 2002), suggesting that my plants maintained a higher winter threshold than plants in these other biomes. Interestingly, in my *Solanum* species, winter thresholds remained high despite the cool winters they experience, suggesting that, even in cooler seasons the potential exposure to extreme high temperatures necessitates maintaining thresholds at least equivalent to tropical species.

Over large scales, the degree to which a species shifts to acquired thermal tolerance—their acclimation ability—increases with the range of temperatures in its native habitat (Cunningham and Read 2003, Zhu et al. 2018). In my study, both species had higher PSII and membrane thresholds in summer, but species had distinct patterns of acclimation (Figure 2.5). *Solanum oligacanthum* shifted photosynthetic thresholds by up to 4°C, in line with other desert species (3-5 °C; Zhu et al. 2018), while *S. orbiculatum* made a 2°C change (Table S2.1). Such species differences in photosynthetic acclimation ability within a single biome have also been recorded in tropical trees (0-3.5 °C; Sastry and Barua 2017). The species disparity in acclimation ability was even greater for membrane stability: *S. oligacanthum* adjusted by 4°C, compared with only 1°C in *S. orbiculatum*. These distinct species patterns of acclimation point to different strategies for tolerating heat stress. For example, this small change in membrane threshold of *S. orbiculatum* may have been all that was required considering the already high winter thermal threshold.

Ranking of threshold temperatures for different physiological responses explains the order in which reversible photoinhibition to permanent damage occurs. The order of ranking among HSPs, PSII and MSI was similar between the species and remained the same from winter to summer. Permanent damage to PSII occurs at > 45°C (Thompson et al. 1989, Yamane et al.

1998), so the 44°C temperature onset of $F_v'/F_m'_{90}$ decline in my species was likely reversible (Kouřil et al. 2004, French et al. 2019). For both species, the peak of HSP expression mostly occurred before the onset of photosynthetic damage, highlighting the importance of HSPs in thermal tolerance. At temperatures exceeding the peak of HSP production, when leaves could no longer produce HSPs, damage to the photosynthetic apparatus began (Figures 2.6 and 2.7). The exception was the expression of chl-sHsp24 in *S. oligacanthum*, which occurred after the onset of PSII damage ($F_v'/F_m'_{90}$) (Figure 2.7). Capacity of recovery was possible up until at most 50°C in *S. oligacanthum* in summer. Complete cell rupture occurs between 55-70°C in mesic plants (Ilík et al. 2018). On average, the membranes of my *Solanum* species became fluid at ~52°C, but the difference between this membrane damage and $F_v'/F_m'_{90}$ varied between 8-11°C, depending on species and season. The difference was similar in conifers (11-13°C (Marias et al. 2017)) and within the range reported by Berry and Bjorkman (1980), but well below the > 16°C difference between F_0 and membrane thresholds for mesic species reported by Ilík et al. (2018). Either way, the large difference between photosynthetic and membrane damage means that under heat stress photosynthesis may be compromised but cells remain intact, implying there is potential for recovery.

As to how HSP expression might change as plants become more thermally tolerant, I offered four scenarios and recorded evidence supporting three of these. One senario was that I would record more HSP in summer than winter, likely because plants were primed for production. This was the case for Hsp70 in *S. oligacanthum* (Figure 2.6). The temperature peak of Hsp70 expression in *S. oligacanthum*, in winter and summer, was 42°C. This temperature has been recorded in other species from contrasting habitats (Knight 2010) and identified as a critical threshold for plants (Berry and Bjorkman 1980). A second scenario was that HSP expression would be lower in summer, perhaps because primed, heat-resistant cellular mechanisms require less protection from HSPs. Lower expression in summer than winter was observed for Hsp70 in *S. orbiculatum* (Figure 2.6) and has been recorded in populations of *Chenopodium album* (Barua et al. 2008). Peak HSP expression itself is known to acclimate with changing growth temperature (Barua and Heckathorn 2004) and usually occurs ~10°C higher than growing temperatures (Vierling 1991, Howarth and Ougham 1993). In *S. orbiculatum* peak Hsp70 temperature was 44°C, approximately 10°C above summer growing temperature (33.8°C mean maximum 30-days prior to sampling; BoM 2018). The third senario was that different kinds of HSPs are expressed in each season. I found that, in both species Chlp-sHsp24 was expressed in summer at high temperatures but not detected in winter, providing evidence for the requirement for priming before expression (Larkindale et al. 2005, Suzuki et al. 2008) and its importance in acquired thermal tolerance (Charng et al. 2006, Davies et al. 2018). This was one of the few studies to consider seasonal change in HSP expression and

has identified a number of ways expression can change highlighting the complexity of the stress response. To establish HSP expression patterns more completely, finer temporal sampling is recommended.

At temperatures beyond optimal, resistance to stress or adjustment to new thermal regimes comes from the repair of heat-sensitive apparatus and maintenance of metabolic homeostasis (Bita and Gerats 2013). Plants with the ability to maintain PSII health at high temperature also have a greater ability to recover from heat stress, as seen in tropical trees (Krause et al. 2010), desert plants (Curtis et al. 2014) and Brassicaceae species (Halter et al. 2016). In the current study, higher PSII and membrane thresholds were generally associated with higher temperatures for recovery in both winter and summer. This suggests that the initial suppression of photosynthetic health was reversible and/or cellular processes remained intact to aid in rapid repair (Sharkey 2005, Ruehr et al. 2019). Recovery thresholds were higher in summer, particularly for *S. oligacanthum*, which recovered 88% of PSII function (R_{Fo}) at temperatures up to 48°C in summer, compared to 46.5°C in winter. The enhanced ability to recover in summer was likely facilitated, in part, by chl-sHsp24, which was only detected in summer and positively correlated with recovery. Indeed, sHSPs are thought to aid in recovery by maintaining acquired thermal tolerance (Charng et al. 2006).

Species differences in thermal tolerance can be explained through the lens of the microhabitat to which they are adapted and where they allocate their resources. Plants from more xeric microhabitats have been shown to have higher photosynthetic thermal thresholds than congenerics from more benign microhabitas (Curtis et al. 2016). Resource-expensive high LMA leaves have been found to be associated with photosynthetic tolerance in tropical forests (Sastry and Barua 2017) and in desert and coastal shrubs (Knight and Ackerly 2003a). It therefore was my expectation that *S. oligacanthum,* from wetter microhabitats within a desert biome and with lower LMA, would have lower photosynthetic thresholds and acclimation potential than *S. orbiculatum*, from relatively drier microhabitats with significantly higher LMA; however, the reverse was the case. Higher tolerance in ‘cooler’ than ‘warmer’ species have been attributed to life history traits, including the ability to shift thresholds (Méthy et al. 1997, Marias et al. 2017), and may help explain higher photosynthetic tolerance in my ‘wetter’ *versus* ‘drier’ species. I propose that *S. oligacanthum* invests in protecting photosynthetic machinery through high photosynthetic thermal tolerances to enable opportunistic rapid growth under favourable conditions. This species is found in ephemeral habitats that are more likely to get pulses of nutrients and water. Based on the leaf economic spectrum (Wright et al. 2004, Reich 2014), I might infer that the low LMA leaves of *S. oligacanthum* have higher photosynthetic rates. Also, this species has greater plasticity, shown by its acclimation capacity and the fact that it drops leaves and readily resprouts following stress (*personal observation*). By contrast,

S. orbiculatum grows in harsher environments, where it invests in building high LMA leaves, therefore maintainance of high membrane thermal tolerance would assist in protecting the investment in that leaf biomass. The difference between photosynthetic and membrane thermal tolerance in *S. orbiculatum* was ≥ 10°C, equating to a relatively large buffer between damage to proteins (photosynthesis) and damage to the whole cell (membranes), suggesting that this species may wait out poor conditions with limited photosynthetic activity, but retain the potential to recover function later. This kind of endurance is evident in another Australian desert species, *Acacia aneura*, which possesses a suite of traits, including high LMA, to survive low water and high temperatures (Eamus et al. 2016). To confirm my predictions, gas exchange, leaf nitrogen and water use efficiency measurements, alongside thermal thresholds, are required.

The selection of congenerics from differing microhabitats has highlighted the different mechanisms species might use to cope with seasonal high temperature: in this case, one develops high photosynthetic thresholds while the other has high membrane thresholds. I showed that even at moderately high temperatures (>44°C), a short heat shock can supress the production of HSPs, leading to inhibition of photosynthetic proteins at temperatures above this. That both species were able to acquire thermal tolerance (although their ability differed) and did so with different HSP expression patterns, demonstrates that there are a number of solutions for acclimation. Processes and tissues have different tolerances and overall plant heat tolerance cannot be evaluated in one metric (Marias et al. 2017). Different strategies used by desert plants (Barron-Gaffery 2013) explains the diversity of plants in the arid zone (Eamus et al. 2016), but also emphasise that some species will be more susceptible to future extreme high temperatures than others.

Acknowledgements

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CHAPTER 3: PLANT STRESS UNDER SPRING OR SUMMER EXTREME HEAT EVENTS

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3.0 Summary

- Heatwaves are becoming more severe and increasingly occurring out-of-season. The timing of heatwaves may affect plants not primed for the event. In the arid-zone, heat stress could be compounded by limited resources meaning increased production of protective heat shock proteins (HSPs) could have downstream effects on reproductive fitness.
- I investigated the response of arid-zone *Solanum oligacanthum* and *Solanum orbiculatum* to the timing of heat stress under differing nutrient conditions. Heat stress events were imposed in open-topped chambers using infrared lamps in spring (out-of-season) and summer. Chlorophyll fluorescence, membrane stability and Hsp70 and chl-sHsp24 expression were used to assess short-term impacts, while biomass allocation, visible damage and flowering and fruiting were measured to determine long-term effects.
- I found that plants fared more poorly following a summer heat stress and expressed more Hsp70 and chl-sHsp24 than in spring. There was a strong influence of nutrient availability on downstream effects of heat stress including fitness.
- The severity of an arid summer, including supra-optimal conditions of high temperature and low water availability/high water demand, was more detrimental to plants in both short-term and long-term effects. Nutrient availability on survival and reproductive output was species-specific, where reallocation of resources was likely based on life-history traits.

Keywords

Chlorophyll fluorescence (F_v/F_m), desert, fitness, heat shock proteins, heatwaves, membrane stability, Solanum, thermal tolerance

3.1 Introduction

To understand the effects of climate change on plants, most studies have focused on imposing a projected CO_2 and/or temperature rise (Nijs et al. 1996, Hovenden et al. 2006, Crous et al. 2018). These studies provide information on species responses to predicted changes in long-term averages. Yet, the increased frequency of extreme events is potentially more influential. Their effects last beyond their short duration (Jentsch et al. 2007) and have greater influence on plant physiology than mean climate (Gutschick and BassiriRad 2003, Jump and Penuelas 2005, Reyer et al. 2013). Observations following heatwaves have reported reduced gross primary productivity of forests (Ciais et al. 2005, Bauweraerts et al. 2014b) and increased mortality (Allen et al. 2010, Harris et al. 2018, Hoffmann et al. 2019). Heatwaves are, and will continue to become, longer, hotter and more frequent (Cowan et al. 2014, Perkins et al. 2014,

Lewis et al. 2017), highlighting the need for understanding species responses to high temperature extremes.

During heatwaves, high air temperatures, when coupled with drought conditions (often the case; Vicente-Serrano et al. 2014, Zscheischler and Seneviratne 2017), contribute to reduced photosynthesis through stomatal closure (Berry and Bjorkman 1980, Tang et al. 2007). Continued high leaf temperatures affect photosynthesis directly by damaging photosynthetic proteins (Havaux 1993a, Law and Crafts-Brandner 1999, Sharkey 2005) or indirectly, through leakiness and oxidative damage to thylakoid and other membranes (Daniell et al. 1969, Bukhov et al. 1999, Djanaguiraman et al. 2018). As photosynthesis declines and respiration rates increase (Atkin et al. 2005a, O'Sullivan et al. 2013), there is less available energy for other processes, such as protection, repair, growth and reproduction (Parsons 1990).

With high temperature, the stress response of plants is stimulated in order to protect from damage and return cells to normal metabolic function. Part of the stress response is the expression of heat shock proteins (HSPs), used to hold and repair denatured proteins (Vierling 1991, Barua and Heckathorn 2004) and to enhance a plant's resilience to future events (Charng et al. 2006, Bita and Gerats 2013). Chloroplastic small HSPs (chl-sHSP) protect thylakoid membranes and PSII (Downs et al. 1999a, Downs et al. 1999b, Haq et al. 2013) and moderate protein aggregation (Vierling et al. 1989). Hsp70 then acts to re-solubilise proteins for re-folding (Żwirowski et al. 2017). Following a sub-lethal stress, plants express greater amounts of HSPs in rapid response to extreme temperatures than with no prior sub-lethal stress (Havaux 1993c, Heckathorn et al. 2002). During stress, HSPs are rapidly up-regulated (Li et al. 1999, Sung et al. 2001) and can comprise up to 10% of soluble protein in leaves (Coleman et al. 1995, Parsons 2005), reducing resources for other processes (Tonsor et al. 2008). Therefore, in addition to directly impairing productivity and function, heat stress also presents a cost in the energy required for protection and repairing damage. This raises the question of how plants allocate resources between protection and survival *versus* growth and reproduction.

Heatwaves shape arid ecosystems (Holmgren et al. 2006) with plants already living close to thermal limits and exceeding thresholds during extreme events (O'Sullivan et al. 2013). Many deserts are typified by poor access to nutrients, due either to water scarcity (Erskine et al. 1996, Handley et al. 1999) or through low nutrient soils (Stafford Smith and Morton 1990). Soil nutrients, particularly nitrogen, influence sHSPs (Heckathorn et al. 1996b, a) so that the effects of heat stress events may be compounded in low nutrient conditions, especially when coupled with drought (Teskey et al. 2015, Ward et al. 2015, Pivovaroff et al. 2016, Harris et al. 2018).

Heatwaves are now more likely to occur out-of-season. Winter warm spells are increasing at a greater rate than summer heatwaves (Cowan et al. 2014) and the heatwave season is starting earlier (Steffen et al. 2014). A-seasonal heatwaves are potentially more damaging as they occur when organisms are not physiologically primed for high temperatures. With priming, plants are able to acclimatise into warmer seasons (Atkin et al. 2000, Atkin and Tjoelker 2003, Aspinwall et al. 2017); however, out-of-season heatwaves are likely to leave some species ill prepared. The rate at which Australian arid-zone plants increase their thermal tolerance thresholds to maximum summer values varies among species (Curtis *et al.*, unpublished), with those slower to increase potentially being most susceptible to out-of-season heatwaves. Under more benign climates, spring heatwaves may be beneficial to plants (De Boeck et al. 2011); however, it is not known whether the same conclusion would be drawn in a more severe climate, such as a desert, where a spring heat stress event can exceed optimal temperatures (e.g. 45.4°C recorded at SA in October; BoM 2019). Considering that priming is important for plant resilience and that the evidence on the impacts of timing of high temperature events is scarce, it remains unclear if the effect of an early heatwave is more severe than a heatwave after seasonal priming.

In this study, I addressed the following questions: 1) Is a spring or summer heat stress more detrimental to growth and fitness outcomes for plants? Due to lack of priming, I predicted that out-of-season heat stress would result in poor fitness outcomes. 2) How does HSP expression after exposure to heat stress events differ at different times of the year? I expected that summer plants, primed for heat stress, would produce more HSPs, as shown in short-term priming experiments (Larkindale et al. 2005, Larkindale and Vierling 2008, Suzuki et al. 2008). 3) How does nutrient availability influence downstream effects of heat stress, i.e. growth and fitness? My expectation was that plants with access to more resources would be able to use nutrient stores to better protect or recover from heat stress damage. I applied these questions to two Australian arid zone *Solanum* species grown under two nutrient treatments. In spring and summer, I imposed a heat stress event of biological relevance and followed the plants through to fruiting.

3.2 Materials and Methods

Site description and species

This study was conducted at the Australian Arid Lands Botanic Garden, Port Augusta, South Australia (32°28'4.35" S, 137°44'36.99" E), where mean maximum monthly temperatures range from 17.9°C in July and 34.2°C in January and mean monthly precipitation ranges from 13.4 mm in January to 25.7 mm in December (BoM 2018). Two arid zone *Solanum* species with differing microhabitat preferences were selected. *Solanum orbiculatum* (Dunal ex Poir.)

subsp. *orbiculatum* is found in drier microhabitats (sandplains and dunes, rocky hills and outcrops; FloraNT 2013) and *Solanum oligacanthum* (F. Muell.), which typically grows in wetter microhabitats (sandy or clayey soils in soaks and creek lines; Bean 2004). Both are annual herbs with hair-covered leaves and long flowering and fruiting periods (Bean 2004, PlantNET 2004, eFloraSA 2013) (Fig. **3.1**).

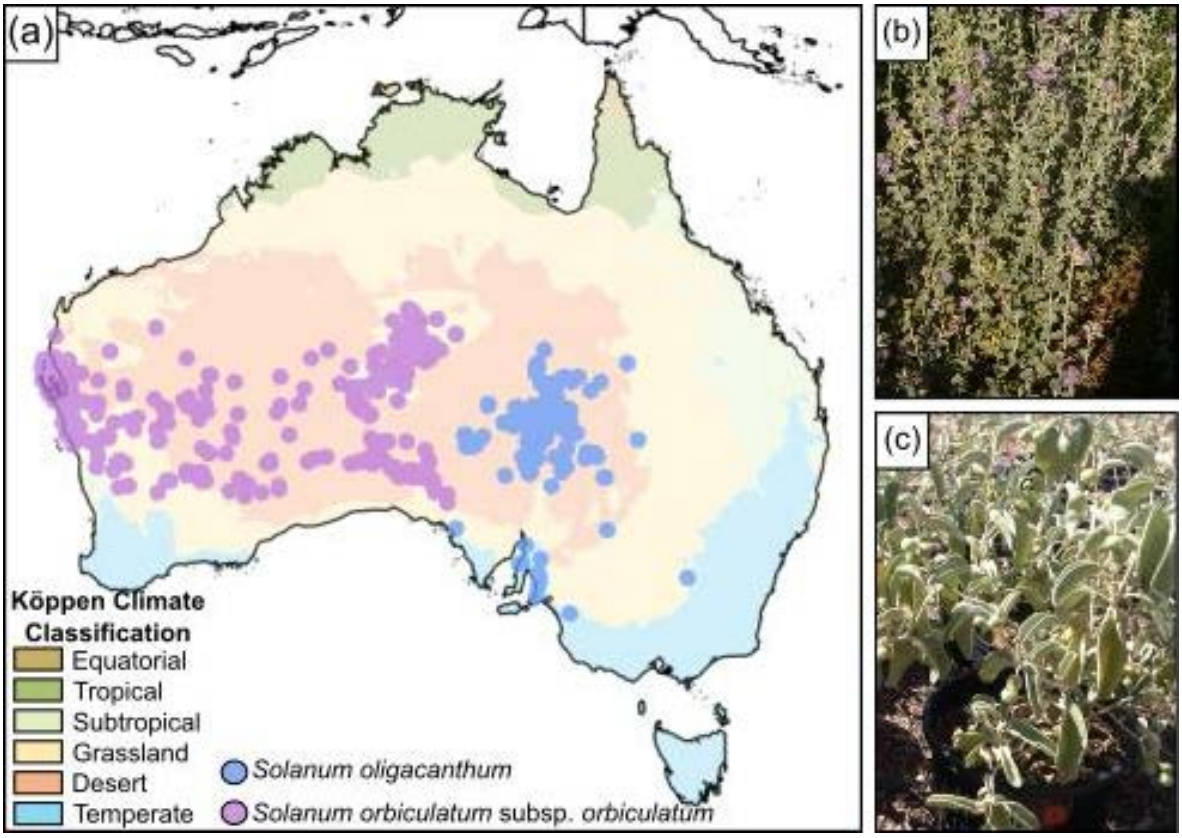

Figure 3.1. Distribution **(a)** and appearance of the two study *Solanum* species, *Solanum orbiculatum* **(b)** and *Solanum oligacanthum* **(c).** Distribution is displayed in relation to the major classes of the Köppen climate classification of Australia (BoM 1990), showing the common arid zone distribution of the two species. Within this broader classification, *S. orbiculatum* (purple points) has a wider distribution and is found in 'drier' microhabitats, while *S. oligacanthum* (blue points) grows in 'wetter' microhabitats. Species distribution map was produced using the Atlas of Living Australia (ALA 2018).

Treatments and heat stress application

Plants for this experiment were grown from cuttings collected from at least five individuals of the living collection of plants at the AALBG, and established in 75 mm tubes for 33 weeks. Cuttings were transferred to 4.5 L pots filled with 3:1 local sandy soil to standard potting mix for four weeks before experiments began (Fig. **S3.1**). Root biomass was collected during pre-heat stress harvests to test that pot size was adequate (Poorter et al. 2012). A two-factor

ANOVA showed root:shoot ratio was significantly higher in summer than in spring ($F_{1,44}$= 7.08, p =0.011) and higher in low *versus* high nutrient plants ($F_{1,44}$= 7.31, p= 0.010) (see Table S3.2 for means). Plants from each species were randomly assigned to either the high nutrient (potting mix with Nutricote Standard Pink slow release fertiliser; Yates, NSW, Australia, N:P:K 19.1:0.0:11.9) or low nutrient (potting mix with no fertiliser) group, applied at time of potting. To verify the effect of nutrient status, I determined total leaf protein concentration (See Methods **S3.1**). Additional fertiliser increased the nitrogen status of fertlised plants, although there was an interaction with species and season (Fig. **S3.2**). Within each nutrient treatment, plants were randomly divided into two groups relating to season of the heat stress: austral spring (October 2016) or summer (Februrary 2017) heat stress (Fig. **S3.1**). These groups were further subdivided into heat stress treatments: ambient plants, receiving no heat stress, and plants subjected to a heat stress event in open top chambers (OTCs). Plants were grown outdoors in full sun but kept well-watered.

My focus is on single extreme high temperature days, periodically recorded in these regions (BoM 2019). To best represent prevailing desert conditions, plants were grown and treated *in situ*, incorporating the naturally high light and low humidity conditions that are difficult to reproduce in growth chambers and heat stresses were imposed over the top of the natural weather. I aimed to replicate what plants would experience during a heat stress event, of equivalent length and severity to the afternoon of a heatwave, when wind speed drops and the leaves of water stressed plants are likely to reach dangerously high temperatures (Vogel 2005, Leigh et al. 2012). The heat stress treatment was applied in two open-top chambers (0.72 m (h) x 0.49 m (w) x 1.09 m (l), with sides enclosed using PVC plastic sheeting). Two ceramic infrared lamps with reflectors (1000 W, 230 V full trough element, 60 kW/m^2; Ceramicx, Ireland, fitted with aluminised steel reflectors and solid state relays) were hung 0.3 m above each chamber and tilted at ~40°, similar to Kimball et al. (2008). Heat stress events were imposed to chambers for 3 h between 13:00-16:00. As leaf temperature can vary considerably from air temperature, my goal was to monitor leaf temperature within chambers, maintaining the temperature of target leaves close to 45°C, which I did by gradually ramping up IR radiation from the lamps over the first hour, then holding temperaturefor the remaining two hours (Fig. **3.2**). Due to chamber size, and to maximise replication, four heat stress events were run on consecutive afternoons, each on a separate set of plants. Shortly before commencing each event, at least one plant from each treatment group was placed in one of two chambers and ambient plants were placed adjacent to the chambers. Ambient plants are referred to as such as they are not true controls, they did not experience chamber conditions. Therefore, I compared plants under predicted future heatwave conditions (e.g. high temperature and vapour pressure deficit (VPD)) to plants under current ambient conditions.

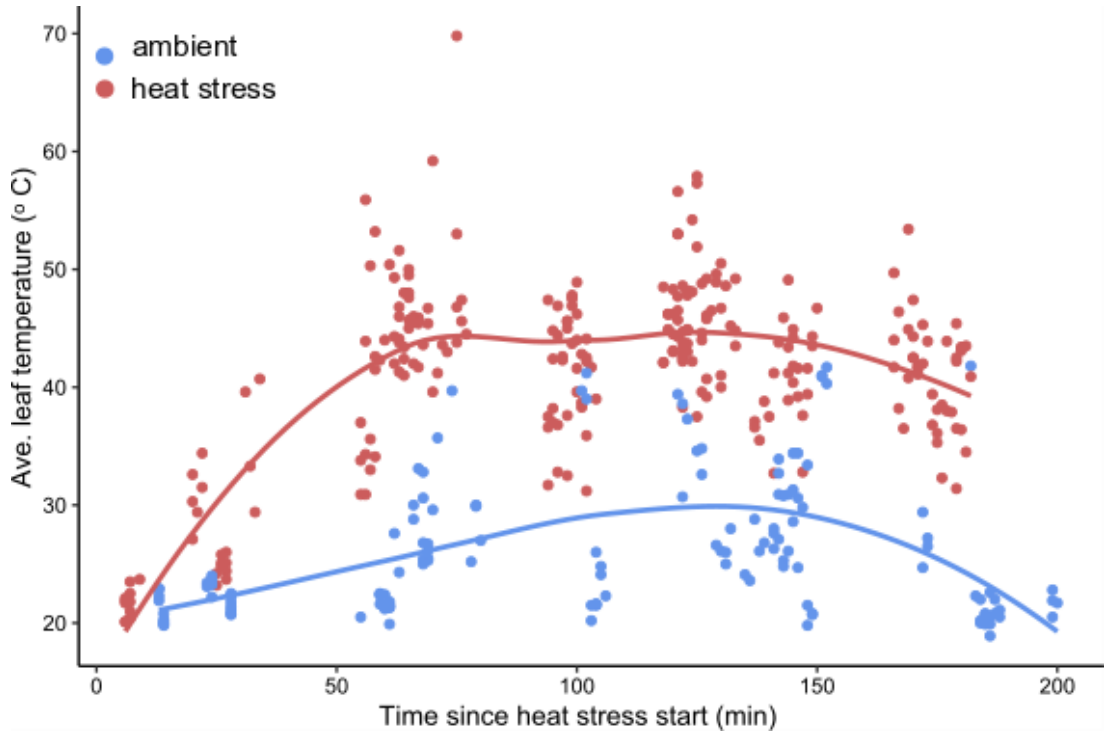

Figure 3.2. Example of ramping of leaf temperatures (red points) during imposed heat stress using IR ceramic lamps in open top chambers (data shown are all measured plants from spring and summer). Lamp temperature in the chambers was ramped over the first hour and then adjusted manually to maintain leaf temperatures at ~45°C until completion of heat stress at 180 min. Leaf temperature was monitored with a thermographic camera and infrared thermometer. Mean leaf temperature is the average of three leaves per plant. The leaf temperature of ambient plants (blue points) outside and adjacent to the chambers was not controlled and depended on the environmental conditions on that day. Solid lines show the loess smoothing of leaf temperatures.

During heat stress treatments, leaf temperature was monitored using a non-contact infrared thermometer (accuracy ± 2.5%°C; IP67; Jaycar, NSW, Australia) and a thermographic camera with emissivity set to 0.95 (accuracy ± 2°C or ± 2% of m.v.; Testo 885-2; Testo SE & CO.KGaA, Lenzkirch, Germany). For leaf temperature analysis, images taken with the camera were used to find temperatures of three target leaves per plant using the manufacturer's software (Testo IRSoft, v4.4). During all heat stress events (except one replicate in spring), ambient air temperature (T_{air}) and humidity in chambers were recorded using climate loggers (DS1923; iButton®, Alfa-Tek Australia) suspended within a double-layer, disc-shaped white plastic shield to maintain air flow around the sensor while reflecting radiation. In addition to chamber measurements, air temperature and humidity were constantly monitored in the area in which the potted plants were grown. Vapour pressure deficit was calculated from temperature and humidity.

Desert plants naturally experience heatwaves under high light conditions and low humidity. Because natural heatwaves often are coupled with low soil water availability (Teskey et al. 2015), irrigation was withheld from all plants six days prior to heat stress. Daily checks of plants for appearance of water stress (wilting) were conducted and pre-dawn leaf water potential (Ψ_L) was measured on the day of the heat stress treatment using a pressure chamber (Model 1505D; PMS Instrument Company, OR, USA). To confirm that experimental plants were water stressed, leaf water potential (Ψ_L) was compared with that of well-watered plants grown alongside experimental plants. In spring, although non-significant Ψ_L was lower in water stressed (-7.5 (-6.2,-8.5) MPa; bootstrap mean and 95% CI) than well-watered (-6.1 (-5.4,-7.0) MPa) *S. oligacanthum* plants and *S. orbiculatum* plants (-6.5 (-5.3,-7.4) MPa and -5.5 (-4.5,-6.5) MPa respectively). In summer, Ψ_L was significantly lower in water-stressed than well-watered plants of both *S. oligacanthum* (-9.0 (-6.9,-11.9) and -5.5 (-5.0,-6.4) MPa respectively) and *S. orbiculatum* (-12.0 (-9.2,-16.2) and -6.7 (-5.2,-9.0) MPa respectively). Heat stress events in nature often occur when wind speed drops, reducing forced convection that would otherwise prevent leaves from overheating (Vogel 2009). To check that experimental heat stress events mimicked such conditions, wind speed inside and outside of the chambers was measured using a digital anemometer (435; Testo, Testo SE & CO.KGaA, Lenzkirch, Germany). Recorded wind speeds were 0.04-1.14 ms^{-1} inside chambers and 0.09-8.9 ms^{-1} outside chambers, with greater variance (SD) outside than in chambers (1.30 and 0.20 ms^{-1} respectively). Wind speed was significantly higher outside than inside chambers (Welch two sample t-test with unequal variance; $t_{390} = 23.527$, $p < 0.001$). During spring, photosynthetically active radiation (PAR) was measured with a Li-190R Quantum Sensor and LI-250A light meter (Li-COR, Lincoln, Nebraska, USA). PAR received in the chambers was ~ 26% lower than light levels outside (independent samples t-test; $t_{14} = 2.57$, $p = 0.02$), however, the mean chamber PAR of 1347 ± 118 µmol m^{-2} s^{-1} was similar to saturating light levels for Australian desert plants (e.g., 1200 µmol m^{-2} s^{-1} PAR for *Acacia anuera* in arid Northern Territory; (Wujeska-Klause et al. 2015).

Evaluating effects of heat stress

To evaluate short-term damage to plants during heat stress, PSII health, membrane stability and the expression of HSPs were measured. The longer-term energetic cost of a heat stress event was estimated from growth and fitness metrics at the whole plant scale. Three leaves on each of three plants per treatment group were sampled from 0.9 m from the heat source.

Photosystem II (PSII) health was assessed via maximum quantum yield (F_v/F_m) of PSII, which was measured pre-dawn using a chlorophyll fluorometer (mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) on the morning of, and after the day of, heat stress treatment. Many plants

had a pre-heat stress F_v/F_m values below 0.83 (Fig. **S3.3**) due to the water stress preceding the heat stress. For this reason, an estimate of damage to PSII was calculated using the equation:

$$D_{PSII} = 1 - \frac{Post\ F_v/F_m}{Pre\ F_v/F_m} \quad \text{Equation 1}$$

from Curtis et al. (2014). This metric accounts for the starting point of the plants prior to the heat stress, including background damage that may be caused by water stress or seasonal differences.

Membrane stability was assessed with a conductivity meter (TetraCon 925; WTW, Weilheim, Germany) to measure the electrolyte leakage from ~ 0.5 g of fresh leaf placed in 15 mL of de-ionised water for 90 min (modified from French et al. (2019)). A membrane stability index (MSI) was then calculated:

$$MSI = 1 - \left(\frac{EC_{90} - EC_0}{EC_{max} - EC_0}\right) \quad \text{Equation 2}$$

where EC_0 was the conductivity of the water 5 min after leaves were placed in the water, EC_{90} was the conductivity measured after 90 min and EC_{max} was the conductivity measured the morning after leaves were heat treated at 100°C.

Following the heat stress treatments, plants were returned to irrigated, full sun growth conditions. Approximately 1-2 months after each seasonal event, a single replicate plant from each treatment group was randomly selected for harvest, which may have included dead plants. At harvest, leaves and stems were separated and oven dried at 60°C until weight was stable. Visual damage to all plants was assigned based on estimated percentage of dead or discoloured leaves on the plant; plant survival also was recorded.

Propagation of all plants occurred at the same time, meaning plants exposed to the summer treatments were older and larger than plants used in spring. For this reason, the influence of heat stress on plant growth was assessed using relative growth rate (RGR) of leaves (g day^{-1}), the instantaneous rate of increase, which takes into account the relative size of the plant (Pérez-Harguindeguy et al. 2013), calculated as:

$$RGR_{leaf} = {(lnM_2 - lnM_1)}/{(t_2 - t_1)} \quad \text{Equation 3}$$

where M_1 and M_2 are leaf mass, harvested at t_1 and t_2 or pre- and post-heat stress, respectively. For estimating M_1, a subset of plants was harvested pre-heat stress and the mean dry mass was used.

To investigate relative aboveground resource allocation, the ratio stem:leaf was calculated. In addition, as an indicator of environmental tolerance and competitive ability (Poorter and De Jong 1999, Poorter et al. 2009) leaf mass per area (LMA, g m^{-2}) was determined. At the time of harvesting, all leaves per plant were scanned and leaf area measured using ImageJ software (National Institutes of Health; Bethesda, MD). Plant level LMA was calculated by dividing total LA by total dry leaf biomass.

Prior to each heat stress treatment, flowers and fruits were removed from the plants so that only those that developed after the heat stress were recorded. To estimate the fitness cost of heat stress, flowers and fruits of all plants were counted and flowers and fruit from harvested plants were weighed (weight included peduncle as accessory costs). Due to many fruit not being fully developed at harvest the mean number of seeds was recorded from a subset of fruit (n = 9-12 for *S. oligacanthum* and n = 12 for *S. orbiculatum*); so it was not possible to ascertain absolute seed numbers. Estimates of seed output $plant^{-1}$ were calculated based on the mean number seeds $fruit^{-1}$ multiplied by the number of fruit on a plant, normalised to per day output due to differences in harvest times. As seed output was estimated, these data were not analysed but graphically represented (see Results). In order to assess whether plants altered the resources directed to flowering or fruiting with respect to treatment, the ratio of flower or fruit mass to aboveground biomass was calculated (flower:AG, fruit:AG (g:g)).

HSP expression

Greater detail on the protein extraction and immunoblotting technique is provided in Methods **S3.1**. Briefly, c. 1 h after each heat stress event ~100 mg of leaves from each of three plants in each treatment group was snap frozen in liquid nitrogen. Protein extraction, SDS-PAGE and immunoblotting followed standard procedures using equal protein sample loading and the primary antibodies, anti-Hsp70 (1:2000 dilution; N27F34; ENZO Life Sciences Inc., Farmingdale, USA) and anti-chlp sHsp (1:2000 dilution; using whole sera). Horseradish peroxidase-conjugated IgG secondary antibodies (1:2000 dilution anti-mouse; 9044 and 1:5000 dilution anti-rabbit; A9169, both from Merck KGaA, Darmstadt, Germany) were used for visualisation.

Statistical analyses

This experiment used a four factor design, each factor with two levels. Species, season, nutrient treatment and heat stress treatment were fixed effects. The heat stress treatment was administered over four consecutive days, with three new plants from every treatment combination used each day. Total number of plants used for this experiment was 2 species x 2 seasons x 2 nutrient treatments x 2 heat stress treatments x 4 replicate heat stress days (n.b. with the exception of high nutrient *S. oligacanthum* in summer, for which there were 3

replicate heat stress days) x 3 replicate plants + pre-heat stress harvest; 2 species x 2 seasons x 2 nutrient treatments x 3 replicate plants = 210 plants overall. As mean air temperature within the two chambers was equivalent and significantly higher than ambient temperature (ANOVA; $F_{2,18} = 5.27$, $p = 0.0158$), I did not consider chamber in the experimental design and the values for any parameter for the three plants was averaged for each stress day, i.e., n=4 replicate heat stress days, except where deaths occurred or high nutrient *S. oligcanthum* in summer. Variables of growth (LMA, RGR_{leaf}, stem:leaf, flower and fruit:AG) were sampled once per heat stress treatment day. Analysis of visible damage and RGR_{leaf} included dead plants, but dead plants were removed from analysis of stem:leaf and LMA.

Using R (R Core Team 2018), the variables visible damage and survival were analysed using binomial logistic regression. The proportion of plants per heat stress treatment day that survived or recorded visible damage (where visible damage >10% = 1, and <10% = 0 or no damage) were used in analyses. All other variables were analysed using ANOVA using the "lm" function, after assumptions were tested and relevant transformations made (see Table 3.1). Models were simplified by hierarchical removal of non-significant interactions (Crawley 2013). Order of removal used AIC values using the 'drop1' function of the 'car' package (Fox and Weisber 2011). Interaction terms were dropped until the model with the lowest AIC value that did not deviate significantly from the maximal model was found. Due to the unbalanced nature of the experiment, Type II sums of squares were used (Langsrud 2003) to preserve the marginality principle. Where there were significant interactions, Tukey HSD in the emmeans package were used to find differences (Lenth 2018).

Data availability

The data that support the findings of this study will be openly available in Dryad on acceptance of the manuscript.

3.3 Results

Heat stress characteristics

Seasonal differences in ambient air temperature and VPD during the experimental period (including the five days prior, during and five days post heat stress treatment) were apparent, with warmer and drier conditions in summer than in spring (Fig. **S3.2**). In summer, a natural heatwave (three consecutive days exceeding the 90th percentile) occurred two days prior to experimentation (Fig. **S3.2b**). During the heat stress treatments in spring, air temperatures in the open-top chambers (Table S3.1; Fig. **S3.2a**) were generally greater than naturally occurring heatwaves in this region at a similar time of year (three days >33°C, 90th percentile maximum temperature data from Port Augusta Airport 2001-2017; BoM 2018). During summer

treatments, imposed heat stress air temperatures in the chambers were similar to typical summer heatwaves (3 days >40°C) (Table S3.1; Fig. **S3.3b**). Mean leaf temperatures of heat-stressed plants reached 47°C, in spring and 50°C in summer, which is comparable to a mean maximum leaf temperature of 52°C, measured in other water-stressed native desert plants at this site during early summer (Cook et al., unpublished).

Despite seasonal differences in ambient conditions between spring and summer, the recorded maximum leaf temperatures reached in chambers during imposed heat stresses in spring and summer did not differ ($F_{1,56}$ = 7.52, p = 0.0008; Fig. **3.3**). There was a significant season by temperature effect, whereby ambient leaf temperatures in summer were higher than those of leaves in spring (Fig. **3.3**). It is noted that in this experiment, the use of a chamber to apply heat stress conditions resulted in differences in conditions between heat stress and ambient plants beyond just altering temperature; VPD, wind and light were altered, so when referring to heat stress I acknowledge all stressors present.

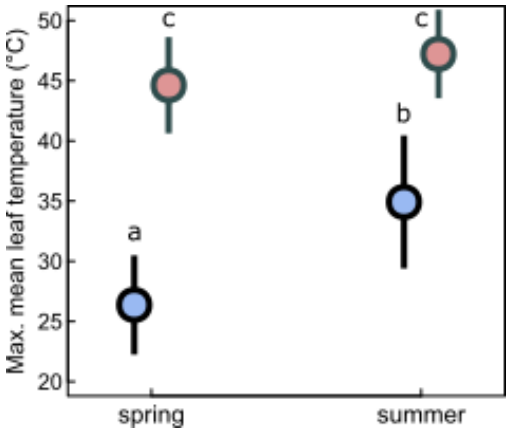

Figure 3.3. Mean maximum leaf temperatures (± SD, n = 4) recorded during a seasonal heat stress experiment on *Solanum oligacanthum* and *S. orbiculatum* in southern arid Australia. Plants were placed in one of two chambers for imposed heat stress (red) using IR lamps or left in ambient conditions (blue). Different letters indicate significant (P < 0.05) differences between the means of treatment responses.

Short-term responses of PSII damage, membrane stability and HSP expression to heat stress

There was significantly more damage to PSII in plants that were exposed to heat stress than ambient plants (Fig. **3.4a**) and significantly more damage to PSII in summer than in spring (Fig. **3.4b**, Table 3.1), irrespective of species or nutrient levels. In terms of membrane damage, there was a significant season x nutrient x heat stress effect (Table 3.1), whereby, plants with reduced access to nutrients incurred little damage to membranes in spring or summer, with only a small increase in damage between ambient and heat-stressed plants (Fig. **3.4c**). For high nutrient plants, there was no difference between ambient and heat-stressed plants in

spring, but following a summer heat stress, significantly more membrane damage was recorded (Fig. **3.4c**). The effect of season on membrane damage differed between species (Table 3.1). There was little membrane damage to *S. orbiculatum* in either season, while *S. oligacanthum* recorded similarly low damage in spring, but greater membrane damage in summer (Fig. **3.4d**).

The way that Hsp70 expression was influenced by nutrient availability depended on both species and season, with significant interactions with both these factors (Table 3.1). Species differences in Hsp70 expression were not apparent when species were restricted in nutrient availability, but when nutrients were available, *S. orbiculatum* expressed significantly more Hsp70 than *S. oligacanthum* (Fig. **3.4e**). Generally, more Hsp70s were expressed in summer than spring, irrespective of nutrient availability (Fig. **3.4f**). When comparing nutrient treatments within a season, similar amounts of Hsp70 were expressed regardless of nutrients in spring, but in summer significantly more Hsp70s were expressed by low than high nutrient-grown plants (Fig. **3.4f**). Similar to Hsp70 expression, there was generally greater chl-sHsp24 expression in summer than in spring; however, there was a season x nutrient x temperature effect (Table 3.1). Generally, though not significanlty so, more chl-sHsp24 was expressed in heat-stressed plants and in high nutrient plants in spring; in summer, low nutrient plants generally produced more chl-sHsp24 (Fig. **3.4e**).

To sumarise and compare responses of these desert annuals, I produced a heat map, where the severity of response for each variable was calculated relative to the treatment group with the strongest mean response for that variable (Table 3.2). With regards to the short-term responses, generally there was greater damage to MSI and more HSP expression in summer, and in heat-stressed rather than ambient grown plants (Table 3.2). The exception to this trend was damage to PSII, where plants in spring were more negatively affected than plants in summer.

Table 3.1 F-values of four factors in models of physiological and growth traits of *Solanum oligacanthum* and *S. orbiculatum;* factors were species, season (spring *versus* summer), nutrient treatment (high *versus* low) and heat stress treatment (ambient *versus* heat stress). Analysis of variance was used for all variables, except damage and survival which were analysed using general linear models. In both analyses, models were simplified by step-wise removal of non-significant interactions. Levels of significance denoted as follows: *** = P < 0.001; ** = P < 0.01; * = P < 0.05 .

	D_{PSII}	MSI	Hsp70	sHsp24	stem: leaf	LMA	RGR_{leaf}	Visible damage	Survival	Flower no.	Fruit no.	Flower:AG	Fruit: AG
species	0.22	**38.22*** **	**20.34*** **	1.46	**16.91*** **	**24.29*** **	0.01	1.59	**10.10** **	72.78	1.14	**19.45*** **	**7.57** **
season	**8.07** **	**12.92*** **	**37.18*** **	**62.53*** **	**14.90*** **	0.14	**58.79*** **	0.80	1.42	4.85	3.82	**19.21*** **	**7.83** **
nutrient	1.39	**5.23***	2.90	0.58	**14.74*** **	0.34	**14.25*** **	0.10	0.17	**129.73*** **	**39.99*** **	1.73	0.21
temperature	**8.74** **	**6.55***	3.35	**16.86*** **	1.26	2.32	**4.98***	**20.43*** **	**16.18*** **	**8.78** **	2.77	0.00	0.07
species * season		**12.44** **	0.05	0.04	0.96	1.92	**4.82***		**4.19***	**30.57*** **	2.14	**11.86** **	**15.53*** **
species * nutrient		2.31	**4.72***	3.75	1.53	0.26	0.12		3.03	3.28		0.26	
species * temperature		0.00	0.56	0.02	1.70	0.26	1.02		0.04	0.37	2.17	0.51	2.12
season * nutrient		**14.63*** **	**8.95** **	0.52	0.13	0.47	**6.84***	1.01	2.33	**5.37***		0.10	3.62
season * temperature		0.24	0.02	0.04	0.07	1.89	1.59	3.13	**6.40 ***	2.05	1.78	0.21	2.45
nutrient * temperature		0.55	0.34	1.29	0.00	3.64	0.20	**4.33***	1.47	3.60		0.55	
species * season * nutrient			2.54		3.07	1.66	1.82			2.76			
species * season * temp.						2.58						**4.64***	
species * nutrient * temp.					2.32				**5.58***				
season * nutrient * temp.		**4.21***	3.23	**4.51***								2.86	
degrees of freedom	1,57	1,50	1,41	1,42	1,51	1,45	1,50	1,54	1,50	1,50	1,54	1,45	1,49
transformation	neg. √	logit	√	√	log_{10}	√				log_{10}	√	√	√

Variable descriptions: D_{PSII}, damage to PSII; MSI, membrane stability index; Hsp70, relative expression of Hsp70; sHsp24, relative expression of chl-sHsp24; stem:leaf, ratio of stem to leaf biomass; LMA, mean leaf mass per area; RGR_{leaf}, relative growth rate of leaves; visible damage, visual damage to plants analysed as a proption, where <10% damage = 0, >10% damage = 1; survival, proportion of plants survived; flower/fruit no, number of flowers/fruits produced per day since heat stress; flower/fruit:AG, ratio of flower/fruit mass to aboveground biomass.

Table 3.2. Heat map of *S. oligacanthum* and *S. orbiculatum* relative responses to heat stress. In this study, response can mean tolerance, protection, damage, survival, growth rate or reproduction. Within each variable, the severity of response incurred during the seasonal heat stress experiment is relative to the treatment group with the strongest mean response/damage (1 = most damage (red), 0 = no damage (blue)) or, in the case of HSPs, the group that had the highest HSP expression (1 = highest HSP (red), 0 = no HSP (blue), based on the assumption that production of HSPs requires energy and therefore a cost to the plant). A sum total close to eleven indicates that plants did poorly across all response measures. The values are shown to two decimal places for ease of viewing.

Species	Season	Nutrient treat.	Heat stress treat.	D_{PSII}	MSI	Hsp70	sHsp	RGR_{leaf}	Vis. Damage	Survival	Flower /day	Fruit /day	Flower:AG	Fruit:AG	sum total
S. oligacanthum	spring	high	ambient	0.31	0.84	0.11	0.00	0.93	0.33	0.58	0.01	0.11	0.05	0.08	3
			HS	0.92	0.85	0.21	0.06	0.94	0.98	0.58	0.01	0.14	0.09	0.25	5
		low	ambient	0.14	0.84	0.14	0.02	0.95	0.00	0.58	0.02	0.31	0.08	0.20	3
			HS	0.82	0.85	0.15	0.11	0.96	0.98	0.64	0.09	0.95	0.09	0.14	6
	summer	high	ambient	-0.12	0.91	0.23	0.29	0.98	1.00	0.66	0.02	0.19	0.40	0.15	5
			HS	0.11	1.00	0.16	0.38	0.99	1.00	0.58	0.02	0.10	0.12	0.01	4
		low	ambient	-0.04	0.85	0.58	0.24	1.00	0.00	0.58	0.07	1.00	0.40	0.67	5
			HS	0.54	0.86	0.92	1.00	0.99	0.85	0.78	0.11	0.69	0.45	0.02	7
S. orbiculatum	spring	high	ambient	0.12	0.83	0.26	0.00	0.93	0.00	0.58	0.05	0.05	0.32	0.01	3
			HS	1.00	0.83	0.57	0.28	0.94	0.63	0.78	0.06	0.08	0.29	0.01	5
		low	ambient	0.41	0.83	0.22	0.03	0.96	0.00	0.58	0.53	0.25	1.00	0.01	5
			HS	0.83	0.84	0.35	0.10	0.99	0.98	1.00	1.00	0.47	0.22	0.01	7
	summer	high	ambient	0.08	0.83	0.72	0.70	0.98	0.38	0.70	0.02	0.08	0.38	0.04	5
			HS	-0.01	0.87	0.75	0.76	0.98	0.63	0.87	0.04	0.21	0.54	0.04	6
		low	ambient	-0.05	0.83	0.69	0.22	0.97	0.33	0.64	0.12	0.77	0.49	0.29	5
			HS	0.85	0.83	1.00	0.58	0.99	0.53	0.70	0.15	0.68	0.47	1.00	8

Variable descriptions: D_{PSII}, damage to PSII; MSI, membrane stability index; Hsp70, Hsp70 expression; sHsp, chlp-sHsp24 expression; RGR_{leaf}, relative growth rate of leaves (RGR_{leaf} included negative values so transformed by adding 1); Vis. damage, proportion of plants showing visible damage; Flower/Fruit:AG, flower/fruit mass to above ground biomass; Flower/Fruit per day, number of flowers or fruits produced per day since heat stress; Survival, proportion of plants that survived the experiment; Sum total, sum of all variables, maximum possible value is 11.

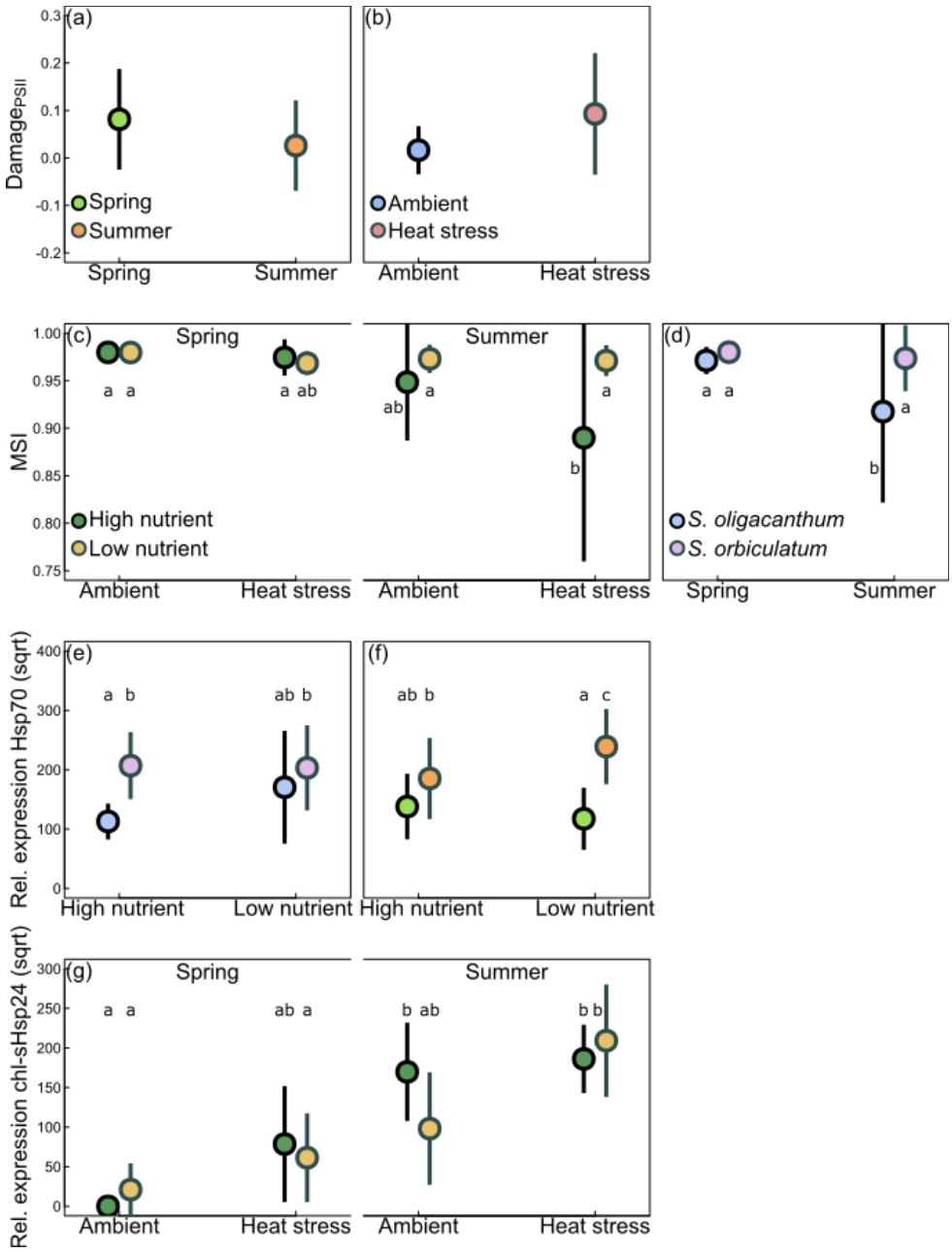

Figure 3.4. Mean (± SD) short-term physiological responses of *Solanum oligcanthum* (pale blue) and *Solanum orbiculatum* (purple) during a heat stress experiment in southern arid Australia. Significant interactions of membrane stability (MSI; a,b), relative expression of Hsp70 (c,d) and relative expression of chl-sHsp24 (e) are plotted. The colours of symbols are indicative of whether plants were grown in high or low nutrients (dark green and yellow respectively) and exposed to ambient (light blue) or heat stress (red) conditions in either spring (green) or summer (orange). Different lower-case letters above symbols indicate significant differences (p <0.05) among the means of treatments. Note that panels c and g represent three-way interactions, d-f show two-way interactions and a and b are main factors.

Growth and allocation of resources

In relation to resources allocated to leaves, *S. orbiculatum* had significantly higher LMA than *S. olicaganthum* (Table 3.1, Fig. **3.5a**). There were no effects of season, nutrient or heat stress treatment on LMA (Table 3.1). *Solanum oligacanthum* allocated greater mass to stems than leaves compared to *S. oribiculatum* (Table 3.1, Fig. **3.5b**). Overall, significantly more mass was allocated to stems than leaves in summer compared with spring (Table 3.1, Fig. **3.4c**) and stem:leaf was higher in low nutrient plants than high nutrient plants (Table 3.1, **Fig. 3.5d**). There was no effect of heat stress treatment on the stem:leaf ratio. Growth rate of leaves differed with the seasons, but depended upon both species and nutrients, with significant interactions with these factors (Table 3.1). Growth of leaves was significantly higher in spring than summer in both *S. oligacanthum* and *S. orbiculatum* (Fig**. 3.5e**). Leaf growth was significantly higher in high nutrient plants than low nutrient plants in spring, and significantly reduced in overall summer relative to spring, such that there was no difference between nutrient treatments in summer (Fig. **3.5f**). RGR_{leaf} was significantly reduced in plants that were heat-stressed compared with their ambient counterparts (Table 3.1, Fig. **3.5g**). Overall, RGR_{leaf} was reduced in summer compared with spring, with low nutrient plants faring more poorly than their high-nutrient counterparts (Table 3.2).

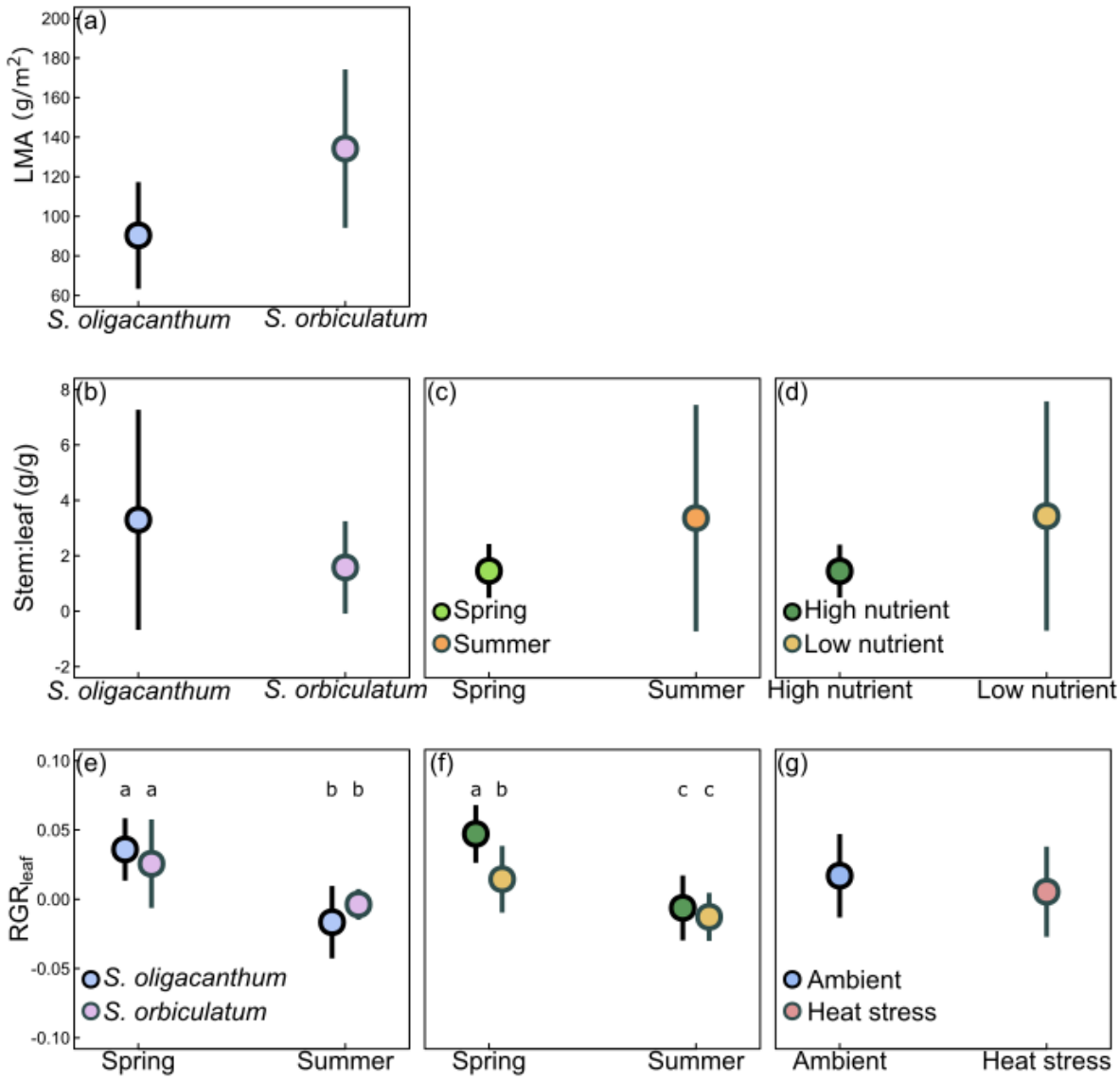

Figure 3.5. Responses of growth and allocation of biomass in two species of *Solanum* subject to heat stress (mean ± SD). *Solanum oligacanthum* and *S. orbiculatum* were grown in high or low nutrients and subjected to heat stress or ambient conditions in either spring or summer. Colours are described in Fig. 3.4. Variables are: LMA (a); stem to leaf ratio (b); and relative growth rate of leaves (RGR_{leaf}, g day^{-1}). Significant interactions are plotted e and f. Different lower-case letters above symbols indicate significant ($P < 0.05$) differences between the means of treatments. Note that panels e and f show two-way interactions and a-d,g are main factors. Means of main factors of aboveground biomass can be seen in Table S3.2.

Visible damage and survival

Both species showed visible damage to leaves and stems and suffered some deaths following heat stress treatment. The influence of nutrient availaiblity on the proportion of plants showing visible damage was determined by heat stress treatment (Table 3.1). Generally greater damage was observed in heat-stressed plants than plants under ambient conditions. Low-nutrient, heat-stressed plants incurred significantly more damage than their ambient-grown counterparts (Fig. **3.6a**). There were three significant interactions explaining the proportion of plants that survived heat stress (Table 3.1). First, survival was determined by species, nutrient availability and heat stress treatment. When *S. oligacanthum* had access to nutrients survival of heat-stressed plants was comprable to ambient plants, under low nutrient conditions and after heat stress survival was minimally reduced compared with ambient counterparts (Fig. **3.6b**). On the other hand, survival of *S. orbiculatum* plants was significantly reduced in heat-stressed plants compared with ambient plants, regardless of nutrient availability (Fig. 3.6b). Second, survival was influenced by heat stress depending upon the season in which the heat stress occurred but post hoc differences could not be determined (Table 3.1, Fig. **3.6c**). Generally, within a season, survival was reduced in heat-stressed plants compared with ambient plants and fewer ambient plants survived in summer compared with their spring equivalents. Finally, species differences were apparent in relation to season but post hoc differences could not be determined (Table 3.1, Fig. **3.6c**). Typically, *S. orbiculatum* had the poorest survival rates, irrespective of season, and *S. oligacanthum* surivival was lower in summer than in spring (Fig. **3.6d**). Generally, visible damage was greatest in high nutrient plants in summer, compared with other treatment groups (Table 3.2). The influence of nutrient status on survival was converse to its influence on visible damage; a greater proportion of high nutrient plants surivived than their low nutrient counterparts, and *S. oligacanthum* appeared to survive better than *S. orbiculatum* (Table 3.2).

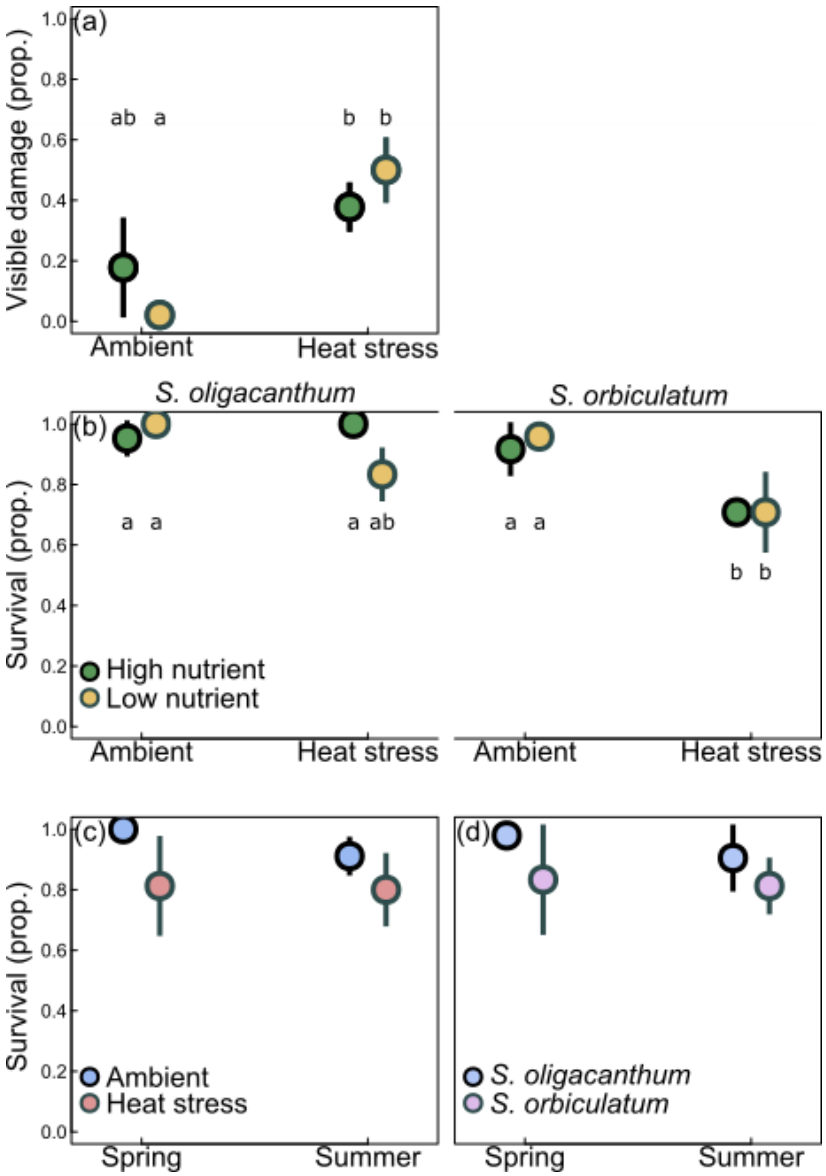

Figure 3.6. Visible damage and survival of desert *Solanum* species following heat stress treatment (mean ± SD). Colours are explained in Fig. 3.4. Proportion of plants with visible damage greater than 10% (a); proportion of surviving plants (b-d). Significant two-way interactions are shown in panels a, c and d and three-way interaction in panel b. Different lower-case letters above symbols indicate significant ($P < 0.05$) differences between the means of treatments. Note that panel b represents a three-way interaction and a,c and d show two-way interactions.

Fitness

The number of flowers produced following heat stress treatment was determined by season and influenced by both nutrient and species (Table 3.1). For plants with access to high nutrients, flower production was high and there was no effect of season; for low nutrient plants, however, the number of flowers produced was significantly reduced in summer compared with spring (Fig. **3.7a**). Species differences were observed in flower production but dependent on season (Table 3.1): the number of flowers produced by *S. oligacanthum* was significantly reduced from spring to summer (Fig. **3.7b**). Generally, *S. orbiculatum* produced fewer flowers than *S. oligacanthum* but unlike *S. oligacanthum*, the number produced increased from spring to summer (Fig. **3.7b**). Plants that were heat-stressed produced significantly fewer flowers than plants under ambient conditions (Table 3.1, Fig. **3.7c**). The biomass allocated to flowering was affected by an interaction of species with season and heat stress (Table 3.1). Regardless of season or heat stress treatment, *S. orbiculatum* allocated similarly low biomass to flowers compared *S. oligacanthum* (Fig. **3.7d**). *Solanum oligacanthum* had notably high flower:aboveground biomass ratios in spring, which then dropped to being signicantly lower in summer (Fig. **3.7d**). Generally, there was a small, non-significant, reduction in fruit production in heat-stressed plants compared with ambient treatment plants; however the greatest effect on fruit production was nutrient availability (Table 3.1). Plants with access to additional nutrients produced significantly more fruit per plant than those in low nutrient soils (Fig. **3.7e**). The allocation of biomass to the fruit production was affected by a species by season interaction (Table 3.1), such that *S. oligacanthum* allocated low amounts of biomass to fruit, irrespective of season, but the amount of biomass allocated to fruit in *S. orbiculatum* was significantly reduced in summer compared with spring (Fig. **3.7f**). Comparison of proportional reproductive output of each species under different treatments clearly shows that *S. orbiculatum* has greater seed output per plant than *S. oligacanthum* (Fig. **3.7g,h**). Seed production of both species was higher when plants had access to high nutrients, compared to low nutrient conditions. Overall, access to nutrients appeared to be the most important factor influencing reproductive fitness, with the contrast between species being greatest in summer, where the difference between low and high nutrient *S. orbiculatum* fitness was less defined than that of *S. oligacanthum* (Table 3.2).

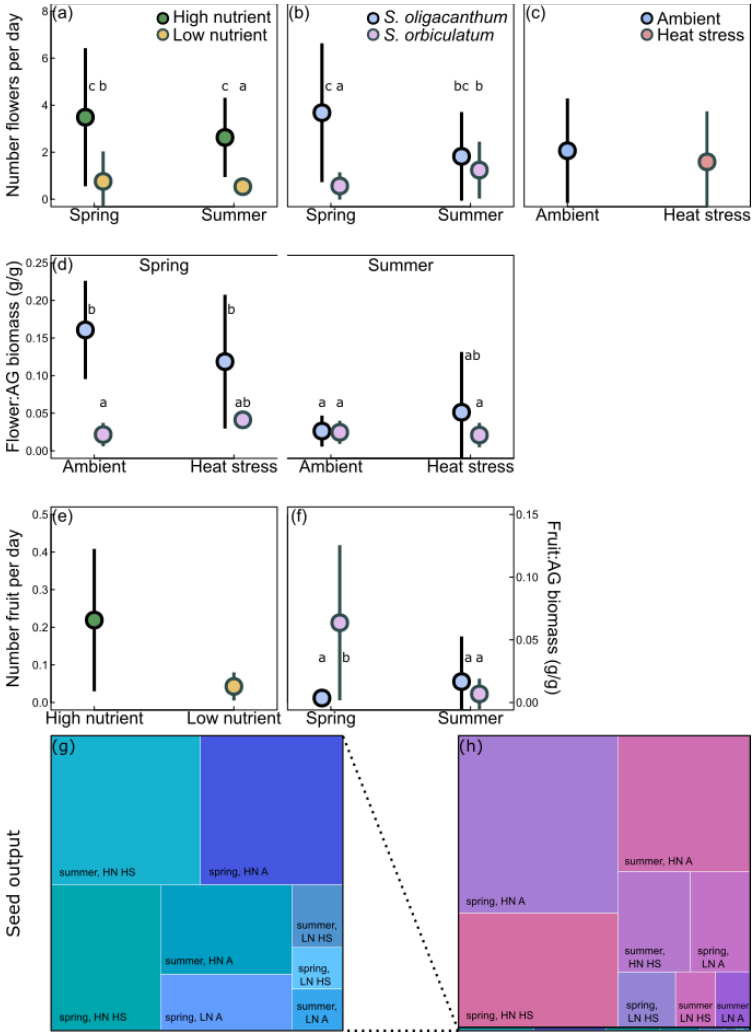

Figure 3.7. Fitness and allocation of resources to reproductive structures of desert *Solanum* species in response to nutrient availability and seasonal heat stress (mean ± SD). Colours are explained in Fig. 3.4. Number of flowers produced per day following heat stress treatment (a-c); flower mass to aboveground (AG) biomass (d); Number of fruits produced per day following heat stress treatment (e); flower mass to AG biomass (f). Note, panels c and e show main factors, two-way interactions are shown in panels a,b,f and a three-way interaction in panel d. Different letters indicate significant ($P < 0.05$) differences between the means of treatments. Relative proportional representation of estimated seed output of *S. oligacanthum* (g) and *S. orbiculatum* (h). Fruit were harvested following heat stress treatment (ambient, A; or heat stress, HS) on plants grown in low (LN) or high (HN) nutrients in spring or summer. The mean number of seeds plant^{-1} was calculated using the mean number of seeds fruit^{-1} x number of fruit plant^{-1} day^{-1}. Note that the panel on the right contains both species, with *S. oligacanthum* represented by the very narrow strip at bottom, which is magnified on the left to show *S. oligacanthum* seed output only. Mean seed output by each species by factor is shown in Table S3.2.

3.4 Discussion

One of the greatest concerns for plants regarding climate change is whether they will be able to tolerate and persist under more frequent and intense temperature extremes, particularly in resource-limited environments such as deserts. In this study, I investigated the impacts on two desert *Solanum* species of nutrient level and timing of heat stress events on plants' short-term tolerance and their reproductive fitness in the longer term. Overall, I found that 1) a heat stress event in summer is worse than a heat stress event in spring; 2) plants responded to heat stress in summer by expressing more Hsp70 and chlp-sHsp24 than in spring; and 3) the influence of nutrient availability on downstream effects of heat stress is species-specific.

Summer is harsh for plants and the worst time for a heat stress event

I expected that spring heatwaves might confer greater damage to plants, which are not primed for high temperature extremes. In contrast, my heat map patterns highlight that, overall, the most damaging time to experience heat stress was in summer (Table 3.2). I suggest that summer was worse because firstly, these desert species maintain a high basal tolerance, resulting in some resilience to high temperatures in spring. Recent records of spring temperatures in these regions have included spikes of up to 45.4°C (BoM 2019). So it follows that basal thermal tolerance for species adapted to this environment must be high to maintain a thermal safety margin (O'Sullivan et al. 2013, Drake et al. 2018). Basal photosynthetic thermal thresholds (T_{50}) of both species were in the range of 44 – 45°C (Chapter 2). Therefore, despite not being primed in spring, my study species generally incurred less damage in spring than in summer, suggesting an inherent resilience to a-seasonal high temperatures.

The second reason plants experiencing a summer heatwave fared worse than in a spring heatwave, is likely due to the combined severity of both heat stress and other stressful conditions pre-, during and post-heat stress being greater in summer. In mesic environments, differential responses to the timing of stress events have been attributed to the event severity. A heat stress in spring can be beneficial if temperatures rise to those optimal for photosynthesis (Marchand et al. 2005, De Boeck et al. 2011), whereas even average temperatures in summer might be supra-optimal. Similarly in desert environments, a brief warm period in spring might stimulate photosynthesis, but not be sufficiently sustained to cause long-term damage. By contrast, ambient desert conditions in summer can exacerbate other stresses like water limitation; in fact, drought alone has a greater effect on plant health than heat stress alone (De Boeck et al. 2011, Orsenigo et al. 2014, Davies et al. 2018). In my study, all my plants experienced water stress, exacerbated by higher VPD (Fig. **S3.4**), such that even in the absence of heat stress, my ambient treatment plants often did more poorly in summer than in spring. In summer, drought stress was likely to be compounded by an element

of root crowding, particularly in low nutrient plants. Therefore, even though leaf temperatures of heat-stressed plants were similar in spring and summer (Fig. **3.3**), the additional severity of background conditions in summer will have compounded the effects of temperature stress.It is likely that the natural heatwave contributed to the poor outcomes for my summer plants. My findings thus provide realistic insight into the susceptibility of desert plant species as the frequency of these kinds of events continues to increase as predicted (Cowan et al. 2014).

Not only are conditions during a summer heat event more severe, but supra-optimal conditions following the event are likely to hamper recovery in the long term. Physiological recovery from heat stress occurs when benign temperatures (Drake et al. 2018, Guha et al. 2018), including cooler nights (Atkin et al. 2005a) and/or access to water (Wang and Huang 2004) return. Although my plants were returned to water immediately following heat stress, long-term effects of drought on tissue damage, survival and fitness can manifest some months after the stress event (Wang et al. 2016, Davies et al. 2018). In my study, the longer-term, damage was most visible and fruiting most reduced after summer heat stress, particularly in *S. orbiculatum* (Table 3.2), suggesting physiological repair was somewhat suppressed, possibly due to lack of adequate recovery conditions. Repeat extreme events are likely in summer and indeed, my summer plants experienced a naturally occurring heatwave in the days before the heat stress treatment. In these desert summers, there is a relative lack of cooler night time temperatures, which, coupled with reduced access to water and/or increased likelihood of experiencing repeated stressfully high temperatures are all likely to reduce opportunities for recovery.

The effects of nutrient status on protection and downstream costs

In terms of mechanisms associated with protection, my expectation was that plants primed for heat stress would produce more HSPs, as shown in laboratory-based priming experiments (Larkindale et al. 2005, Larkindale and Vierling 2008, Suzuki et al. 2008). In this study, one of the first to investigate seasonal (i.e. longer term) HSP expression under natural conditions, both my desert *Solanum* species expressed greater amounts of Hsp70 and chl-sHsp24 in summer (Fig. **3.4f,g**). Hsp70 was detected in all samples but expressed in higher amounts in heat-stressed plants (Table 3.2), presumably because this protein is required for both basal (Preczewski et al. 2000, Kumar and Wigge 2010) and acquired thermal tolerance (Sung et al. 2001, Mittler et al. 2012). Like many small HSPs, chl-sHsp24, was expressed during stress and hardly detected in spring, supporting the notion that expression is required not only for protection but also acquired thermal tolerance (Charng et al. 2006, Hu et al. 2015, Davies et al. 2018). That this small protective protein was detected in high amounts in ambient plants in summer (Fig. **3.4g**) points to the stressful combination of drought and high temperatures.

I would generally expect that plants with access to more resources will be better able to use nutrients to protect against damage (Heckathorn et al. 1996a) or recover (Zhao et al. 2008). My species illustrate different responses to summer heat stress, depending on whether this occurred under high or low nutrient conditions. In *S. oligacanthum* plants with access to high nutrients that were summer-stressed expressed low amounts of Hsp70 and chl-sHsp24. These plants avoided damage to PSII, but incurred increased membrane instability (Fig. 3.4c,d) and visible damage and had reduced growth rate (Table 3.2). When under low nutrient conditions and heat-stressed, this species expressed high amounts of both Hsp70 and chl-sHsp24, which initially appeared beneficial in protecting membrane stability (Table 3.2). The long-term outcomes for *S. oligacanthum* under high and low nutrient was stark, high nutrient plants were able to survive and reproduce, while low nutrient plants did not (Table 3.2, Fig **3.7e**). Overall, for both species, those with access to high nutrients generally fared better than plants in low nutrients, even if they incurred damage or expended resources on protection during heat stress (Table 3.2). Although high nutrients may have afforded plants greater potential for protection via HSP expression, the high amounts may reflect greater damage because expression, while greater, was insufficient to mitigate against the stress incurred by these relatively more robust plants. Whatever the reason, protein expression is resource intensive, bearing potential long-term costs, especially for nutrient-stressed plants (Table 3.2). The final number of fruit produced per plant was determined only by nutrient status (Fig. **3.7e**). This result suggests that if these species have access to nutrients the cost of damage and repair can be mediated in terms of fitness. These findings suggest that nutrient status affects the overall longer-term outcome for my study species, especially following summer heat stress.

Potential drivers for species-specific responses and fitness

Ultimately, predicting persistence of species under altered occurrences of extreme events requires an understanding of the fitness costs of heatwaves. The resources allocated to reproduction and the ability to produce fruit is a critical component of future survival. Overall, short-term responses to seasonality of heat stress were similar between my two species (Table 3.2). However, there was disparity between species longer-term responses of survival and reproductive allocation. *Solanum orbiculatum* produced more, smaller seeds per fruit than *S. oligacanthum* (1.5 ± 0.2 mg *versus* 7.3 ± 0.7 mg in size, respectively and Table S3.2). Irrespective of the difference in seed size, overall seed production per plant was far greater for *S. orbiculatum* than *S. oligacanthum* (Fig. **3.7h**). These species-specific outcomes suggest the potential role of adaptation to microhabitats being important in heat stress responses.

To endure heat stress events, *S. oligacanthum* appears to utilise a strategy of die-back and resprouting of ephemeral shoots (Fig. **S3.7**), a trait suitable for its preferred microhabitat, which tends to be prone to inundation and used by stress-avoiders (Kassas and Girgis 1970).. This species showed a strong tendency for vegetative resprouting, with lower LMA, higher stem:leaf ratio (Fig. **3.5a,b**) and greatly reduced growth rate of leaves in summer compared with *S. orbiculatum* (Fig. **3.5e**). In a similar way that fire resprouters allocate more biomass to storage than reseeders (Pate and Bell 1999), the ability to store resources following severe damage may explain why *S. oligacanthum* had greater survival and maintained flowering after heat stress. Many resprouted *S. oligacanthum* had developed leaves during the experiment (Fig. **S3.5**) and the high survival rate of this species infers the potential to reproduce later (Geber 1990, Friedman and Rubin 2015). Extended longevity via resprouting may be an adaption to stochastic events in arid environments where reproductive potential is stored for rare and episodic recruitment events (Nano and Clarke 2011 and references therein). Through vegetative propagation, *S. oligacanthum* avoids the bad and can exploit the better conditions in the dynamic microhabitat in which it grows.

Solanum orbiculatum, which typically grows in resource-poor areas such as sandy dunes and plains, expresses traits reflecting this environment; for example, high LMA leaves. Like *S. oligacanthum*, *S. orbiculatum* is said to resprout following fire (Nano and Clarke 2011), but I did not observe resprouting following severe heat stress damage. In response to extreme events in summer, *S. orbiculatum* appears to dedicate meristematic activity to immediate reproduction rather than vegetative growth and delayed reproduction, with reduced RGR_{leaf} (Fig. **3.5e**) and increased flower numbers (Fig. **3.7b**). Coupled with its low survival, lack of resprouting in *S. orbiculatum* highlights the importance of high seed output for its ongoing persistence. The energy requirement for reproduction could put this species at risk of death if recovery conditions are not suitable. Extreme conditions are said to induce more clonal over sexual reproduction (Abeli et al. 2012). Given that summer heat has a marked negative impact on *S. orbiculatum*, there is likely to be strong selection to shift phenological patterns. However, as with many such species living in extreme regions, the prospects for adaptation outpacing currently poor survival under heat stress are fair at best.

Little work has looked at the timing of heat stress events (De Boeck et al. 2011, Wang et al. 2016) and few heat tolerance studies consider plants from desert environments. My findings suggest that, for species that possess high basal thermal tolerances, like those in deserts, a-seasonal heat stress events occurring in spring pose less risk than they might for species in more benign environments. However, repeated events during the height of summer have implications for fitness and survival, especially in low nutrient conditions. While it would be useful to be able to generalise about how all species will endure increased summer heat

stress, the species-based differences found in this study highlight the risk in doing so. To best understand and manage productivity and survival in harsh environments, attention should be directed to plant responses based on ecologically relevant signatures, such as life history and microhabitat.

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Author Contribution

Together with A. Leigh, S. Valenzuela and K French, K Milner designed the experimental procedure and methods. K Milner undertook the field work, analysis and led the writing of the paper. S. Valenzuela supervised the analysis of heat shock proteins, while Leigh and French supported the field work. D. Krix helped with the analysis.

CHAPTER 4: SEASONAL CHANGES IN FUNCTIONAL PROTEIN GROUPS, IN CONTRASTING DESERT PLANT SPECIES

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4.0 Abstract

To cope with warming seasonal temperatures, plants have the ability to upwardly adjust thermal thresholds. Adjustment to high temperature is of particular importance in the desert, where plants can be exposed to extreme high temperatures in summer. In this study, molecular tools were used to uncover the changes occurring at the level of proteins, making change in thermal tolerance possible. I predicted that proteins with functions in lipid metabolism and photosynthesis should be altered to maintain functioning under new thermal regimes, while proteins involved in secondary metabolism, redox homeostasis and heat shock proteins (HSPs) should be highest in summer, when high temperatures are most extreme. Australian desert species; *Acacia ligulata*, *Myoporum montanum* and *Solanum oligacanthum*, were grown in a common garden in semi-arid southern Australia and sampled in winter, autumn and summer. In all species thermal thresholds of PSII health (measured via chlorophyll *a* fluorescence) and membrane stability (assessed via electrolyte leakage), were highest in summer, however *A. ligulata* did not adjust membrane thresholds to the higher seasonal temperatures. Common to all species, rather than alter amounts of light harvesting proteins or Rubico, these plants appeared to use control of Rubisco activity to match photosynthesis to prevailing conditions. Lipid re-modelling and re-balancing of redox homeostasis was detected, and in contrast to predictions, a secondary metabolism protein for isoprene production was lowest in summer. Small HSPs were highlighted as important for tolerance of photosyntheis and membranes, with increased expression detected in all species. Acquired thermal tolerance is a multi-gene response, my exploration of seasonal change in proteins has identified some of the ways in which plants adjust with warming temperatures and expands our knowledge of proteins that naturally occur in the environment.

4.1 Introduction

Plants are adapted to the environmental conditions they frequently experience and one of the greatest climatic factors driving plant distribution and traits is temperature (Moles et al. 2014). Acute change in temperature affects rates of key physiological processes, such as photosynthesis (Drake et al. 2015, Crous et al. 2018) and respiration (Atkin et al. 2000, Atkin and Tjoelker 2003). Rate changes are often the first adjustments plants make to new temperatures, however adjustment to chronic/seasonal change can take days to weeks for gene expression and enzyme activity to alter capacities so that appropriate physiological rates are maintained in the long-term (Tattersall et al. 2012). In order to maintain optimal functioning, plants adjust their temperature optima for photosynthesis and respiration (Berry and Bjorkman 1980, Lin et al. 2012) and their thermal thresholds (Zhu et al. 2018, Curtis et al. In review).

Over the expanded temporal scale, seasonal change results, in part, from alteration to the leaf proteome.

As part of the acclimation process, particularly in environments with limited resources or high stress, the partitioning of resources must be allocated carefully to maximise growth and fitness outcomes. For example, plants should maximise instantaneous photosynthesis by directing leaf N to the limiting step, which may be the raw products (C, water or light), the activity or amount of the photosynthetic machinery or removal of end products (sugar) (Warren and Adams 2004). To optimise carbon assimilation in winter, when temperature may limit rates of photosynthesis and light levels are low, leaf N may be directed proteins involved in light capture or enzymatic activity (e.g. Rubisco regeneration, Yamori and von Caemmerer 2009). In summer, when water is potentially scarce but temperature and light are not limiting, we might expect to see more Calvin cycle (carbon capture) proteins such as Rubisco or Rubisco activase because stomatal closure associated with dry conditions reduces internal CO_2 concentrations (Galmés et al. 2005) and high temperatures reduce Rubisco specificity (Yamori et al. 2006, Galmés et al. 2015). But how resources might be allocated across the whole suite of leaf proteins necessary for maintainance of photosyntheis is not known. It follows that a careful but dynamic balance would be maintained, to direct limited resources to where they can benefit most under given circumstances.

The ability to withstand or to acclimate to higher than optimal temperatures results from prior exposure to non-lethal temperatures, repair of heat-sensitive components and prevention of further injury during stress via maintenance of metabolic homeostasis (Hemme et al. 2014). Despite adjustments to better suit prevailing conditions, upper temperature thresholds are crossed during hotter months (Chapter 3), especially under a warming climate. Damage during heat stress includes protein denaturation (Feder 1999, Eisenhardt 2013), loss of membrane integrity (Daniell et al. 1969, Bukhov et al. 1999) and high production of damaging reactive oxygen species (ROS) (Mittler 2002) (Figure 4.1). Acclimation and stress response include a range of physiological alterations (Figure 4.1). Membranes are composed of three types of macromolecules: fatty acids, sterols and proteins, the proportions and types determining the thermal stability (Nomura et al. 1999, Wang et al. 2017). As they are the major component of membranes, saturation level of fatty acids is crucial for maintaining optimal fluidity, with increased saturation improving heat tolerance (Raison et al. 1982, Murakami et al. 2000, Larkindale and Huang 2004). Upregulation of HSPs offers improved thermal tolerance (Vierling 1991, Preczewski et al. 2000, Knight and Ackerly 2003a). Small HSPs (sHSPs) aid in the maintenance of electron transport (Downs and Heckathorn 1998, Coleman et al. 1999, Downs et al. 1999b) and hold denatured proteins for re-folding by the larger HSPs (Hsp70, Hsp90 and Hsp100) (Al-Whaibi 2011, Bita and Gerats 2013, Wu et al. 2013). Stress protein

upregulation is costly and this is likely why we do not see high levels of protective heat shock proteins (HSPs) all year round (Chapter 3). Redox homeostasis must be maintained to avoid the damaging effect of free radicals. Stress-induced ROS production in chloroplasts (Foyer et al. 1994) and mitochondria (Møller 2001) cause membrane lipid peroxidation (Havaux 2003). ROS detoxification protects against high temperature (Larkindale and Knight 2002, Suzuki and Mittler 2006). Therefore, plant stress tolerance is closely linked to an increased capacity to scavenge and detoxify ROS (Foyer et al. 1994, Wang et al. 2014b, Sgobba et al. 2015) via antioxidants including glutathione and ROS-scavengers peroxidases (Takahashi and Murata 2008). Proteins involved in secondary metabolism of heat tolerance include those related to terpenoids (isoprenes, monoterpenes), phenolics (anthocyanins) and alkaloids. These compounds are generally upregulated during heat stress to convey tolerance (Rivero et al. 2001, Wahid et al. 2007). For example, isoprenes confer protection during heat and oxidative stress (Sharkey et al. 2008, Harrison et al. 2013).

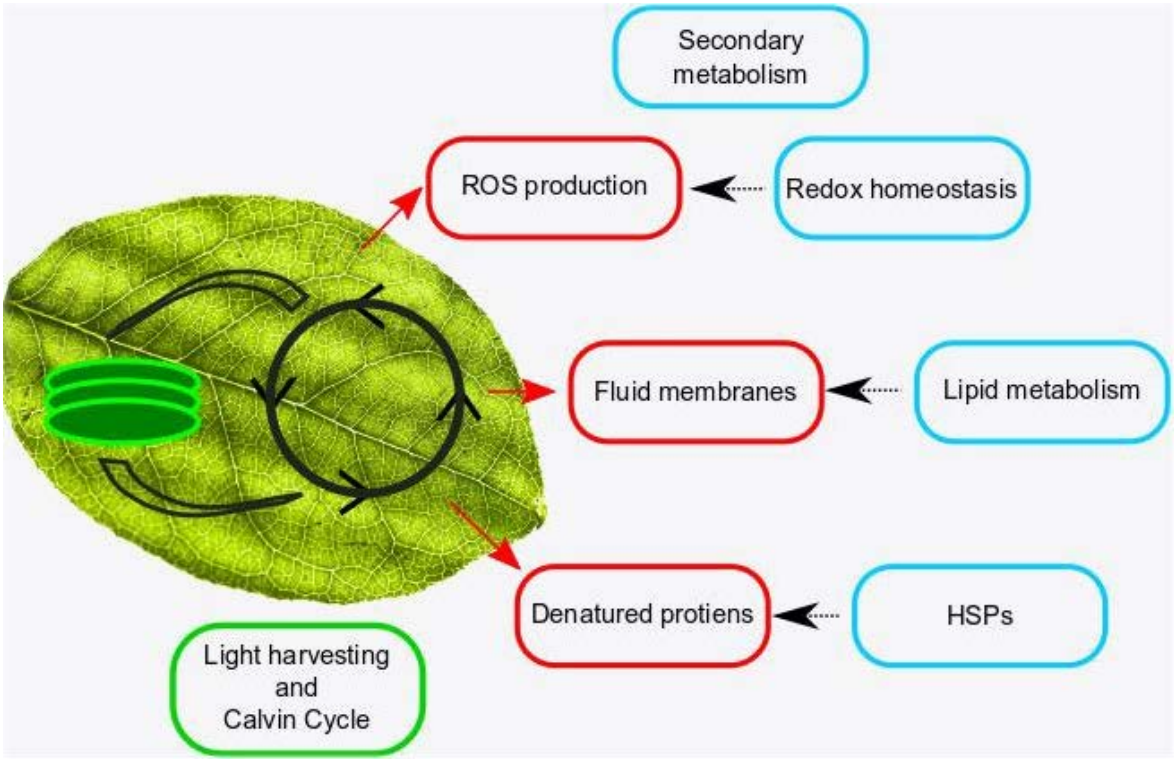


Figure 4.1. Proteins required for the stress response and involved in acquired thermal tolerance (blue boxes) of photosynthesis (green box) and membranes. When temperatures cross thermal thresholds, stress occurs, including increased reactive oxygen species (ROS) production, and damage to proteins and membranes (red boxes). Photo: Annie Spratt.

In Australian deserts, where soils are nutrient poor, plants typically have long-lived leaves (Orians and Milewski 2007). Rather than plants dropping leaves and building new ones to

better suit the season, many alter cellular make-up for temperature resilience. We therefore might expect that plants from these environments alter their proteome profile seasonally to optimise allocation to thermal protection versus growth and reproduction for changing circumstances. In this study, my aim was to link physiological threshold measurements to changes in absolute amounts of protein to achieve a mechanistic explanation of acquired thermal tolerance. I selected three Australian desert species representing different functional types and microhabitats within the arid zone. My focus was on specific protein functional groups: photosynthesis, lipid metabolism, environmental stimuli response (HSPs), redox homeostasis and secondary metabolism. My first expectation was that leaf N would be directed to those proteins involved in photosynthesis that result in maintenance or increased rates of carbon assimilation under the new seasonal conditions. For the other functional groups, I predicted increases in proteins required for acquired thermal tolerance to deal with stress and recovery as the temperature rises from winter to summer. For example, expectations were for increased saturated fatty acid synthesis, more HSPs, antioxidants and ROS scavengers and isoprenes. Finally, given their functional differences, I predicted that each species would have a different expression patterns in relation to warming with season.

Proteomics have been used for some time to examine plant responses to the environment. Early methods used 2-D gels coupled with mass spectrometry to identify proteins that changed between control and experimentally imposed conditions (for example, Bedon et al. 2012, Pinheiro et al. 2014, Wang et al. 2017) and these methods are still used today. More recently, gel-free shotgun proteomics has become the preferred method (e.g. in a special edition of *International Journal of Molecular Science*, shotgun proteomics is used in 21 out of 27 research articles (Komatsu 2019)), but the experimental design remains the same: differential relative expression is investigated, generally in economically important species. With improved shotgun proteomics, including SWATH acquisition, a highly effective extraction method and quantification of the leaf proteome (mg m^{-2} leaf area)(Aspinwall et al. 2019), I was able to explore subtle seasonal changes in the leaf proteome. SWATH acquisition is data independent, allowing quantitative MS/MS for every peptide in a sample. The method I used was further improved by setting narrow width precursor isolation windows (swaths) at lower mass to size ratio (m/z), where there is a high abundance peptides (Gillet et al. 2012). Species from the extreme desert environment have not been investigated using this novel technique. By applying this highly accurate approach to desert plants across seasons, my aim was to determine how adjustments to the proteome might enable species to increase thermal tolerance, while optimising primary metabolism under altered seasonal conditions.

4.2 Method

Species selection and growth conditions

To provide understanding of how desert plant species adjust to season, three species from different functional groups were selected: *Acacia ligulata* A.Cunn. ex Benth., a widespread nitrogen-fixing shrub, growing predominantly on sand dunes; *Myoporum montanum* R. Br., a widespread shrub preferring wetter sites; and *Solanum oligacanthum* F.Muell., a restricted perennial herb that seasonally flooded watercourses (Figure 4.2). Plants were grown in a common garden setting on sandy soils *in situ* in a desert environment in southern arid Australia (at the Australian Arid Lands Botanic Garden, Port Augusta, South Australia; 32°28'2.58"S, 137°44'38.70"E). *Myoporum montanum* (parent source unknown, but occurs locally) and *S. oligacanthum* (original plant material sourced from Mungerannie South Australia) were grown from cuttings collected on site, both growing in the local sandy soil in the common thermal environment. *Acacia ligulata* was grown from seed (Goldfields area, Western Australia, closest town Kalgoorlie) although naturally occurring *A. ligulata* grows adjacent to the study plot. Water was supplied to the plants via sub-surface irrigation during establishment. Each row was planted with six individuals of each species, randomly assigned to a position (at 1.5 m spacing), with each row repeated three times, resulting in 24 plants per species. Plants were grown for seven months to allow for root and shoot establishment before measurements and sampling.

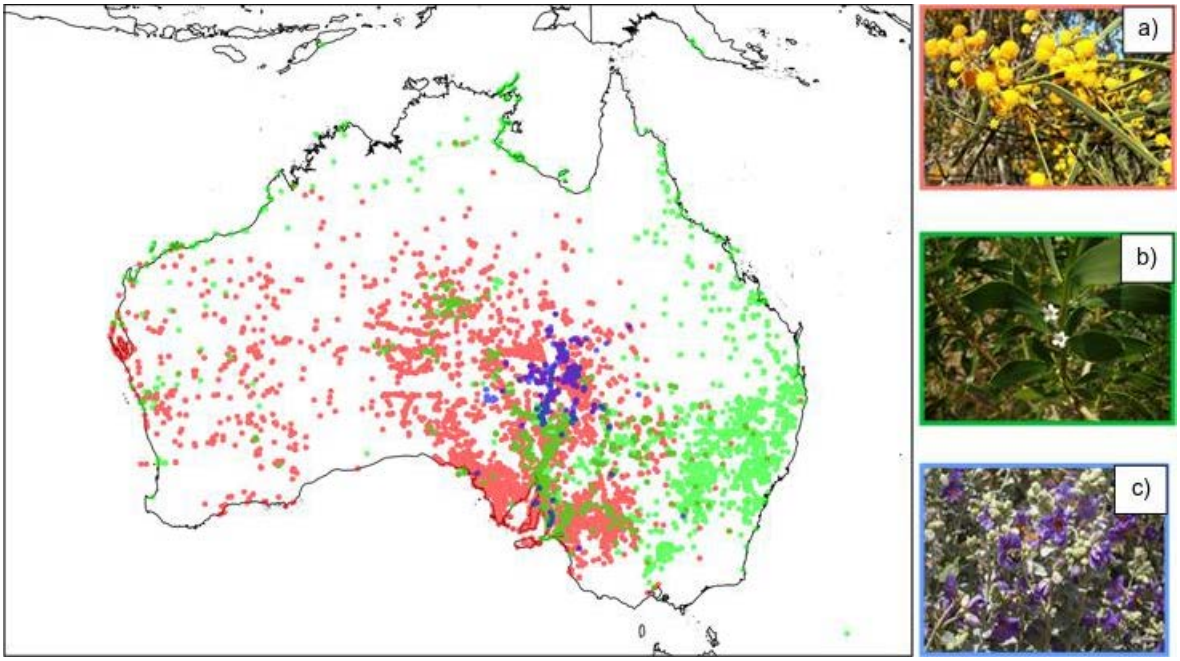


Figure 4.2. Species distributions according to occurrences recorded in Atlas of Living Australia (ALA 2018). The shrubs *Acacia ligulata* (red points; a) and *Myoporum montanum* (green points; b) are widely distributed, while the herb or sub-shrub *Solanum oligacanthum* (blue points; c) has a narrow distribution.

Measurement of thermal thresholds

As this is an exploratory study of leaf proteins expressed seasonally by desert plant species, my aim was to link proteomics to physiology. For this reason, thresholds of PSII and membrane thermal tolerance were assessed. Sampling was conducted once each in winter (June 2016), spring (September 2016) and summer (January 2017) for all three species. (Figure 4.3). Due to the time taken to undertake thermal assays, species were sampled on separate days. Enough fully-expanded leaves for all assays were collected from the north side of five plants pre-dawn on the day of the assay. Where possible, leaves of the current season were collected, however, most Australian desert plant species do not have a defined growth season, with growth flushes tending to be opportunistic and based on rainfall so may occur year-round. Leaves were kept in dark, in moist zip-lock bags prior to sampling.

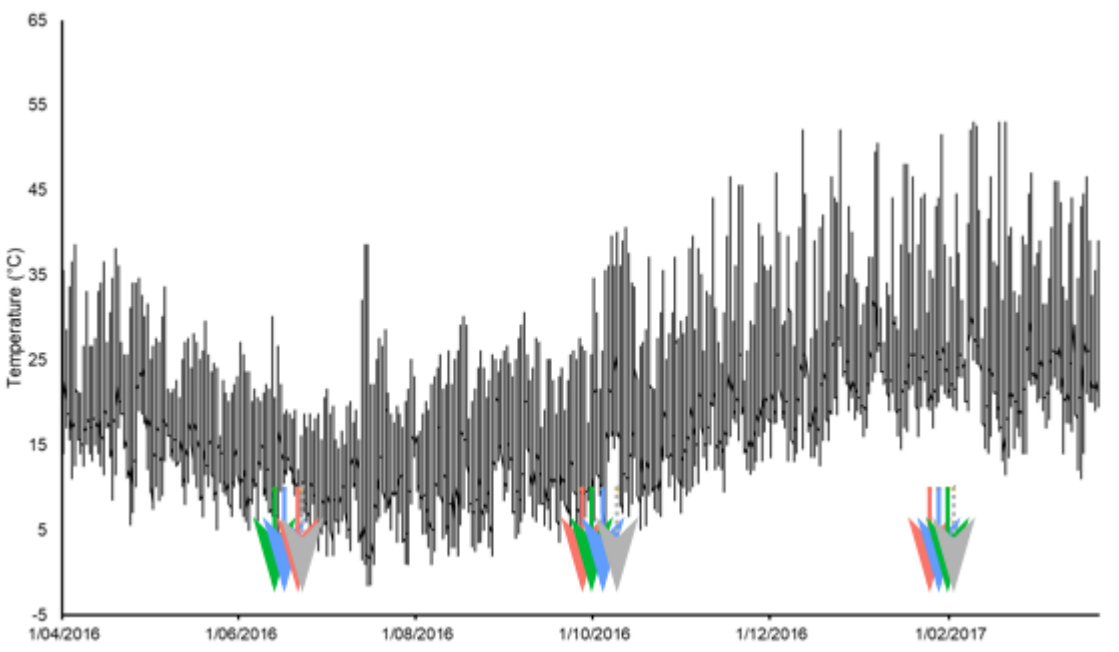


Figure 4.3. Hourly air temperature (°C) in Port Augusta, South Australia. Arrows indicate sampling points of protein samples (grey arrows), species thresholds (colours follow Figure 4.2). Air temperature collected using i-Button placed in the experimental garden.

Membrane stability threshold

Membrane stability index (MSI) was calculated as the inverse of relative electrical conductivity (Rollins et al. 1962, French et al. 2019).

$$MSI = 1 - \left(\frac{EC_{90} - EC_0}{EC_{max} - EC_0}\right)$$

Leaves were placed in five replicate tubes (n = 5 plants) with deionised water (~0.5 g leaf weight: 15 ml water) and electrolyte leakage was measured at various points using a conductivity sensor (TetraCon925; WTW, Weilheim, Germany). Initial electrical conductivity (EC_0) was measured 5 min after leaves were placed in tubes. Leaves were then exposed to temperature in the following sequence; 15 min at 28°C then 15 min at a treatment temperature (28, 44, 46, 48, 50, 52, 54 extended to 56 in spring and summer ± 0.2°C) and then 90 min at room temperature after which EC_{90} was taken. Tubes were placed in oven for 1hr at 100°C and left overnight, EC_{max} was read the following day.

Photosynthetic thermal threshold

The use of chlorophyll fluorescence (particularly maximum quantum yield; MQY or F_v/F_m) for measurement of photosynthetic thermal thresholds is well-established (Knight and Ackerly 2003a, Curtis et al. 2014). The method used follows Curtis et al. (2014). Briefly, ten leaves (n = 5 plants, 2 leaves per plant) were exposed to one of six of treatment temperatures (28,

42 (winter only), 44, 46, 48, 50, 52 (summer only) ± 0.2°C) in temperature-controlled water baths. Leaves were first exposed to 28°C for 15 min, followed by 15 min at their treatment temperature. Following the treatment temperature, leaves were left to 'recover' at 28°C for 90 min. While in the temperature baths leaves were kept under sub-saturating light (~280 µmol photons m^{-2} s^{-1}). Leaves were left overnight (in the dark) and F_v/F_m was measured (using a MINI-PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany) the following morning.

Identification and quantification of leaf proteomes

Fully expanded leaves were collected from the northern side of plants pre-dawn and placed in moist zip-lock bags (see Figure 4.2 for sample collection dates). Leaf material between 80-100 mg, and of known leaf area, was snap frozen in liquid nitrogen. For each species, three biological replicates, with three technical replicates.

Protein extraction

Protein extraction followed the method developed previously by Van Sluyter (Aspinwall et al. 2019). Leaf tissue was ground using a 6 mm Zirconox Satellite bead (Klausen) in a Qiagen TissueLyser (20Hz, 1.5 min, 6 times). Samples were chilled in LN_2 between each beating. Once ground the first stage of extraction aims to remove phenolics and pigments from the leaf tissue. Samples were kept chilled on dry ice for the following steps because low temperatures prevent solubilisation of protein in organic solvents and oxidation reactions are inhibited. Ground leaf material was washed twice in 1.5 mL Chloroform:Ethanol buffer (98% 1:1 Chloroform:Ethanol with 50 mM ammonium acetate, 1% 2-Mercaptoethanol, 0.5% 1 M diethyldithiocarbamate, 0.5% 0.4 M dithionite) and centrifuged (4300 g, -20°C). Hexane (1.5 mL) and then ethanol solution (1.5 mL 98% ethanol, 50 mM ammonium acetate and same reducing agents as Chloroform:Ethanol buffer) were used for a third and fourth wash respectively. The washed pellets were suspended in 0.7 mL borate-citrate buffer (0.4 M boric acid, 0.3 M trisodium citrate, 1.0 M LiCl, 50 mM glycine, 50 MM EDTA, 1% lithium dodecyl sulphate, 20 uM E-64, 1 mM benzamidine HCl, 2% 2-Mercaptoethanol, 2 mM sodium sulphite; pH 9.0 with NaOH) and immediately snap frozen in LN_2.

The second stage of the procedure results in the extraction of proteins, using phenol, from solvent washed leaf tissue. Ovalbumin was added to frozen samples (2.5 µg per cm^2 leaf area)—for downstream protein quantification—followed by 800 µL phenol (with 2% 2-Mercaptoethanol, 0.1 mg/mL butylated hydroxytoluene). Samples were shaken until thawed and immediately sonicated at 80°C for 10 min. Following sonication samples were mixed and placed in an ice bath for 10 min. To separate the phases samples were centrifuged at 14,500 g for 3 min. The top phenol phase was transferred to 15 ml Falcon tubes. The addition of

phenol was repeated a further two times with sonication at room temperature for 5 min, centrifugation and removal of the top phase. The aqueous phase and pellet was frozen (for potential extraction of cell wall-bound proteins). Cold glycine buffer with LiCl (4 mL; 50 mM glycine, pH 9.5 with NaOH, 1 M LiCl, 5 mM EDTA, 1% 2-Mercaptoethanol, 1 mM sodium sulphite, 0.5 mM sodium isoascorbate) was added to the phenol phases, mixed and centrifuged at 1500 g for 5 min. The aqueous top layer was discarded. To the bottom layer 4 mL cold glycine buffer without LiCl was added and treated as before. Samples were snap frozen in LN_2 and stored at -80°C at this point.

Proteins were precipitated from the phenol phase by filling the 15 mL tubes with cold 1:1 ethanol:diethyl ether with 0.1 M ammonium acetate. Tubes were left on ice overnight. Pellets were resuspended in 4 mL Ethanol:ether (with 1% water, 1% glycerol) by rasping and vortexing. Samples were centrifuged at 1500 g for 5 min and supernatant was discarded. Addition of further 4 mL of Ethanol:ether solution was followed by centrifugation as previously stated and the supernatant discarded. Pellets were then partially dried by standing upside down for 5 min, then on their side for 5-10 min. Pellets were then suspended in 700 µL of urea buffer (8 M urea, 50 mM lactic acid, 0.2 M LiCl, 1 mM EDTA, 2% lithium dodecyl sulphate, 10 mM Tris(2-carboxyethyl)phosphine hydrochloride, 5 mM cysteine, 1 mM sodium sulphite, 0.5 mM sodium isoascrobate) by vortexing. Suspension was aided by the addition of two 3 mm Zirconox beads (Klausen). Pellets were then sonicated at room temperature for 10 min so the mixtures resembled cloudy suspensions. To the suspensions 8 µL of N-methylmorpholine was added and vortexed until the protein went into solution. For cysteine alkylation 10 µL of 50% 2-vinylpyridine in methanol was added, followed by gentle vortexing, then incubation for 1 hr at room temperature. To cease reactions 5 µL of 2-mercaptoethanol was added and gently vortexed. At this point 4 µL of sample was diluted with 96 µL of water in a 96-well plate for protein quantification. Samples were snap-frozen in LN_2 and stored at -80°C. To determine protein amounts in each protein sample; samples and an 8-point BSA standard curve were run in triplicate using the FluroProfile Protein Quantification Kit (Sigma).

ASPEX – acetate solvent protein extraction

Acetate solvent protein extraction (ASPEX) was used to prepare samples for in-solution digestion for LC-MS/MS by removing substances that interfere with digests or mass spectrometry (Aspinwall et al. 2019). Aliquots of 50 µg protein were made to which 250 µL of 5 pmol/µL ApoMyoglobin, 67% methanol, 25% chloroform and% water solution was added. Tubes were then mixed by inversion (10 times). Immediately after mixing 500 µL of cold 10 M ammonium acetate was added and mixed as before. Samples were centrifuged at 15 000 g for 1 min. The top aqueous phase was removed carefully so as not to disturb the precipitated protein at the interphase. Ice cold water saturated diethyl ether (500 µL) was added to the

lower phase and mixed by inversion to precipitate proteins. Following precipitation of the proteins, 100 µL of 25% Trifluoroacetic acid in ethanol was added to protonate the acetate so that the small amount of residual aqueous phase became miscible with the diethyl ether phase, and mixed 10x. Following centrifugation at 4°C for 10 min at 15 000 g the supernatant was discarded. Once the solvents were removed the protein pellet was washed in 800 µL of Triethylamine solution (0.1 M Triethylamine, 0.1 M acetic acid, 1% water and 1% DMSO in 1:1 ethanol:ether) by vortexing (1 s) and centrifuged as before. The supernatant was again discarded and the protein pellets were ready for digests. At this stage the pellets were stored at -20°C.

Protein digestion

This protein digestion method used a staggered addition of Lys-C followed by trypsin to digest the 50 µg of sample cleaned by ASPEX. The mild detergent RapiGest (Waters) was used to improve access to proteolytic sites for enzymatic cleavage. Sample pellets were almost completely air dried before 25 µL of 40 ng/uL Lys-C (Wako), 0.3% RapiGest in 0.2 M N-methylmorpholine was added. Samples were placed in a Thermomix at 45°C, 1200 rpm for 15 min followed by sonication at 40-45°C for 45 min. Samples were cooled in a cold block for approximately 5 min to avoid evaporation and condensation. Trypsin (5 µL; Promega) was added before spinning to collect liquid and then samples were mixed by briefly vortexing. Samples were incubated overnight at 37°C. To stop the digest 6 µL of 12.5% Trifluoroacetic acid was added and incubated at 37°C for 45 min. Samples were then chilled on ice, centrifuged at 4°C for 10 minutes, > 15 000 g. The supernatant was used for mass spectrometry and was stored at -20°C.

Mass Spectrometry

In preparation for LC-MS/MS supernatants were diluted in 3% ACN, 0.1 formic acid in PCR plates (Thermo) and loaded into autosampler at 8°C. Analysis of samples followed Cain et al. (2019) with modifications (Aspinwall et al. 2019) on a TripleTOF 6600 System (SCIEX Singapore) with SWATH 2.0 acquisition. All mass spectrometry was performed by Van Sluyter. Data independent acquisition (SWATH) is a method to acquire MS data in predefined m/z windows across an entire LC-MS/MS (tandem mass spectrometry) analysis for consistent quantification across samples (Larance and Lamond 2015).

Protein identification and quantification

Databases were built by Van Sluyter at Macquarie University, for each of my three study species, using available genome sequences of the most closely related species. Transcript data from three *Acacia* species were contributed by Dr Adam Carroll, ANU. For *S. oligacanthum* a Uniprot query (taxonomy: "*Solanum lycopersicum* (tomato) (*Lycoperscion*

esculentum) [4081]") resulted in 35,987 sequences. Since *Myoporum montanum* has fewer well-studied close relatives, a composite database was created. Sequence sources included Scrophulariaceae species from 1kp project (Matasci et al. 2014) included SIBR (*Celsia arcturus*), XXYA (Verbascum sp.), EJBY (*Anticharis glandulosa*), and XRLM (*Buddleja lindleyana*), GRFT (Buddleja sp). A Uniprot search of Lamiales proteins at 90% identity (Uniref query: "uniprot:(taxonomy:lamiales) AND identity:0.9"), which gave 79,112 sequences, many from a *Mimulus guttatus* sequencing project (Hellsten et al. 2013), and the chloroplast sequence of *Scrophularia dentata* (Ni et al. 2016) were downloaded. These three sources were concatenated giving a total of 132, 579 sequences.

SWATH ion libraries, of the three species, were created by Van Sluyter according to Aspinwall et al. (2019) using ProteinPilot loaded with data-dependent acquisition (DDA) runs of pooled samples and the database sequences. Using the Paragon method, the software takes the sequence of amino acids and "digests" them to create an ion library. Search settings in ProteinPilot were based on sample preparation e.g. vinyl pyridine for alkylation, digestion with Lys C and Trypsin, and denatured urea buffer to look for potential modifications to the peptides. At peptide FDR of 1%, *S. oligacanthum* had 9,667 distinct peptides, *A. ligulata* had 8,999 distinct peptides and *M. montanum* had 7,805 distinct peptides. SWATH ion libraries were created by importing ProteinPilot results into the SWATH micro app in PeakView 2.2 (Sciex).

For quantitation and identification of proteins the ProteinPilot group files were loaded into PeakView Software (v2.2) using the SWATH ion libraries. For SWATH data analysis peptide confidence thresholds from from ProteinPilot corresponding to 1% peptide FDRs were used. XIC window was 8.0 min with width of 50 ppm.

Proteins were quantified according to Aspinwall et al. (2019)except that the top two ions of the top two peptides were used instead of the top three peptides (Ludwig et al. 2012). This corrects for issues with abundant and large proteins. Inclusion of ovalbumin meant that the amount of ovalbumin spiked into samples based on the leaf area of the sample gives a target protein amount in mg leaf area^{-1} and is required because the relationship between MS signal and amount of protein is non-linear and the area under the curve is unitless.

The general protein quantification calculations involved: 1) finding target protein areas (top2/top2); and 2) calculating target protein amounts relative to ovalbumin proteins as standards. Top2/top2: The sum of the top two most intense ions gave the peptide area. Then for the target protein the protein area was the average of the two peptides with the greatest area. 2) For each sample, the averaged peptide areas of two ovalbumin peptides (following top2/top2) were found. Areas of target protein were found relative to the averaged ovalbumin

area. Target protein areas were then converted to moles using the number of moles of ovalbumin per leaf area. Finally the amount of target protein (mg m^{-2}) was found using the molecular weight of proteins/peptides multiplied by the moles. The R package 'Peptides' (Osorio et al. 2015) was used to find the molecular weight of amino acid sequences. R scripts (authored by James Lawson, Macquarie University) were used to identify the top two ions and peptides and for calculation of non-isotopically-labelled proteins.

Mercator (X4 v1.0; (Lohse et al. 2014)) was used by Van Sluyter to assign proteins to MapMan BINS; hierarchical functional protein categories using the protein identifiers. Total proteins is all proteins identified with accension numbers..

Data analysis

Temperature thresholds of MSI and F_v/F_m

MSI and F_v/F_m were used to construct a temperature-decay curve of membrane stability and PSII health respectively. Curves, for each replicate plant (n=5), were constructed and the threshold temperature at 12% decline in health found using plcfit (Duursma and Choat 2017) in R (R Core Development Team 2010). To compare thresholds among species and seasons ANOVAs were conducted, with logical comparisons e.g. among species within a season or within a species among seasons. If significant, Tukeys *post hoc* comparisons were made to find where differences lay.

Protein analysis

Protein lists were modified before analysis. Table 4.1 shows numbers of proteins identified, removed and analysed. First, there was the removal of all single proteins with an average (across all samples of each species) of < 1 mg. Proteins were then summed into higher functional categories. Unassigned proteins that were not in the top 150 averaged ranked proteins per species were removed. The remaining unassigned proteins were assigned functional protein groups where possible using UniProt. Proteins were grouped on a hierarchical basis and examined at the level of two hierarchical levels for the entire proteome (Table S4.1). For the functional groups of proteins of interest (PoI) (1, 5, 9, 10, and 26) proteins were grouped into complexes or up to four hierarchical levels. The final list of protein groups used is presented in Supplementary information (Table S4.2). Due to the importance of photosynthesis and in particular Rubisco, the absolute amounts and percentage of these proteins relative to total protein were compared using 2-way ANOVA in R.

Table 4.1. Number of proteins identified, removed and used for downstream analysis for three Australian desert plant species: *Acacia ligulata*, *Myoporum montanum* and *Solanum oligacanthum*.

Species	Total ID	Singles & complexes	<1 mg	Total unassigned	Unassigned removed	Functional groups at 2 levels	Functional groups of PoI
A. ligulata	1560	1473	199	350	323	107	87
M. montanum	1164	1043	140	158	154	95	72
S. oligacanthum	1102	1041	136	193	172	108	82

Total proteins is all proteins identified with accesion numbers. Proteins and complexes is the number of quantified proteins and complexes. Proteins <1 mg are the total number of individual proteins removed from data set with a mean <1 mg. Total unassigned proteins are proteins with unknown function, some of these were manually assigned to functional proteins groups the rest were removed from analysis. Data were arranged to group proteins at two levels of hierarchy. There were a total of 122 groups of proteins at two levels of hierarchy, but some were not detected in all species.

Data were log transformed for multivariate analysis in PRIMER 6.0 (v6.1.14) (Clarke and Gorley 2006) and Bray-Curtis distances were used to create the resemblance matrix. PERMANOVA+ (v1.0.4) (Anderson et al. 2008) was used to test main effects and the interaction of different groupings of proteins among species and season. If there were positive main effects of season or species * season these were queried using pair-wise t-tests. The Monte Carlo P statistic was used due to the small sample size meaning necessary conditions for applying other tests may not be satisfied (Hope 1968). Any significant seasonal differences were investigated in SIMPER to identify the proteins that contributed the most to the dissimilarities between seasons. PCA was run in PAST with log transformed data (Hammer et al. 2001).

PatternHunter in MetaboAnalyst 4.0 (Chong et al. 2018, Chong and Xia 2018) was used to perform correlation analysis of protein expression against a given pattern of expression over seasons. The patterns tested were based upon the possible pattern that thermal tolerance thresholds could make with changing of the season. Spearman's R was used and only significant results displayed. Proteins that did not change with season were identified using one-way ANOVA and a $-\log_{10}(p) < 0.1$ in MetaboAnalyst. Species were analysed separately and data were generalised logarithm transformed.

4.3 Results

Temperature thresholds of PSII and membranes

The thresholds of PSII health and membranes, show upwards shifts with the warming of seasons. *Acacia ligulata* showed a small 1.5°C increase in MSI thresholds between winter and summer and photosynthetic thresholds were significantly higher in summer than winter, with spring being intermediate (Table S4.1, Figure 4.4). For *M. montanum*, membrane thresholds were significantly higher in summer than winter, with spring not differing from those two seasons (Table S4.1, Figure 4.4a). Photosynthetic thresholds for this species did not shift between winter and spring, but increased by 4.5°C in summer (Table S4.1, Figure 4.4b). Both membrane and photosynthetic thresholds of *S. oligacanthum* moved in the same way, with little change between winter and spring and then a significant shift in summer (Table S4.1, Figure 4.3). Species did not differ from one another in their winter thresholds (Table S4.1), which were approximately 44°C for PSII and 50-53°C for membranes. In spring, PSII thresholds did not differ among species (Figure 4.4b), but *M. montanum* had significantly higher membrane thresholds than the other species (Figure 4.4a). In summer, *M. montanum* had acquired significantly higher membrane thermal tolerance than *A. ligulata*, while *S. oligacanthum* was intermediate (Table S4.1; Figure 4.4a). Summer PSII thresholds were significantly higher in *M. montanum* than both *S. oligacanthum* and *A. ligulata*. *Solanum oligacanthum* was the only species to shift both MSI and F_v/F_m thresholds in a similar way across the three seasons (Figure 4.4).

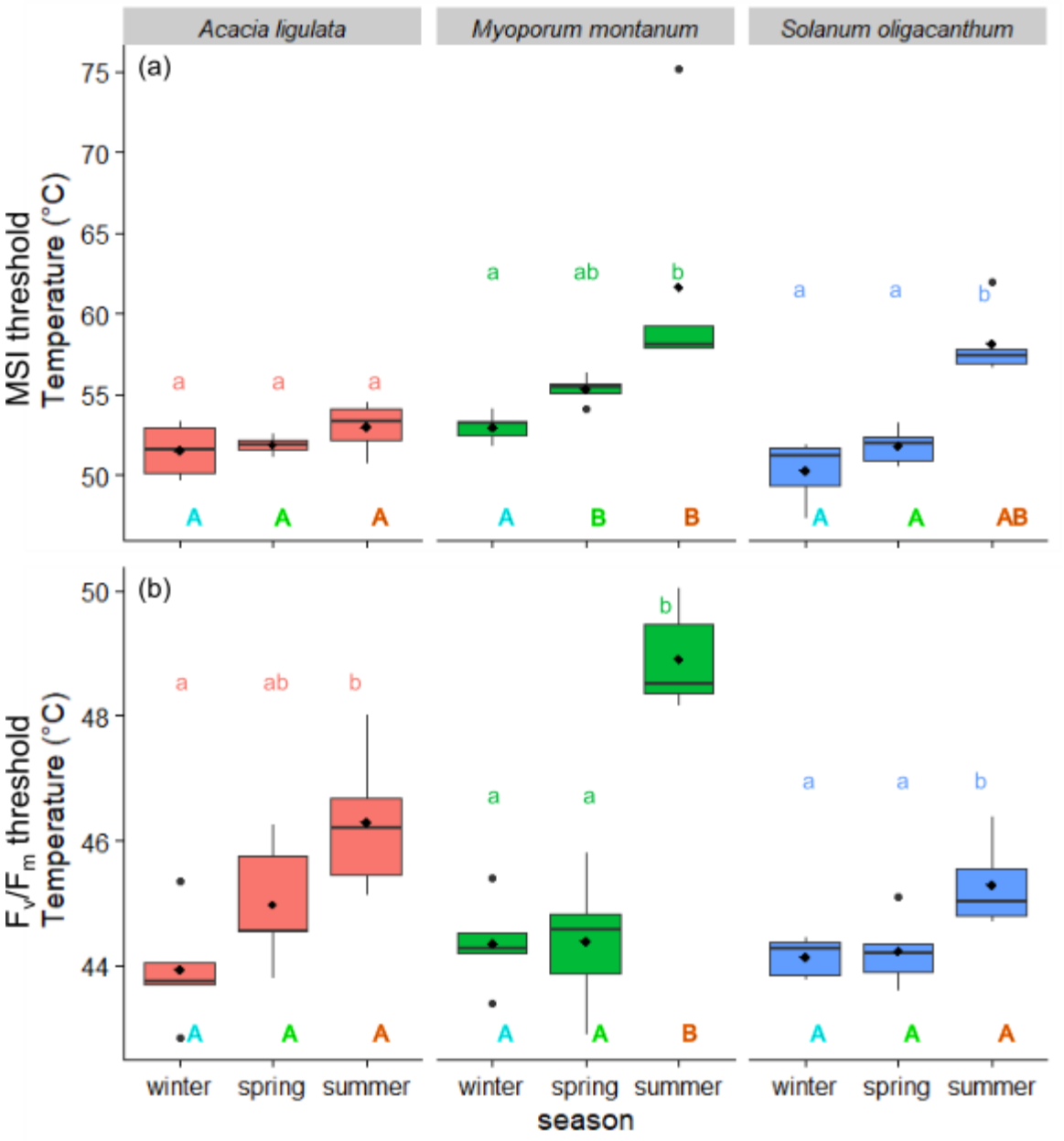


Figure 4.4. Seasonal membrane stability (MSI, a) and PSII efficiency (F_v/F_m, b) threshold temperatures of three species of desert plants (*A. ligulata* in red, *M. montanum* in green and *S. oligacanthum* in blue). Box and whisker plots (in the style of Tukey: interquartiles with whiskers extending to lowest and highest datum within 1.5*IQR of lower and upper quartiles respectively). Small black diamonds represent the mean. Different letters show groups with significantly different means ($p < 0.05$). Lower-case letters above plots indicate tests within a species among seasons and upper-case letters below plots indicate differences within a season among species (winter in light blue, spring in light green, summer in orange).

Seasonal leaf proteomes

There were species, but no seasonal, differences in both the amount of total protein and Rubisco and likewise for the proportion of total photosynthetic proteins and Rubisco as a percentage of total protein (Table S4.2). There was significantly more protein in *M. montanum* leaves than in *S. oligacanthum* leaves (Figure 4.5a). The percentage of the entire proteome dedicated to photosynthesis was >60% for all species, but differed among species (Table S4.2; Figure 4.5b). Absolute amounts of Rubisco were significantly greater in *M. montanum* than in *A. ligulata*, with *S. oligacanthum* expressing intermediate amounts (Figure 4.5c). In absolute terms, the percentage of Rubisco in leaves did not vary a great deal among species, but was significantly higher in both *M. montanum* (38.7 ± 1.0%) and *S. oligacanthum* (36.4 ± 0.8%) than in *A. ligulata* (32.5 ± 0.9%) (Figure 4.5d).

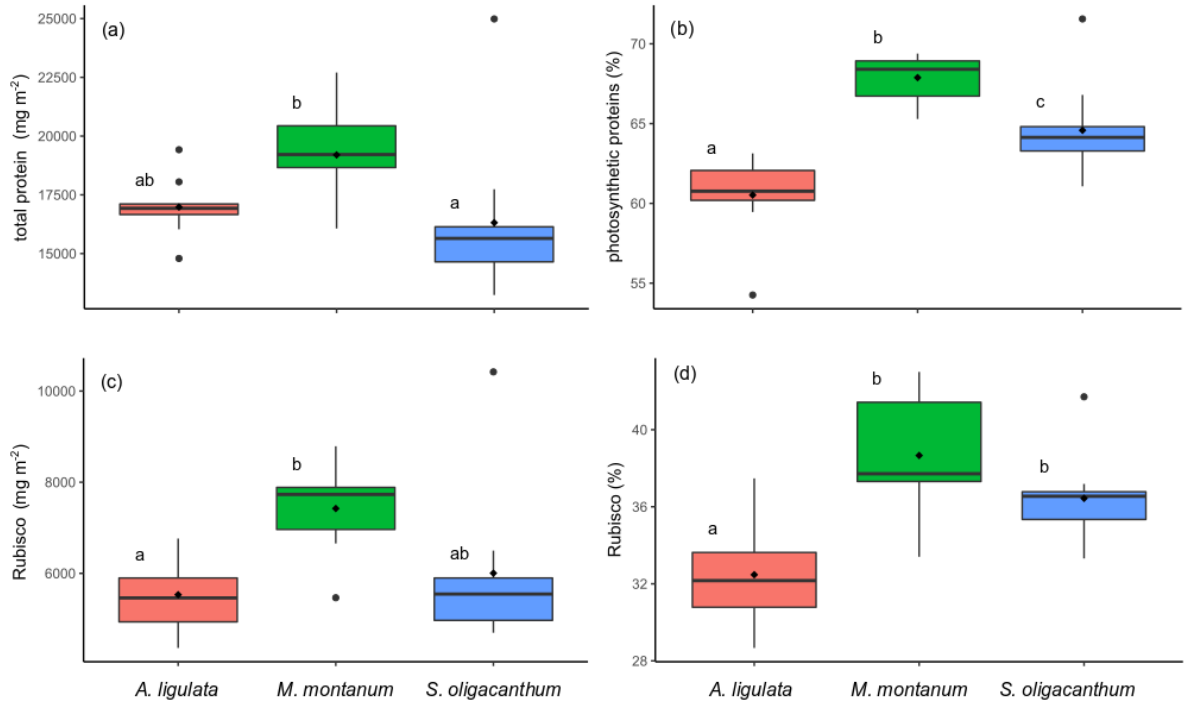


Figure 4.5. Species comparison of total and photosynthetic proteins within the leaf proteome of *Acacia ligulata*, *Myoporum montanum* and *Solanum oligacanthum*. Amounts (mg m^{-2}) of total protein (a) and total Rubisco (c) and the make-up (percentage of total protein) of photosynthetic protein (b) and Rubisco (d) are shown. Each protein group was analysed separately, and different letters signify significant differences ($p < 0.05$) among means. Boxplots explained in Figure 4.4.

As expected, the proteomes of the three species were significantly different from one another. The simplest exploration of the proteome, when the entire proteome was examined with

grouping of proteins at the second hierarchical level, found a significant species*season interaction (PERMANOVA; Pseudo-$F_{4,18}$ =1.86, Monte Carlo p = 0.006), where the differences among species were large, compared with seasonal changes (Figure 4.6). Many of the species differences were driven by proteins that were not detected in all species. For example, protein degradation (19.1) was only detected in *M. montanum* and proteins involved in RNA biosynthesis (15.3), external stimuli response (26.35) and cell wall proteins (21) were only detected in *A. ligulata* and *S. oligacanthum* (for full details of protein numbers see Table S4.3). Pairwise t-tests identified that the *M. montanum* proteome differed significantly between seasons (Table S4.5), while the other two species showed no seasonal differences.

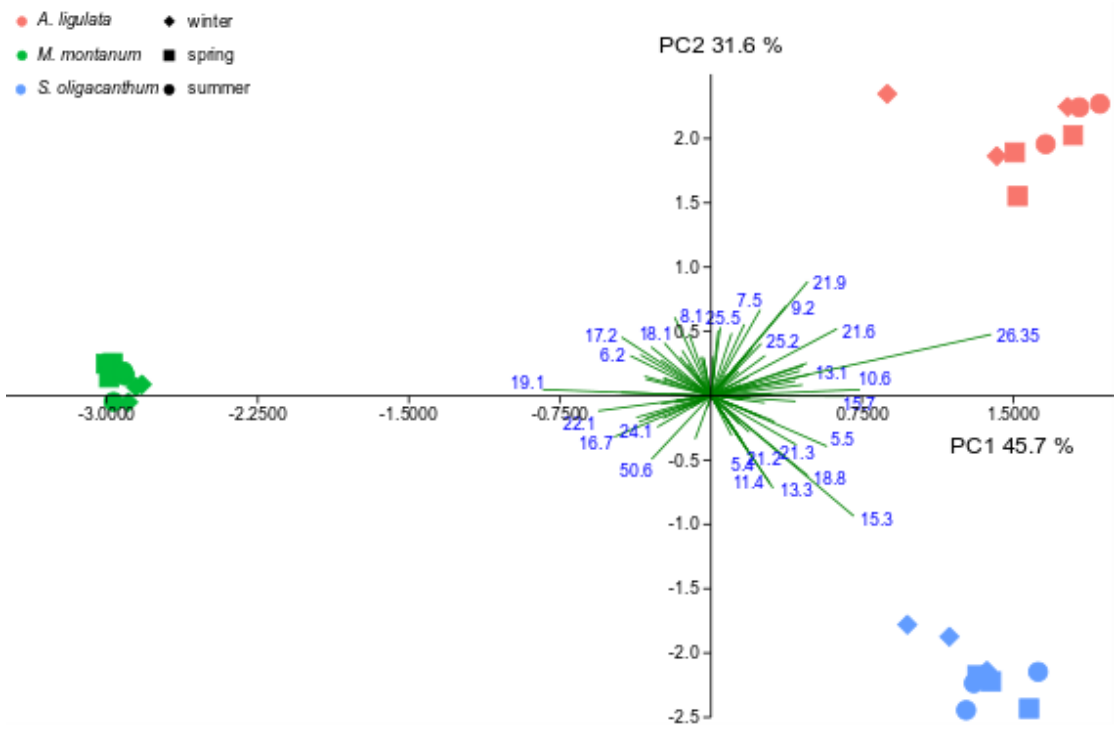


Figure 4.6. Principal Component Analysis (PCA) of the leaf proteomes of three desert plants. These first two axes (PC1 and PC2) explain 77.3% of the variance. Species are shown as coloured symbols (*Acacia ligulata* (red), *Myoporum montanum* (green) and *Solanum oligacanthum* (blue)) and symbol shapes represent season (winter (diamond), spring (square) and summer (circle)). Variables are proteins (blue numbers) grouped at two levels of hierarchy according MapMan BINs (see Table S4.3 for protein function). Green lines show the strength of the influence of a protein on the principal component.

A-priori selection of proteins to explore in detail included proteins involved in photosynthesis (1), lipid metabolism (5) and stress response and protection (secondary metoblism (9)), redox homeostasis (10) and environmental stimuli response (e.g. HSPs; 26)). Investigation at this level again revealed large species differences (Table 4.2); however, because seasonal

change in proteins was the focus of the current study, these differences were not explored further. Seasonal differences were found to be signficant for all functional protein groups, with no signficant interactions (Table 4.2). Pair-wise comparisons showed signficant differences in the amount of proteins expressed in winter and spring in all but one case: secondary metabolism proteins (Table 4.2). Also, there were signficant differences among all seasons for proteins involved in redox homeostasis (10) and HSPs (26).

Table 4.2. Pseudo-F and p-values (Monte Carlo; MC) of PERMANOVA for species and seasonal expression of selected protein functional groups: photosynthesis (1), lipid metabolism (5), secondary metabolism (9), redox homeostasis (10) and external stimuli response heat shock proteins (HSPs; 26). Where main seasonal effects were signficant, pair-wise t-tests identified where seasonal differences lay (bold p-values).

		main test			Pair-wise test			
protein group		*df*	Pseudo-F	P(MC)	pair-wise comparison		t-test	P(MC)
1	Species	2	43.40	0.001	winter	spring	1.75	**0.045**
	Season	2	21.78	**0.016**	winter	summer	1.46	0.073
	sp x se	4	11.10	0.233	spring	summer	1.17	0.236
5	Species	2	74.24	0.001	winter	spring	2.18	**0.010**
	Season	2	2.92	**0.019**	winter	summer	1.63	0.069
	sp x se	4	1.68	0.105	spring	summer	1.36	0.166
9	Species	2	333.03	0.001	winter	spring	1.49	0.108
	Season	2	2.46	**0.043**	winter	summer	1.62	0.066
	sp x se	4	1.26	0.268	spring	summer	1.58	0.087
10	Species	2	65.04	0.001	winter	spring	1.91	**0.015**
	Season	2	4.29	**0.001**	winter	summer	2.37	**0.003**
	sp x se	4	1.65	0.075	spring	summer	1.86	**0.015**
26	Species	2	46.67	0.001	winter	spring	2.14	**0.017**
	Season	2	10.62	**0.001**	winter	summer	4.01	**0.001**
	sp x se	4	1.91	0.128	spring	summer	3.09	**0.003**
	residuals	18						

The top three proteins driving seasonal differences in each functional protein group were identified. Calvin cycle proteins drove differences in photosynthetic functional groups from winter to spring (Figure 4.7a). The greatest driver of difference being Rubisco activase. Functional proteins driving seasonal difference in lipid metabolism were reduced in abundance from winter to spring and included proteins for photosterols, fatty acid synthesis and lipid degredation (Figure 4.7b). The proportion of proteins involved in redox homeostasis were more complicated in the way they drove seasonal differences (Figure 4.7c). Proportion of chloroplastic thioredoxin (10.5.6) peaked in spring, with a greater proportion in winter than summer (Figure 4.7c). Chloroplastic thioredoxin-like protein (10.5.35) increased in proportion from winter to spring to summer (Figure 4.7c). The proportion of chloroplastic thioredoxin

reductase (10.5.2) was in highest in winter, the only redox protein with its greatest proportion in winter. Non-chloroplastic thioredoxin was in highest proportions in summer than winter or spring (Figure 4.7c). The sHSP family was the greatest driver of difference in external stimuli response and proprtions increased from winter to spring to summer (Figure 4.7d). Hsp90s were in higher proportions in summer than in winter, but peaked in spring (Figure 4.7d). Hsp70s were also most abundant in spring, but in similar abundance in winter and summer (Figure 4.7d).

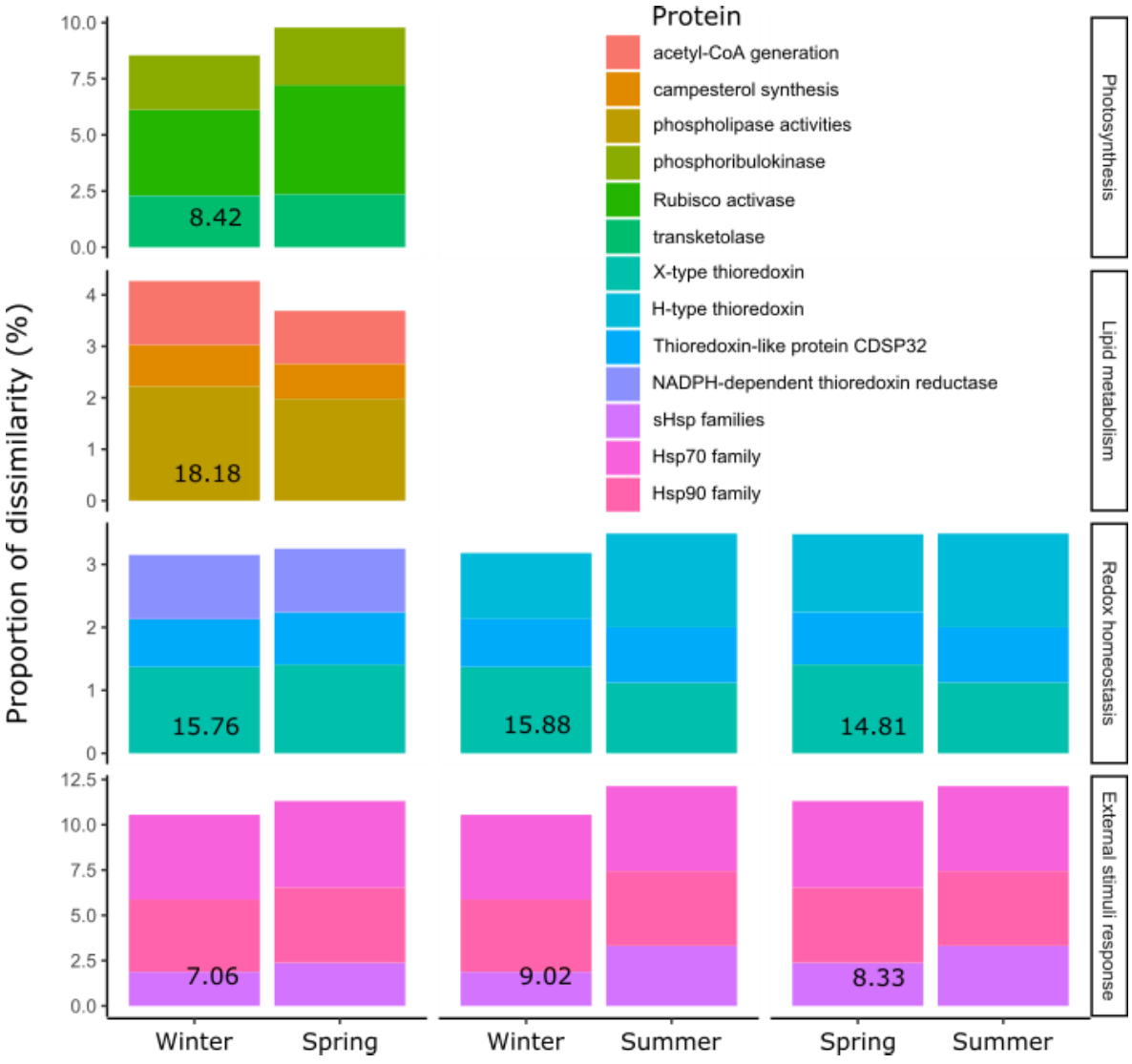
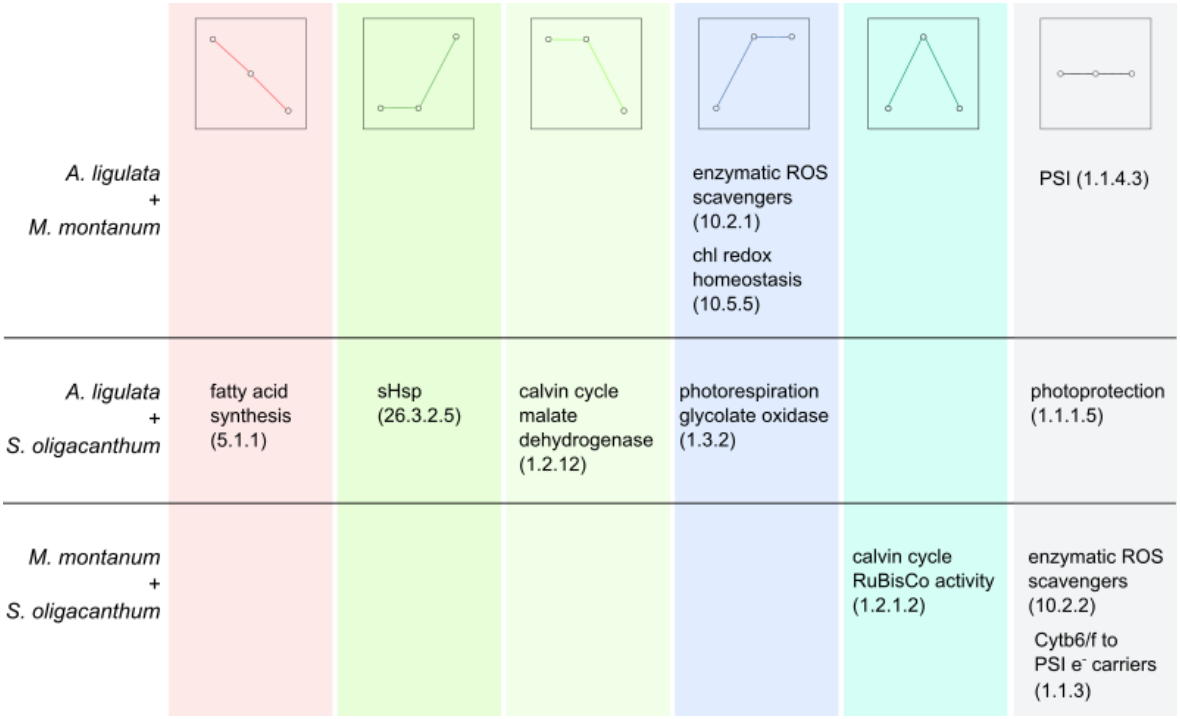


Figure 4.7. The proportion of the top three most influential proteins in each functional protein group contributing to dissimilarities amongst seasons in Australian arid zone plants using SIMPER analysis. Average dissimilarities between seasons are given in the left hand coumn but see Table S4.4. for complete SIMPER output. Functional protein groups are: photosynthesis (a), lipid metabolism (b), Redox homeostasis (c) and external stiumli response (d).

Considering my study species grow in the same broad climatic zone, it was of interest to identify the proteins that changed in similar ways over seasons. There were a number of siginificant correlations between seasons and protein expression that were shared between two species, but none of the proteins explored had a universal expression pattern for all species. Shared expression patterns between *A. ligulata* and *M. montanum* included no change in Photosystem I (PSI) maintenance and assembly (1.1.4.3), while proteins involved in redox homeostasis (10.2.1 and 10.5.5) increased in spring and remained high in summer (Table 4.3). *Acacia ligulata* and *S. oligacanthum* had more protein expression patterns in common than other species pairs (Table 4.3). For these two species, proteins involved in fatty acid synthesis (5.1.1) and the Calvin cycle (1.2.12) declined from highs in winter to lows in summer, while sHSPs (26.3.2.5) and photorespiration proteins (1.3.2) were highest in summer, with no seasonal change in proteins involved in photoprotection (1.1.1.5) (Table 4.3). For both *M. montanum* and *S. oligacanthum*, proteins involved in Rubisco activity, specificially Rubisco assembly (1.2.1.2) peaked in spring, while those involved in ROS scavenging (10.2.2) and electron transport between Cytochrome b6 and PSI (1.1.3) did not change.

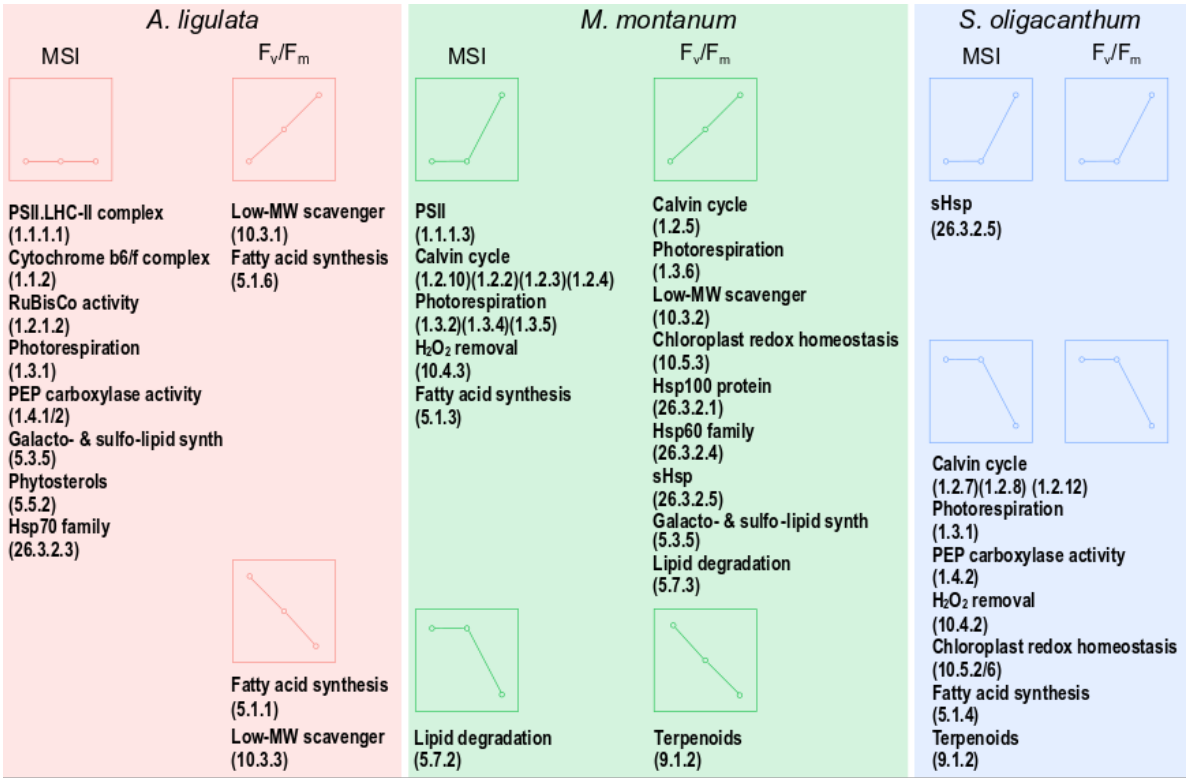
Table 4.3. Protein expression patterns over seasons shared by *Acacia ligulata*, *Myoporum montanum* and *Solanum oligacanthum*. Each box across the top of the Table shows the expression pattern with season; for example, the first box shows a decrease in protein from winter to spring to summer. Only proteins with significant Spearman's R correlations shared between at least two species are shown. The name of the functional group for the protein is given followed by the protein BIN number from MapMan (see Table S4.6 for list of proteins). Colours are not representative of any metric, simply a visual aid.



For each of the species, the proteins that follow the same expression pattern as the shifts in thresholds with season are presented in Table 4.4. Photosynthetic thresholds of *A. ligulata* steadily increased from winter to summer and two functional protein groups also moved in this direction: low molecular weight scavengers (10.3.1) and fatty acid synthesis (5.1.6). Proteins that declined over the same period were another group with the low molecular weight scavengers (10.3.3) and fatty acid synthesis (5.1.1) (Table 4.4). Interestingly *A. ligulata* did not increase membrane thermal tolerance and expression of proteins that did not change among seasons included those involved in lipid metabolism, such as phytosterols (5.5.2) and galactolipid and sulfolipid synthesis (5.3.5), and the Hsp70 family (Table 4.5). *Myoporum montanum* had significantly higher membrane and photosynthetic thresholds in summer than winter but the way in which each threshold increased differed. Membrane thresholds remained low in spring and then jumped in summer, as did a number of proteins involved in hydrogen peroxide removal (10.4.3) and fatty acid synthesis (5.1.3) (Table 4.4), while lipid degradation proteins (5.7.2) were negatively correlated with this pattern (Table 4.4). Thresholds of PSII

steadily increased and so did the functional proteins responsible for photosynthesis (1.2.5, 1.3.6), redox homeostasis (10.3.2, 10.5.3) and lipid metabolism (5.3.5, 5.7.3) as well as HSPs (26.3.2.1/4/5) (Table 4.4). Both thresholds of *S. oligacanthum* remained similar in winter and spring and then increased in summer and the only proteins to follow this path were sHSPs (Table 4.4). Proteins negatively correlated with this pattern included photosynthetic proteins involved in the Calvin cycle (1.2.7, 1.2.8) and photorespiration (1.3.1), redox homeostasis (10.4.2, 10.5.2/6) and fatty acid synthesis (5.1.4) (Table 4.4). Terpenoids (9.1.2) were negatively correlated with expression patterns of F_v/F_m in both *M. montanum* and *S. oligacanthum* (Table 4.4).

Table 4.4. Proteins that correlate with threshold adjustment patterns across season for *Acacia ligulata*, *Myoporum montanum* and *Solanum oligacanthum.* Proteins with significant positive or negative correlations that match the changes in membrane and photosynthetic thresholds are given. Analyses were conducted separately for each species. For protein details, see Table S4.6, for a full list of proteins with significant correlations see Table S4.7.



4.4 Discussion

In this study, my aim was to link physiological threshold measurements to changes in absolute amounts of protein to achieve a mechanistic explanation of acquired thermal tolerance. The winter thresholds of my three study species—from different functional types and microhabitats within the arid zone—were similar. Despite comparable winter thresholds, species differences in thresholds acquired in summer and how these higher thresholds were reached became apparent as the seasons warmed. Species differences were reflected in protein expression patterns; however, considering that species were from the same broad climatic zone, here I focus on similarities in specific protein functional groups. Generally, I found tight regulation of Rubisco and its activity, an increase HSPs with warming temperatures and some complex responses of saturated fatty acid synthesis, antioxidants and ROS scavengers and isoprenes.

The study species had similar winter thresholds of membranes and photosynthesis. The similarity in winter thresholds, both among these three study species and also those between *S. oligacanthum* and *S. orbiculatum* (Chapter 2), are likely due to adaptations to the same broad environmental conditions (Knight and Ackerly 2002, Cunningham and Read 2006, Gunderson et al. 2010). It was only when species acquired higher thermal tolerance in the summer that differences became apparent. In summer, *Myoporum montanum* possessed the highest photosynthetic threshold of all species and equally highest mebrane threshold—shared with *S. oligacanthum* with *A. ligulata* shifting thresholds only moderately (Figure 4.5). Differences in acclimation ability have been associated with habitat (Cunningham and Read 2003, Zhu et al. 2018), but also species within habitats (Sastry and Barua 2017, Sastry et al. 2018). The timing of thermal threshold shifts through seasons is likely to reflect different strategies for optimising function at a given time. Although all species in the current study had significantly higher PSII thresholds in summer than in winter, the ways each species reached maximum thresholds in summer differed. Both *M. montanum* and *S. oligacanthum* maintained similar thresholds in winter and spring, then there was a significant jump to summer, whereas the shifts in *A. ligulata* were gradual, so that no seasonal threshold was significantly higher than the previous season. I note that the limited number of sampling points in what is naturally a variable climate may not provide the potential variation in shift across season or the full association of protein expression associated with thermal thresholds. Species differences in the temporal path of photosynthetic thermal tolerance with season have been shown in four *Pinus* species and desert plants (Froux et al. 2004, Curtis et al. In review). Identification of the temperature cues species are responding to in order to adjust optimal function at a given temperature has not been fully determined. For example, the temperature optima of photosynthesis have been found to correlate with both the mean air temperature in the thirty days preceding measurements (Kumarathunge et al. 2019) and the mean maximum

temperature in the preceding ten days measurement (Slatyer and Ferrar 1977). Similarly, the temperature cues that trigger a specific thermal threshold change have not been definitively determined although my study suggests that these are species-specific. Even within a species, the cues for membrane *versus* photosynthetic thresholds appeared to vary. For example, in *A. ligulata* and *M. montanum*, the thresholds of membranes and photosynthesis did not change in the same way from winter to summer.

Examination of the leaf proteome allowed us to identify if certain proteins were associated with the physiological changes with seasons, and whether they could be contributing to species differences in thermal thresholds. A result of note was the neglible change in the proteome and membrane thermal tolerance of *A. ligulata*. This species had slightly more total leaf protein than *S. oligacanthum*, but significantly less of those proteins were dedicated to photosynthesis (Figure 4.4). This species had the highest LMA (results not shown) of all species, meaning high allocation to leaf structure. As nitrogen fixer, *A. ligulata* may be less conservative with where nitrogen is allocated, i.e. not directing a high proportion to photosynthesis but investing in leaf structure. Also, *A. ligulata* did not acquire higher membrane thermal tolerance, so it does not appear that leaf nitrogen was being allocated to protective mechanisms to stabilise proteins in membranes. Furthermore, there was an absence of change in Hsp70 expression or proteins involved in synthesising lipids in the thylakoid membrane (5.5.2). From the proteins analysed, it appears that *A. ligulata* is less plastic in its ability to adapt its leaf proteome to seasonal conditions than the other two species, this suggests high LMA leaves may not be plastic. These results suggest that *A. ligulata* directs nitrogen to as yet unidentified proteins, and this lack of upregulation may make it susceptible to high temperature damage.

While I did find increased thermal tolerance of PSII, not all proteins involved in photosynthesis changed with season. Many of the light harvesting proteins, e.g. those associated with PSI (1.1.4.3) in *A. ligulata* and *M. montanum* and electron carriers (1.1.3) in *M. montanum* and *S. oligacanthum* (and see Tables 4.4 and S4.6 for more), remained unchanged with season. Similarly, although differing among species, the amount of Rubisco did not change with season. In fact, the percentage of Rubisco in leaves of these study species was only ~30%, which is at the lower end of estimates of 30-50% of leaf protein (Kung 1976, Parry et al. 2003). In plant species from drier habitats or with long-lived leaves, the specificity of Rubisco for CO_2 over O_2 is high due to the selective pressure of low internal CO_2 (Galmés et al. 2005) and there is an increased activation state compensating for lower amounts of Rubisco (Galmés et al. 2011). A higher activation state is linked with higher photosynthetic thermal tolerance (Scafaro et al. 2012). Given my study species are from warm, nutrient and water-limited regions and considering that Rubisco is an N-intensive molecule, the same selective pressure may explain their low percentage of Rubisco.

The reason for the lack of seasonal difference in the amount of Rubisco detected in my species is more complex. Rubisco abundance and activity is said to be carefully regulated to match photosynthesis (Parry et al. 2008, Pinheiro et al. 2014), suggesting that Rubisco activity is controlled to match photosynthesis for the prevailing conditions. Rubisco can be inactivated by binding of xylulose-1,5-bisphosphate (XuBP), a side product of Ribulose (Schrader et al. 2006), which increases under high temperature (Salvucci and Crafts-Brandner 2004). Alterations in Calvin cycle enzymes may help to prevent inactivation of Rubisco and I found evidence for such alterations in my study. For example, XuBP is catalysed by two Calvin cycle enzymes (1.2.7, 1.28), both of which were lower in *S. oligacanthum* in summer, whereas aldolase (1.2.5) is known to suppress XuBP (Schrader et al. 2006), which was higher in *M. montanum* in summer. Abundance of Rubisco activase, responsible for activating Rubisco by removal of inhibitors like XuBP, was the most influential in explaining differences between winter and spring for all species. Greater Rubisco activase in spring was also seen in *Nicotiana tabacum* (Yamori and von Caemmerer 2009). Rubisco activase is temperature sensitive (Crafts-Brandner et al. 1997, Yamori et al. 2006), but thermally stable forms maintain Rubisco activity at high temperature (Scafaro et al. 2016). My findings therefore suggest that increased Rubisco activase in spring and suppression of XuBP in summer may have increased the efficiency of Rubisco, offsetting any lack of increase in summer. Although photorespiration, the result of oxygenation of ribulose 1,5-bisphosphate via Rubisco, results in CO_2 loss rather than gain, it has a protective role as an electron sink, using excess light energy during CO_2-limited photosynthesis (Osmond and Grace 1995, Osmond et al. 1997). Glycolate oxidase (1.3.2), a protein involved in photorespiration, was highest in all species in summer and over-expression has been found to improve photosynthesis in high temperature and light (Cui et al. 2016). The two roles of Rubisco in carbon assimilation and photorespiration are important for utilising light energy before it causes photoinhibition.

Fatty acid saturation enhances the thermal tolerance of not only membranes, but also processes such as photosynthesis and respiration, which occur within membranes (Murakami et al. 2000, Wang et al. 2017, Zhu et al. 2018). I found a number of proteins involved in fatty acid synthesis and degradation associated with membrane or photosynthetic threshold increases in summer. In *M. montanum*, there was increased fatty acid synthesis (acetyl-CoA generation 5.1.3) and fatty acid degradation (5.7.3), in *A. ligulata*, increased mitochondrial fatty acid synthase (5.1.6) and in *S. oligacanthum*, fatty acid synthesis (acetyl-CoA carboxylation 5.1.4) declined. Acetyl-CoA is a carbon source for *de novo* fatty acid synthesis in plastids (Ohlrogge and Browse 1995, Lin and Oliver 2008) and *de novo* synthesis is the only way to increase saturated fatty acids. Increasing the proportion of saturated fatty acids means degradation of unsaturated fatty acids is also required in times of lipid remodelling

(Hemme et al. 2014). UDP-sulfoquinovose synthase (5.3.5), which synthesises a thylakoid membrane lipid (Benning 1998), was increased in *M. montanum* but did not change in *A. ligulata.* Remembering that the membrane threshold for A. ligulata did not increase significantly in summer, the corresponding lack of increase in UDP-sulfoquinovose synthase suggests a role for this lipid in thermal tolerance. Regulation of synthesis and degradation proteins of lipid metabolism suggests re-shuffling of the fatty acid profiles within these species with respect to season.

HSPs are known for their role in stress and enhanced thermal tolerance and my detailed proteome quantification generally confirmed the expectation of more HSPs under warmer months. *Myoporum montanum*, with the highest summer membrane and photosynthetic thresholds, showed peak expression of Hsp100s, Hsp60s and sHSPs in summer. Also, acquired photosynthetic thresholds of *A. ligulata* and *S. oligacanthum* corresponded with peak sHSPs expression in summer (Table 4.4). sHSPs were one of the few protein groups in this study with similar response in all species, as well as being the most influential driver for the seasonal dissimilarities (via SIMPER analysis, Figure 4.7), identified in the external stress stimuli functional protein group (26). These proteins also changed the most between seasons, relative to the other proteins in this group. The importance of sHSPs has been recognised in other plant studies; for example, chloroplastic-sHsp24 in *Solanum* genotypes (Preczewski et al. 2000), chloroplastic-sHsp in C3, C4 and CAM plants (Shakeel et al. 2012) and sHsp20 populations of *Eucalyptus grandis* (Maher et al. 2019). For the other two influential proteins identified, the large HSPs, Hsp70s and Hsp90s, had abundances that peaked in spring. In *Pinus sylvestris*, peak expression of HSPs were proposed as marking acclimation of photosynthetic apparatus to season, with Hsp70 being one of the markers for autumn and spring and sHsp17.6 (Korotaeva et al. 2012). Findings for my desert species support the potential use of Hsp70s as a spring marker and sHSPs as indicative of summer tolerance. Interestingly, functional proteins of secondary metabolism were not prevalent in analyses, the only protein group of significance being part of the methylerythritol phosphate (9.2.1), part of the isoprene synthesis pathway (Cordoba et al. 2009). In both *M. montanum* and *S. oligacanthum*, this group was lowest in summer, potentially because of increased protection from HSPs, reducing the need for protection via isoprenes. Recent research found that isoprene and sHSP production were inversely proportional to each other (Aspinwall et al. 2019). Despite the apparent cost of upregulating HSPs, their importance in maintaining function at high temperature suggests the investment is worthwhile but may be a trade-off with production of protective volatiles like isoprene.

Maintenance of ROS homeostasis comes through the activity of ROS scavengers and the accumulation of antioxidants. The abundance of different chloroplastic thioredoxins peaked in

different seasons in my study: NADPH-dependent thioredoxin reductase (10.5.2) in winter, X-type thioredoxin (10.5.6) in spring, and thioredoxin-like protein in summer. The distinct peak expression period for these proteins is likely due to their differing roles in photosynthetic reactions. Thioredoxin reductase (10.5.2) reactivates thioredoxins (Nikkanen et al. 2017) and more of this protein may be necessary in winter due to slower enzyme rates, a common strategy at cool temperatures. Thioredoxins have different targets; for example, thioredoxin X-type protects against oxidative stress (Collin et al. 2003), while thioredoxin-like proteins control the redox status of proteins of the thylakoid and lumen (Motohashi and Hisabori 2010). As well as shared seasonal responses, I also found other proteins of ROS homeostasis to be species-dependent in their seasonal expression. Activities of the antioxidant peroxiredoxin (10.5.3), mediated by thioredoxin X-type (Collin et al. 2003), steadily increased with temperature only in *M. montanum.* In *A. ligulata,* low molecular weight scavenger proteins for ascorbate biosynthesis (10.3.1) and glutathione metabolism (10.3.3) had high and low expression in summer respectively. Elsewhere it has been found that, despite species-differences in redox activities of dismutase, peroxidase and glutathione reductase in mitochondria and chloroplasts, plants can achieve comparable levels of enhanced thermal tolerance (Wang et al. 2014b). Likewise, in my study, expression of proteins with functions in ROS homeostasis were variable among species, but this variation did not influence increased thermal tolerance with warming temperatures. However, ROS have signalling roles (Mittler et al. 2011) and ROS homeostasis forms part of a wider suite of regulation and protection; for example, ascorbate is linked to yet another protective mechanism, the xanthophyll cycle (Eskling et al. 1997).

It is important to interpret the plant proteome in context. As discussed, many of the proteins examined here have multiple roles. Also, what constitutes heat stress is generally experimentally determined and not necessarily reflective of naturally occurring events. Much of the inference for regulation of these proteins here has come from studies based not on seasonal change but imposition of heat stress (Hemme et al. 2014, Wang et al. 2017, Maher et al. 2019). Prior to sampling of my species, the highest temperature reached was a maximum daily mean of ~46°C (Figure 4.3), this crossed the summer thresholds of 12% decline in PSII health in *S. oligacanthum*. This has implications for the proteins detected because the way temperature treatments are applied can result in different gene expression (Kumar and Wigge 2010). Acquired thermal tolerance is a multi-gene response (Halter et al. 2016); therefore, further exploration of my seasonal proteomes will identify other proteins that explain how plants cope with temperature. However, in its current state this work increases the knowledge of proteins that naturally occur in the environment and expands my knowledge of how proteins are regulated in relation to season.

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CHAPTER 5: THESIS SYNTHESIS

5.1 Summary and ecological implications

In the short-term, the way plants respond to a warming world will determine whether vegetation is a sink or a source for CO_2 (Cleverly et al. 2016, van Gorsel et al. 2016). In the longer-term, increasing temperatures under climate change will drive changes in plant species distributions (Aitken et al. 2008). Under climate change, heatwaves are increasing in frequency, intensity and duration, with 75% of daily hot extremes attributable to climate change (Fischer and Knutti 2015) and warm-dwelling species expected to do more poorly in a warming world (Feeley et al. 2007, Crous et al. 2018). Despite this, little work has looked at the timing of heat stress events (De Boeck et al. 2011, Wang et al. 2016) and few heat tolerance studies have considered plants from desert environments, perhaps due to the assumption that they possess high tolerance and will cope with new extremes.

5.1.1 Same broad climate, same basal thresholds, different acquired thresholds acquired differently

While it would be useful to be able to generalise about how all species will persist under high temperatures, the species-specific differences identified in my thesis highlight the risk in doing so. In Chapters 2 and 4, I demonstrate that in terms of winter thresholds, these species possess similar tolerance to high temperature. Interestingly, winter thresholds of these desert species, which experience a broad range of seasonal average temperatures, are similar to species from warm climates with a narrower range (e.g. tropical species (Sastry and Barua 2017, Zhu et al. 2018, Huang et al. 2019)). While counterintuitive, this convergence of plants adapted to different biomes highlights the danger of generalising about potentially complex adaptive strategies. For example, some mechanisms of heat tolerance may also aid in protection at cool temperatures; also, desert plants are adapted to the highly variable conditions, where multiple stressors may be present at any time. Average habitat temperatures are therefore likely to be only one indication of future species tolerance and persistence.

Only when temperatures become extreme, do differences in species ability to cope become apparent. Chapters 2 and 3 demonstrated that, in many respects, *S. orbiculatum* was less tolerant of both longer (3 hr) and shorter (15 min) heat stress events than *S. oligacanthum*. The danger of susceptibility to high temperature was highlighted in Chapter 3, where lack of mechanisms to manage heat stress resulted in negative reproductive outcomes for *S. orbiculatum*. Some of the mechanisms for surviving heat stress identified in *S. oligacanthum* were likely unrelated to physiological tolerance to heat, but to strategies of avoidance or redirecting resources to growth and delaying reproduction. Also, the fact that the two species I studied with higher LMA, *S. orbiculatum* (Chapters 2 and 3) and *A. ligulata*

(Chapter 4), appeared less plastic in their response to seasonal temperature change has implications for other species. Debate continues as to whether species with high LMA possess high thermal tolerance, with studies finding positive (Knight and Ackerly 2003a, Sastry and Barua 2017) or no relationships (Zhang et al. 2012). My results suggest a trade-off between flexibility in enzyme/protein alterations versus the benefits that come from investment in high LMA leaves.

5.1.2 Timing of heat stress

In light of the literature identifying how heatwave characteristics have changed with climate change, in Chapter 3 I imposed extreme heat stress in two seasons to two desert *Solanum* species. My findings suggest that for these species, possession of high winter thermal tolerances means heat stress earlier into spring may not be a great concern if conditions quickly return to normal. Outcomes may be different and likely very poor if this heat stress was imposed on plants from more mesic environments, with low basal tolerance. For desert species, although seasonal change in heatwaves may not be problematic, change in other heatwave characteristics will likely have negative effects. My findings that heat stress is damaging in summer is of great concern because of the predicted increase in severity and frequency of heatwaves (Cowan et al. 2014, Perkins-Kirkpatrick and Gibson 2017). I imposed only a single heat stress event of three hours duration on plants, while many heatwaves are prolonged suggesting that the results presented here may be conservative in their estimation of the impact of extreme heat events on plants.

5.1.3 The importance of heat shock proteins

I chose to focus my PhD work on wild plants under natural thermal conditions. This adds greatly to the body of work in HSP expression in plants. Firstly, because species other than crops are rarely considered and secondly, the imposition of priming and stress treatments is often conducted in very controlled, short-term experiments. I measured seasonal HSP expression in response to either a 15 min stress (Chapter 2) or an extreme heat event of 3 hours (Chapter 3) or their natural occurrence in the environment (Chapter 4). As HSPs expression is costly (Krebs and Feder 1997), questions arise as to the circumstances under which plants make cellular adjustments to increase thermal tolerance, i.e. to membranes (Gombos et al. 1994, Wang et al. 2017) or thermally tolerant proteins (Scafaro et al. 2016), thereby suppressing the need for HSP expression. In Chapter 3, both *Solanum* species expressed greater amounts of Hsp70 and chl-Hsp24 in summer and generally more of these proteins were detected in low than high nutrient plants. These results emphasise the importance of HSPs as last-resort protective mechanisms, where the cost of not expressing HSPs and incurring damage must be greater than the cost of expressing them. In Chapter 2,

where leaves were exposed to a 15 minute temperature stress, and in Chapter 4, where no experimental temperature stress was applied, the expression of sHSPs necessary for acquired thermal tolerance (Charng et al. 2006, Hu et al. 2015, Davies et al. 2018), was greatest in summer, equating to increased photosynthetic thermal tolerance in all species. The outcomes regarding how larger HSPs were expressed with season were harder to interpret due to additional roles during normal cellular functioning. The contrast in how species expressed large HSP and acquired thermal tolerance was apparent in Chapter 4, *M. montanum* had high expression of HSP100s, HSP90s, HSP70s, HSP60s and enhanced membrane tolerance in summer, but *A. ligulata* did not upwardly adjust expression of HSP70s, nor membrane tolerance. In Chapter 2, there were contrasting responses in the two *Solanum* species in relation to how Hsp70 was expressed between winter and summer; *S. oligacanthum* expressed more Hsp70 with no adjustment to the temperature of peak expression, while *S. orbiculatum* expressed less Hsp70 and peak expression was delayed until a higher temperature. Careful control of Hsp70 expression may be a necessary adaptation to the resource-limited microhabitat in which *S. orbiculatum* grows but could leave it susceptible to heat stress. The results regarding large HSP expression in Chapter 2, when heat stress was applied experimentally, and in Chapter 4, in relation to environmental conditions, could indicate that high LMA species limit HSP expression for reasons of resource conservation.

5.1.4 The importance of more than heat shock proteins

The first two data chapters considered the expression of just two proteins, Hsp70 and chlp-sHsp24, in two species, at two time points. We know that plants have a high diversity of HSPs and there are other proteins with roles in thermal tolerance; chaperones are only part of the heat stress response and many more changes occur over the longer-term (Larkindale and Vierling 2008, Suzuki et al. 2008, Mittler et al. 2012, Jung et al. 2013). For this reason, in Chapter 4, I conducted a comprehensive exploration of the proteome to identify leaf proteins that change over time. Given the diversity and complexity of the leaf proteome, analysing all of them was beyond the scope of this thesis. Nevertheless, some key insights from my work are that the amount of light harvesting proteins and Rubisco do not appear to change with season. Rather, photosynthesis is regulated by other components of the Calvin cycle. Proteins involved in secondary metabolism were expected to increase with season; however, I found that the reverse was true: the single protein involved in isoprene synthesis was negatively correlated with season. Both isoprene and HSPs offer thermal protection (Sharkey and Schrader 2006, Sharkey et al. 2008), so it may be that in my study plants, the production of sHSPs may be favoured over isoprene synthesis. Another finding was that proteins involved in lipid metabolism were quite variable across seasons. This is likely because maintaining optimal fluidity of membranes under different temperatures requires synthesis and

degredation of fatty acids to establish the required level of lipid saturation (Lin and Oliver 2008, Hemme et al. 2014). Futher exploration of my dataset will involve the entire leaf proteome and exploration of co-expression networks (Langfelder and Horvath 2008) to detect proteins that are upregulated or downregulated together, suggesting they are involved in the same processes. In doing this, I aim to identify commonalities in protein expression among species, thereby ascertaining the suite of proteins important for function at high temperatures.

5.2 Future directions

5.2.1 Threshold shifts: what are the cues, how much can they change and what does a maximum threshold look like?

The ability to acquire photosynthetic thermal tolerance into warmer seasons is shared by different species in a range of environments (Hüve et al. 2006, Zhu et al. 2018, Curtis et al. In review). In Chapters 2 and 4 of the current work, I found differences in the way each species reached higher thermal thresholds from winter to summer (e.g. Figure 4.4), suggesting they differ in upper thresholds and may respond to different cues in the environment. The time frame of the response or which aspect of temperature (e.g. minimum, maximum, sum) triggers the change is not well understood. The conditions important for changing thresholds have been variously identified as the average temperature in the preceding 5-6 days before measurement (Hüve et al. 2006), with both 10-12 days (Bjorkman et al. 1980) and 30 days (Zhu et al. 2018) suggested as necessary for full acclimation to new conditions. Elucidating the temperature cues that trigger threshold shifts is crucial to understanding acclimation ability.

As discussed in Chapter 1 (1.2.6), species from low latitudes are suggested to do poorly in regards to acclimation ability because changes in their habitat temperature are minimal (Cunningham and Read 2002, 2003, Zhu et al. 2018). Studies on plasticity of thermal tolerances can be flawed if thresholds are not measured during the most stressful periods. This issue is highlighted by Godoy et al. (2011), who found native and introduced species to not differ under well-watered conditions, but under drought conditions invasive species have higher thermal tolerance. Further, it appears that some processes in plants may be more plastic or have a greater temperature range than others. Comparing studies, it appears that photosynthetic optimal temperatures can shift by 10°C (Slatyer and Ferrar 1977), but PSII thermal thresholds may only shift by 5°C (Zhu et al. 2018, Curtis et al. In review). This has implications for how we interpret research findings for plants in different environments: high optimal temperatures do not necessarily equate to higher acclimation. There is clearly more to be done to understand the plasticity of different processes and the extent to which upper thresholds are biologically constrained.

There is concern that some plant species from warm climates may be functioning above their thresholds, e.g. in temperate and tropical trees (Doughty and Goulden 2008, Mau et al. 2018). In Chapter 4, I noted that desert summer temperatures crossed the threshold (T_{12}) of *S. oligacanthum*. Plant thresholds in the tropics and deserts will be exceeded more often under climate change (O'Sullivan et al. 2013). Identifying maximum thermal thresholds may help understand where species will be able to persist under higher temperatures.

Addressing future thermal tolerance questions

In designing experimental work to address these key gaps, I would consider a range of approaches. Species selection could include either those with large distributions using a collection of provenances, or a selection of species from contrasting habitats. To really understand temperature effects on plants, it is crucial to control (or control for) leaf temperature, as it can be higher or lower than air temperature due to wind speed (Vogel 2009, Leigh et al. 2012) water availability and stomatal conductance (Drake et al. 2018, De Kauwe et al. 2019) or radiation load (De Boeck et al. 2016). If leaf temperature is unknown, then differences in thermal thresholds or photosynthetic optima among species may be due to leaf level response to different temperatures, rather than real differences in physiological tolerances (Curtis et al. 2019). This method requires simultaneous measurement of leaf temperature, PSII thresholds (chlorophyll fluorescence, T_{50}) and temperature optima of photosynthesis (T_{opt}) and potentially respiration. To help clarify timeframes required for change, I propose two linked experiments for quantifying temperature cues, both of which require close, potentially daily, monitoring of T_{50} and T_{opt}. The first is a controlled experiment using plants in a temperature-controlled glass house, maintained to achieve a desired leaf temperature, with parameters measured daily until stable. Temperature would then be stepped up and held until T_{50} and T_{opt} are stable. This could be repeated as many times as necessary until the maximum thresholds and optima are obtained. The second experiment would be conducted in the field with close monitoring of leaf temperature and air temperature alongside photosynthetic parameters. In order to test maximum thresholds in the field, it would be necessary to identify the time of year in which the combination of temperature, water limitations and light is the most extreme, with repeated sampling at this time. Regressions should be tested among T_{50}, T_{opt}, leaf and environmental air temperatures. Regression/gradient experiments are useful in identifying thresholds and tipping points (Kreyling et al. 2014). With these two methods, I could gain a clearer indication of time to acclimate to new temperatures, the environmental temperature of importance (e.g. minimum, maximum) and identify maximum thresholds. The use of species from different environments or populations should help to explain ability to acclimate.

5.2.2 Closely linking electrolyte leakage to physiology of the leaf

Temperature related decline in chlorophyll fluorescence is related to physiological factors including reduced photosynthetic capacity (Calvin cycle limitations), onset of irreversible tissue damage, including membrane damage, and separation of components of PSII (Schreiber and Berry 1977, Bilger et al. 1984, Yamane et al. 2000). We know that for most plants, a healthy maximum quantum yield of PSII (F_v/F_m) is ~0.83 (Maxwell and Johnson 2000). With this knowledge, chlorophyll fluorescence parameters are regularly used to establish the threshold temperature of PSII health. Common applications include the temperature at which there is a 50% decline in F_v/F_m (Knight and Ackerly 2002, Curtis et al. 2014, Drake et al. 2018) and the initiation temperature of the rise in minimal fluorescence, F_0 (Bilger et al. 1984, Ilík et al. 2000). I used the 12% decline in light- and dark-adapted F_v/F_m in Chapters 2 and 4, respectively, and the rise in F_0' in Chapter 2. The use of these parameters allowed me to identify the temperature onset of temperature-induced photoinhibition and identified species and seasonal differences.

In addition to photosynthetic chemistry, I was also interested in other processes involved in plant responses to high temperature stress. Declines in photosynthesis may be due to membrane instability, therefore establishing the temperature thresholds of membranes informed about whole plant thermal tolerance. In many studies, relative electrolyte conductivity (EC, or the inverse membrane stability index (MSI); see Chapter 3 for method) is measured at a single temperature or following a stress treatment. For example, comparative studies expose a number of species to a stress treatment, determine the EC of a detached leaf (alongside other parameters) and declare one species most thermally tolerant (Chapter 3, Agarwal et al. 2002, Kim et al. 2012). While helpful in determining differences among species in a study, a single point measurement does little to explain mechanisms or establish where thresholds lie. Expanding the range of temperatures tested improves the method and allows for calculation of a temperature threshold (Chapters 2, 4, Ilík et al. 2018, French et al. 2019). When curves are fit in this way, provided experimental parameters are comparable, species in different studies can be compared, making the data more powerful.

The values produced using EC are seldom queried in physiological terms, nor whether temperatures applied are of biological relevance to a plant. It has been suggested that 50% electrolyte leakage is useful in measuring species differences in tolerance (Marcum 1998). But in terms of leaf function, what does this value signify? Is it a point of repairable or irreparable damage to the leaf? In Chapter 2, I used the 12% decline in PSII and membrane health as this point is similar to the inflection point on the F_0-temperature rise response marking the onset of damage to PSII (Bilger et al. 1984). Directly comparing these two 12%

points, I found an 8-11°C difference between the PSII and membrane thresholds, depending upon species and season (Figure 3.3). Therefore, the temperature that caused 12% damage to membranes caused >50% damage to PSII (Figure S3.2), recalling that temperatures beyond the T_{50} cause irreversible damage to PSII. Interestingly, in order to fit a curve to obtain a threshold, it is necessary to test at a range of stress temperatures, which can provide some useful insights, but may not be biologically relevant to the question at hand. For my study species in summer, 15 minutes at 56°C was not high enough to induce much leakage from membranes (Figure S3.2 a,b). Although not a lot is known about leaf temperatures reached in the field, work conducted in our laboratory (results not shown) suggest that leaf temperatures above 56°C are infrequent. Imposing temperatures well beyond what is experienced naturally may be useful for determining upper limits for survival but will not inform how plants respond to natural thermal regimes. Therefore, in terms of membrane stability it would be useful to establish links between EC values and physiological functioning of leaves. Also, are these values common to all species? Early work determining the physiological impacts of temperature using chlorophyll fluorescence compared the F_0-temperature curves to necrosis data (Bilger et al. 1984) and it seems that a similar method could be applied to membrane damage. With this in mind, a pilot study I conducted during my PhD investigated attached leaves of *Myoporum montanum*. To measure longer-term effects of temperature stress, attached leaves were immersed in temperature baths for 15 min stress treatment. Based on the MSI index, there was little damage incurred to membranes at temperatures ≤50°C (Figure 5.1). Necrosis was visible in leaves from 50°C (there was some damage to growing tips at 48°C, not shown) and complete necrosis at 54°C (Figure 5.1). Despite complete necrosis of leaf tissue leaves at 54°C, MSI was not close to the 50% decline previously defined as a threshold for comparison of species (Figure 5.1).

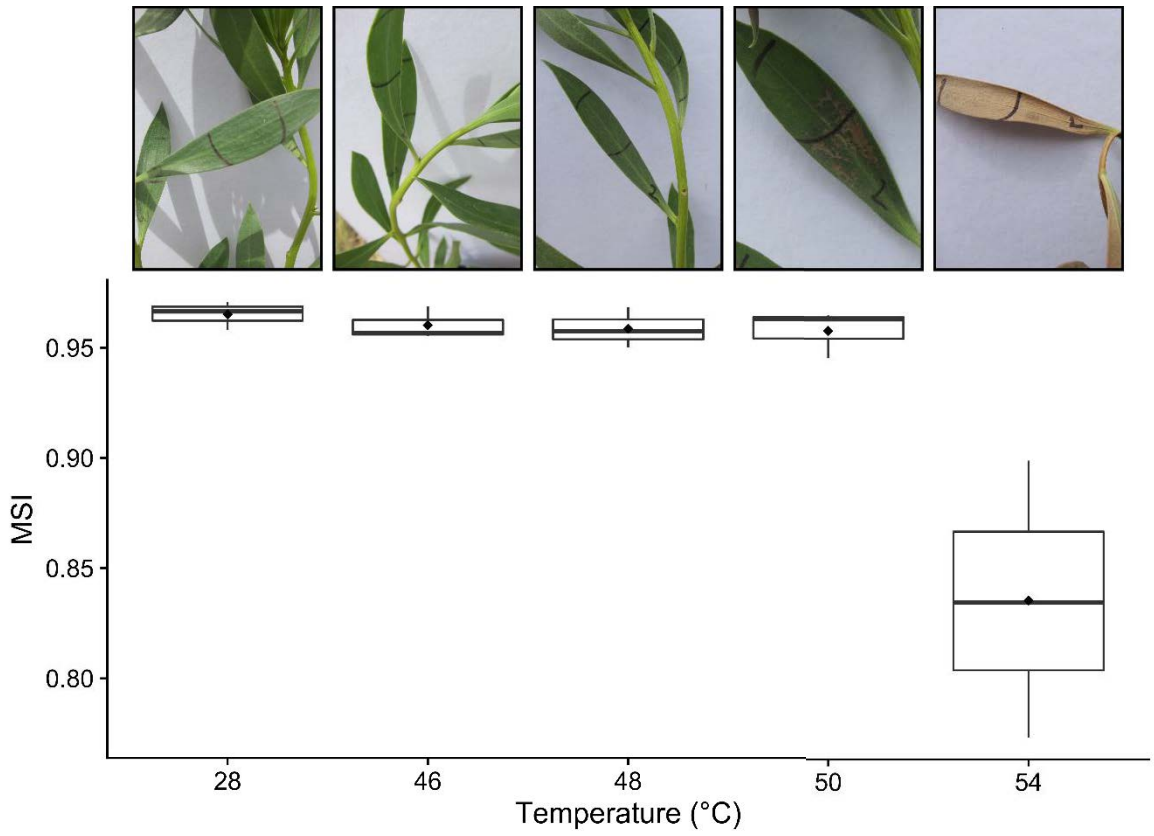

Figure 5.1. Visual damage and membrane stability index (MSI) of *Myoporum montanum* leaves. Heat stress treatment was applied to whole plants by submerging in temperature baths for 15 min. Temperatures used were above and below the T_{50} (PSII threshold) of ~48°C, plus a control and an extreme high temperature. For MSI, a leaf was detached and electrical conductivity measured 300 min after heat stress. Photos were taken the day after treatment. Tukey boxplots show the mean and variance of three replicate experiments.

Considering that EC is such a simple method of measuring thermal tolerance, it would be useful to have it closely linked to physiological parameters and ensure it is comparable among species and studies. Based on the preliminary results described above (Figure 5.1), I propose the following approach to explore these ideas and whether a useful EC threshold can be determined. The experiment would use approximately five species from differing environments (e.g. arid, tropical and alpine/cool temperate). Attached leaves would be tested using the gradual heating methods described in Ilík et al. (2018), removing leaves from the treatment as they reach a predefined temperature. This has the benefit of measuring electrolyte leakage and F_0 simultaneously. Gradual heating is different to shock treatment; therefore, the use of temperature baths for whole plants is also necessary. Following heat treatment, detached leaves should be removed for measurements of MSI. Further parameters to consider measuring include staining for cell survival and H_2O_2 detection as described in Hüve et al.

(2011) and detection of malondialdehyde (MDA) as an estimate of lipid peroxidation (Wang et al. 2014b). In using a suite of parameters, we can identify thresholds that link with the survival or visible signs of necrosis of attached leaves.

5.2.3 Heatwaves and phenology

One of the obvious directions for future studies into effects of heatwaves is to manipulate the way heat stress is applied; it is less obvious to decide which characteristics of heatwaves to manipulate. In Chapter 3, I found repeated events during the height of summer will likely have implications for fitness and survival. Therefore, a key focus of future work should be on repeated extreme events during stressful seasons and heatwaves of multiple days of high day- and night-time temperatures, eventually to include a greater number of species (De Boeck et al. 2018). Also, my research findings underline the vital importance of understanding how these kinds of stress events affect reproduction. Pollen is the most thermally sensitive tissue in a plant (Frank et al. 2009, Bita and Gerats 2013, Hatfield and Prueger 2015) and the whole plant is more susceptible to heat during the reproductive phase. For example, in prairie species, a heatwave during the reproductive phase has a greater impact than heatwaves occurring in an earlier growth phase (Wang et al. 2016). Considering that the timing of heatwaves is changing, one would expect that plants might shift flowering time to avoid the worst of the stress.

In Chapter 3, I identified that summer was the costliest time for the *Solanum* species to experience heat and that *S. orbiculatum* was more vulnerable than *S. oligacanthum*, in part because the latter is able to die-back and re-sprout. These *Solanum* species flower over a long period and with climate change, this may not be the best strategy. Will there be an alteration in phenology or a switch from sexual to vegetative reproduction? Given the species-specific stress responses of *S. oligacanthum* and *S. orbiculatum*, I would expect to see *S. orbiculatum* flower earlier to avoid severe summer heat and *S. oligacanthum* to continue with more vegetative growth; two predictions that could be monitored in the field. Understanding the response of these two species has implications for other species. For example, the way *S. oligacanthum* responds is important because ~25% of central Australian species also have the potential for clonal growth (Maconochie 1982). If plants turn to vegetative growth as a response to avoiding extreme events, as suggested by Abeli et al. (2014), then genetic diversity is under threat. Conversely, if *S. orbiculatum* lacks the ability to alter flowering time; this might reveal low plasticity or genetic variance (Hoffmann and Sgro 2011) putting it at risk from rapid change, an implication that may extend to non-clonal species with low phenological plasticity.

5.3 Conclusion

The work presented in this thesis enhances our knowledge of how plant species from extreme environments use thermal tolerance and protective mechanisms to cope with stressfully high temperatures and the potential downstream effects on reproductive fitness. This work underlines the importance of small HSPs in acquired thermal tolerance in plants. It also takes this knowledge beyond the laboratory and commercially important species and acknowledges the ecological role for these protective proteins in wild species in extreme environments. It was encouraging to uncover that species in the desert may be able to withstand out-of-season heatwaves due to high winter thresholds and that there are mechanisms of avoidance that could contribute to the persistence of species that possess these life-history traits. There are, however, implications for species with less plasticity in their ability to acquire thermal tolerance, due to their suite of life-history traits. The species differences found in this work highlight that, while understanding basal thermal tolerance might be important for knowledge of longer-term evolutionary adaptation to environmental conditions, if we want to understand species persistence with climate change, the focus of research should be on stressfully high temperatures and the associated acquired tolerance. In particular, efforts should be directed towards species responses to extreme high temperatures applied in biologically relevant ways and gaining an understanding of the variety of ways species reach high temperature tolerance. The emphasis here should be on how resources are allocated based on life history and microhabitat differences and the nature of trade-offs for production of HSPs or delayed reproduction.

Appendix

Supporting information for Chapter 2

Table S2.1. Temperature thresholds of photosynthesis and membranes in two Solanums in winter and summer. Thresholds of effective quantum yield (F_v'/F_m'), membrane stability (MSI), minimal fluorescence (F_0') and Recovery of F_0 (R_{Fo}) found using sigmoidal curves fit with Bayesian models. Thresholds were set at 88% of low temperature asymptote. Thesholds were compared between species (*Solanum oligacanthum* and *S. orbiculatum*) within season, or within season between species. Threshold differences were considered significant one threshold higher in >95% of 20 000 iterations and are indicated in bold. Confidence intervals of 2.5 and 97.5% are contained within parentheses.

		Threshold temperatures (°C)								
		Species comparison				**Seasonal comparison**				
		S. oligacanthum	*S. orbiculatum*	Difference	P (S.oli> S.orb)		winter	summer	Difference	P (summer > winter)
MSI	winter	52.3 (52.0/52.7)	54.3 (54.2/54.3)	2.0 (1.6/2.3)	**0.999^**	*S. oli*	52.2 (51.8/52.8)	56.2 (55.8/56.3)	3.9 (3.3/4.4)	**0.999**
	summer	56.1 (55.8/56.3)	56.3 (56.3/56.3)	0.1 (0.0/0.5)	0.606^	*S. orb*	55.3 (54.5/56.3)	56.3 (56.3/56.3)	1.0 (0.0/1.8)	0.927
F_0'#	winter	46.6 (44.4/48.3)	45.7 (43.5/46.8)	0.9 (-1.4/3.5)	0.787	*S. oli*	46.5 (44.1/47.7)	50.4 (49.4/51.0)	3.9 (2.3/6.3)	**0.999**
	summer	50.4 (49.5/51.0)	48.1 (45.9/49.0)	2.4 (1.1/4.6)	**0.999**	*S. orb*	45.8 (44.0/46.8)	48.0 (45.9/49.1)	2.3 (-0.1/4.3)	**0.972**
F_v'/F_m'	winter	43.8 (43.2/44.3)	44.0 (43.2/44.7)	0.2 (-0.7/1.1)	0.667^	*S. oli*	43.8 (43.3/44.2)	47.0 (45.7/48.2)	3.3 (1.8/4.6)	**0.999**
	summer	47.1 (45.7/48.3)	46.2 (45.4/46.9)	0.8 (-0.7/2.5)	0.835	*S. orb*	44.1 (43.2/44.7)	46.3 (45.4/47)	2.2 (1.1/3.4)	**0.999**
R_{Fo}	winter	48.0 (43.9/50.0)	46.5 (45.1/47.6)	1.5 (-2.6/4.0)	0.880	*S. oli*	47.9 (46.6/49.6)	51.6 (48.2/52.2)	3.7 (-0.1/5.5)	**0.970**
	summer	50.7 (46.5/52.2)	48.0 (46.8/48.9)	2.7 (-1.6/4.7)	0.943	*S. orb*	46.6 (45.9/47.3)	48.1 (46.7/49.0)	1.5 (-0.1/2.7)	**0.966**

Models were run as one-tailed tests with the assumption that *S. oligacanthum* had higher thresholds than *S. orbiculatum* or summer thresholds were greater than winter. If this was not the case curves were rearranged and model re-run.

^ Comparison was *S. orbiculatum* threshold > *S. oligacanthum* threshold.

If 95% CI contains the upper limit of 56.3°C, it may likely be an underestimate of the threshold.

Threshold was 12% of low temperature asymptote due to the temperature dependent rise in F_0.

Table S2.2. Spearman Rank correlation coefficients and *p* values between chlorophyll *a* fluorescence and physiological parameters of *Solanum oligacanthum* and *S. orbiculatum* following 15 min heat treatment at six temperatures in winter and summer.

		winter							summer						
		R_{Fo}	**Hsp70**	**sHsp24**	**MSI**	**$F_v'/F_m'_{90}$**	**$F_0'_{90}$**	**$F_v/F_{m\ ON}$**	**R_{Fo}**	**Hsp70**	**sHsp24**	**MSI**	**$F_v'/F_m'_{90}$**	**$F_0'_{90}$**	**$F_v/F_{m\ ON}$**
S. oligacanthum	**R_{Fo}**	-	0.111	ND	**0.005**	0.208	0.872	0.156	-	0.111	0.623	0.156	0.111	0.957	0.111
	Hsp70	0.71	-	ND	0.072	**0.019**	0.623	**0.042**	-0.71		0.208	0.329	0.787	0.266	0.787
	sHsp24	ND	ND	-	ND	ND	ND	ND	0.26	-0.6		0.111	0.872	**0.042**	0.872
	MSI	0.94	0.77	ND	-	0.111	0.957	**0.042**	0.66	-0.49	0.71		0.208	0.469	0.208
	$F_v'/F_m'_{90}$	0.60	0.89	ND	0.71	-	0.704	**0.005**	0.71	-0.14	0.09	0.6		0.872	**0.000**
	$F_0'_{90}$	0.09	0.26	ND	-0.03	-0.20	-	0.623	-0.03	0.54	-0.83	-0.37	0.09		0.872
	$F_v/F_{m\ ON}$	0.66	0.83	ND	0.83	0.94	-0.26	-	0.71	-0.14	0.09	0.6	1	0.09	
S. orbiculatum	**R_{Fo}**	-	0.704	ND	0.111	0.397	**0.019**	0.397		0.957	0.21	**0.005**	**0.042**	**0.042**	**0.042**
	Hsp70	-0.2	-	ND	0.957	0.156	0.704	0.156	0.03		0.62	0.872	0.957	0.544	0.957
	sHsp24	ND	ND	-	ND	ND	ND	ND	0.6	0.26		0.156	0.072	0.704	0.072
	MSI	-0.71	0.03	ND	-	0.787	0.156	0.787	0.94	-0.09	0.66		0.005	0.111	**0.005**
	$F_v'/F_m'_{90}$	0.43	0.66	ND	-0.14	-	0.21	**0.00**	0.83	0.03	0.77	0.94		0.266	**0.000**
	$F_0'_{90}$	-0.89	-0.2	ND	0.66	-0.6	-	0.208	-0.83	0.31	-0.2	-0.71	-0.54		0.266
	$F_v/F_{m\ ON}$	0.43	0.66	ND	-0.14	1	-0.6	-	0.83	0.03	0.77	0.94	1	-0.54	

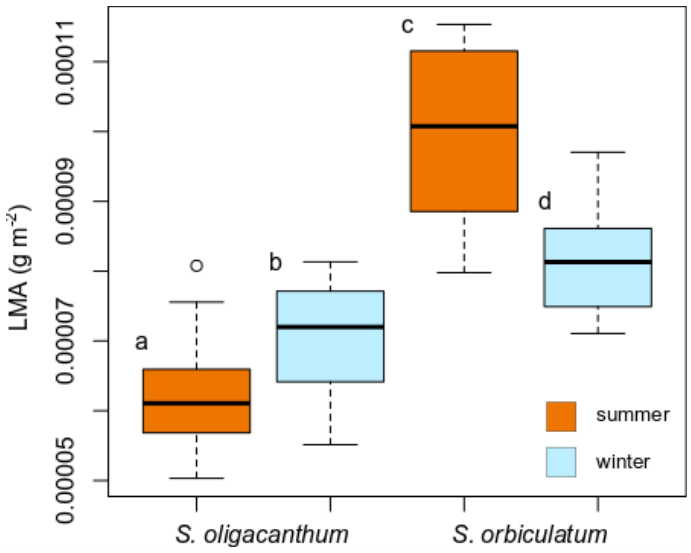

Figure S2.1. Leaf mass per area (LMA; g m^{-2}) of *Solanum oligacanthum* and *S. orbiculatum* in winter and summer. Different letters signify significant differences ($p < 0.05$) among groups.

Methods S2.1 Polyclonal antibody production, validation and optimisation

A rabbit polyclonal antibody against a small chloroplastic HSP (~21 kDa) (chl sHsp) was raised for this experiment based on the amino acid sequence found in the consensus region III of the protein published by Downs et al. (1998); Table S2). A synthetic peptide of 28 amino acids in length was synthesised (Mimotopes, Melbourne, Australia; Table S2.3) and conjugated to keyhole limpet hemocyanin (KLH) to improve the immune response. The peptide-KLH conjugate was used as the antigen for injection into rabbits. The polyclonal antibodies were raised in rabbits (WEHI Antibody Facility, Melbourne, Australia) and whole serum collected from the animals was used throughout the experiments. Antibodies that are raised against a synthetic peptide work well for immunoblotting because they recognise the target protein when it is denatured (Bordeaux et al. 2010).

Table S2.3. Sequence of UniProtKB – P31170 (HS25P_ARATH) heat shock protein 21, compared with the highly conserved amino acid sequence from consensus region III (methionine-rich domain) published by Downs et al. (1998) (underlined section). The leucine, at position 89, in P31170 sequence has been replaced by a methionine in the sequence by Downs et al. (1998).

0 MASTLSFAAS	10 ALCSPLAPSP	20 SVSSKSATPF	30 SVSFPRKIPS	40 RIRAQDQREN	50 SIDVVQQGQQ
60 KGNQGSSVEK	70 RPQQRLTMDV	80 SPFGLLDP*L*S	90 PMRTMRQMLD	100 TMDRMFEDTM	110 PVSGRNRGGS
120 GVSEIRAPWD	130 IKEEEHEIKM	140 RFDMPGLSKE	150 PDNCEKDKIK	160 DVKISVEDNV	170 LVIKGEQKKE
180 DSDDSWSGRS	190 VSSYGTRLQL	200 AELKNGVLFI	210 TIPKTKVERK	220 VIDVQIQ	

A series of immunoblots were produced to ensure the serum reacted with the protein of interest, a chloroplastic sHsp of ~21 kDa and to determine optimal dilution. In all instances where immunoblots were produced after the PVDF membrane was exposed to proteins, membranes were blocked in blocking buffer (5% skim milk in TBST) for 1 h at room temperature. The membrane was incubated with primary antibodies in blocking buffer overnight at 4°C, washed in TBST and then incubated with secondary antibody (1:5000, anti-rabbit IgG, A9169; Merck KGaA, Darmstadt, Germany) for 2 h at room temperature. Proteins were visualised using chemiluminescence (Clarity™ Western ECL substrate) and an Amersham Imager 600 (GE Healthcare, Little Chalfont, UK).

Testing the specificity of the antibody first required a series of dot blots. Dot blots, on PVDF membranes, were loaded with 1 µl pure peptide then incubated in either antisera from the pre-immune, first- or kill-bleed, in a range of dilutions. As expected, polyclonal antibodies in the

first- and kill-bleed, but not pre-immune serum, recognised the pure peptide (Figure S2.2). Second, to find the optimal dilution and test that the anti-chl sHsp21 was detecting a protein of the appropriate size, two protein samples from *S. orbiculatum*—one heat shocked at 50°C for 3 h, the second heated for 3 h following natural priming—were separated by electrophoresis before immunoblots were incubated in the following concentrations: 1:1000, 1:2000, 1:5000 and 1:10 000. The antibody is specific if it is observed in a single band at the molecular weight for the target protein; however, polyclonal antibodies are more likely to show nonspecific binding than monoclonal antibodies (Burns 2009). A protein of ~24 kDa was detected on all immunoblots (Figure S2.3) confirming our anti-chl sHsp21 detected a protein of the expected size. Antibodies raised against the same peptide used in here have previously been shown to detect a chl-sHsp between 21-30 kDa in size in various plant species (Downs et al. 1998). Further, a dilution factor of 1:5000 was chosen because it gave the best signal with minimal background staining.

In testing optimal dilution of anti-chl sHsp21, *S. orbiculatum* leaves heat-stressed in two ways were run: heated at 50°C for 3 h (sample 1) or heat stressed for 3 h following natural priming (sample 2). Sample 1, which worked well as a positive control for Hsp70 (e.g. Figure S2.5), did not appear to express chl-sHsp, whereas sample 2 did (Figure S2.3). After selection of the optimal dilution, a final immunoblot was produced comparing reactivity of pre-immune and kill-bleed sera (Figure S2.4) and, as with the dot blots, there was no protein detected using the pre-immune serum.

The optimal amount of protein for loading was determined using a standard curve similar to the methods of Taylor et al. (2013). A sample of *S. orbiculatum* heated for 3 h following priming was loaded using a 2-fold serial dilution from 80 to 0 µg and the resulting immunoblot was probed for Hsp70, chl-sHsp24 and then total proteins. Intensities of bands were plotted to find the linear range of detection, from which the optimal amount of protein was taken. Based on the curves produced using a serial dilution of total protein, the optimal amount of sample ranges between 20-40 µg for Hsp70 (Figure S2.5) and 15-30 µg for chl-sHsp24 (Figure S2.4). There is a linear relationship (R^2 = 0.9742) between amount of protein loaded on the gel, versus intensity of band on blot (Figure S2.6).

Inconsistent loading and transfer of proteins can lead to inaccuracies of the final amount of target protein detected during immunoblotting. Therefore, it is important to normalise protein amounts in each lane of each membrane. The protein CLIC1 was trialled as an option for the single protein detection method for normalisation, as it has been found previously to show little variation in sample tissue (Valenzuela et al. 1997). Following detection of Hsp70, membranes were incubated in anti-CLIC 1. Due to the large disparity in abundance of CLIC1 and Hsp70,

shown in Figure S2.7, the signals were not in the same linear range of detection (Aldridge et al. 2008), meaning CLIC1 was not an appropriate protein for normalisation in the case. CLIC1 also appears to be a similar size to chl sHSP21. For this reason, normalisation was achieved by using total protein values, determined by staining the membranes using Amido black. Previously, normalisation used detection of a loading protein or housekeeping protein; however, experimental treatments may result in differential expression of housekeeping proteins (Greer et al. 2010, Rocha-Martins et al. 2012). More recently, methods to detect total protein per lane using densitometry have been used and found to show better correlation with fold change in protein load (Taylor et al. 2013).

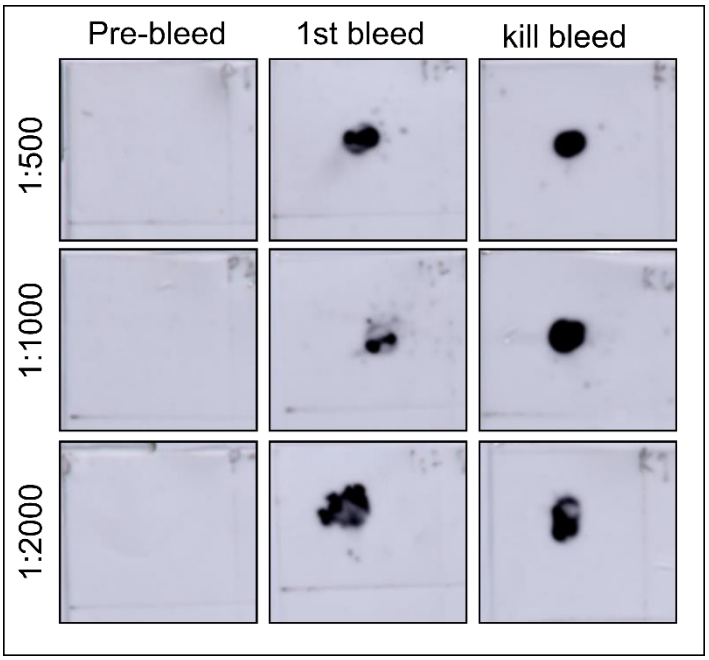

Figure S2.2. Dot blots showing the reactivity of antibody raised against consensus region III of chloroplastic small heat shock protein Hsp21. Each column represents a serum tested (pre-bleed: before rabbit was given peptide; first-bleed: after rabbit was injected with peptide; and kill bleed: after a booster of peptide) and each row represents a different dilution of primary antibody (1:500, 1:1000 and 1:2000). Each dot blot was loaded with 1 µl of synthetic peptide, membranes were blocked, then incubated with appropriate dilution of sera, washed, incubated with secondary antibody and imaged.

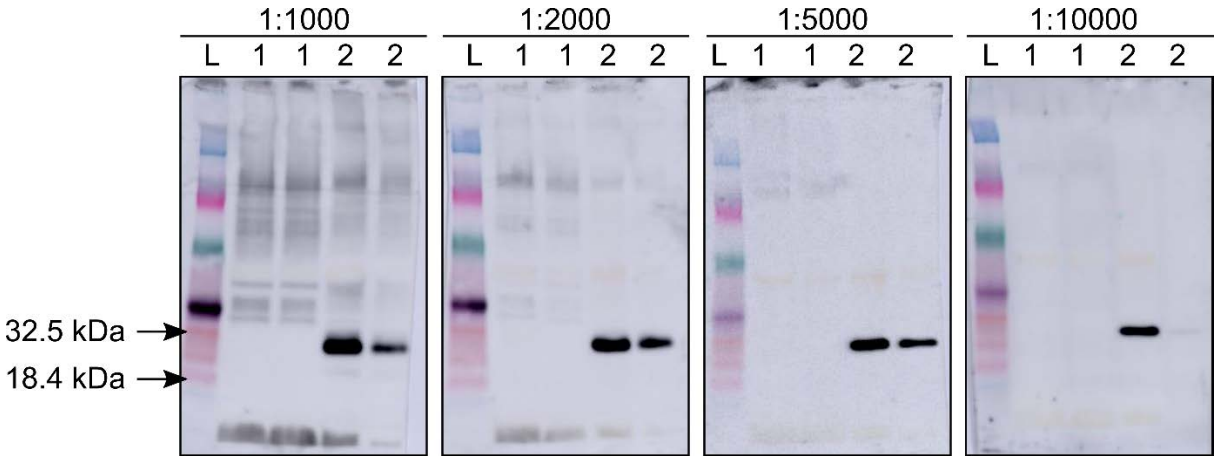

Figure S2.3. Trial for optimal dilution of anti-chl-sHSP21 in So*lanum orbiculatum*. Each immunoblot was loaded with a protein standard ladder (L) and two samples under different heat stress treatments: sample 1 was heated at 50°C for 3 h; sample 2 was heated for 3 h at 50°C following natural priming. Intensities of bands within an immunoblot differ due to unequal loading.

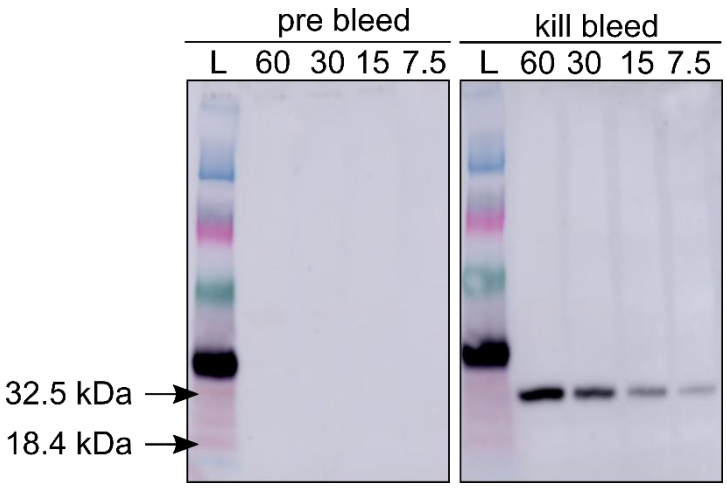

Figure S2.4. Immunoblot for test of reactivity of pre-immune and kill bleed serum used at the optimal dilution of 1:5000. There was no reaction of proteins with pre-immune serum (left), whereas chl-sHsp24 is detected on the immunoblot incubated with kill serum (right). Protein extracted from leaf of *Solanum orbiculatum* heated for 3 h at 50°C following natural priming was loaded in a 2-fold serial dilution 60 to 7.5 µg.

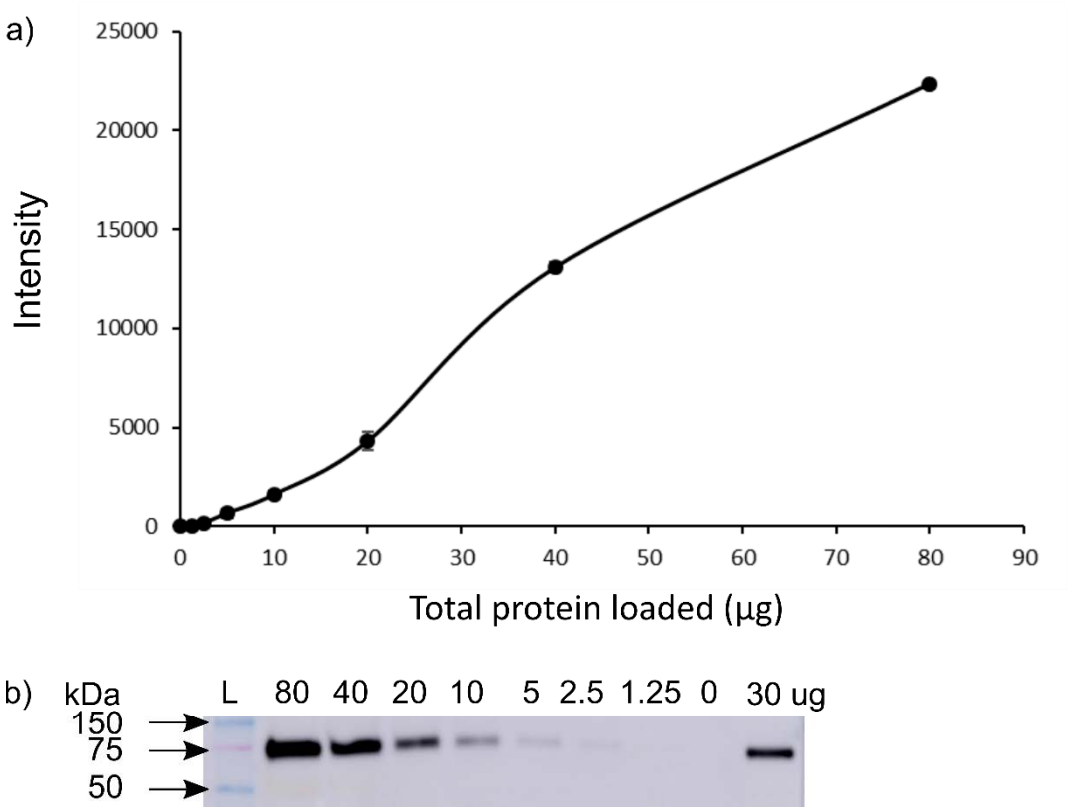

Figure S2.5. Optimisation of total protein loading amount for detection of Hsp70. Mean standard curve (error bars show SE, n = 2) of Hsp70 intensity *versus* total protein loading (a) and example membrane probed with Hsp70 antibody (b).

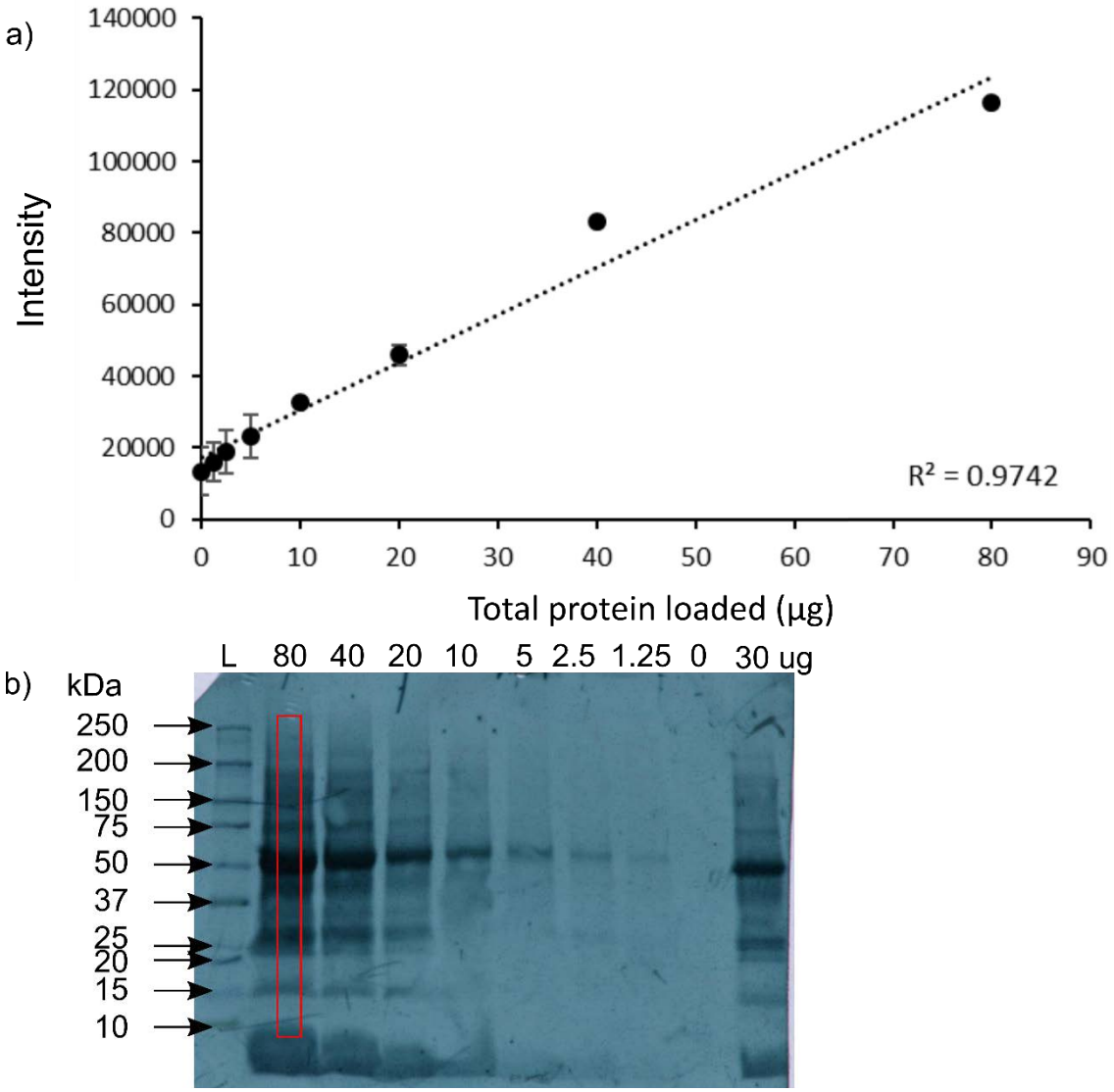

Figure S2.6. Total protein standard curve. Standard curve of mean (± SE; n = 2) total protein loading intensity, produced using 2-fold serial dilution starting at 80 ug of leaf protein sample (*S. orbiculatum* primed and heated for 3 h) (a). Example image of membrane stained with Amido black (b). Total protein intensity in each lane was estimated by selecting a narrow strip as shown in example red rectangle.

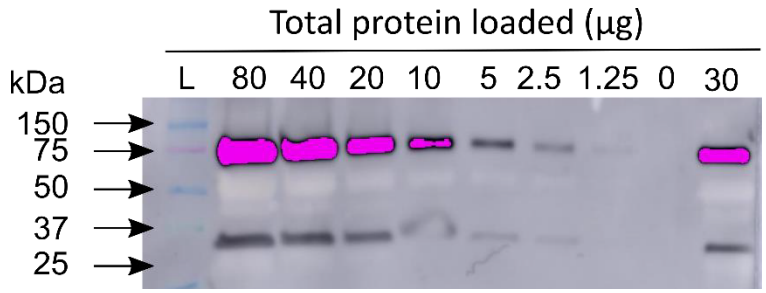

Figure S2.7. Example immunoblot of heat-treated total leaf proteins probed for CLIC1. The protein detected is ~28 kDa in size. To illustrate the differences in expression of Hsp70 and CLIC1 they are both shown taken from the same image, hence Hsp70 is oversaturated.

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Supporting information for Chapter 3

Methods S3.1. Additional methodological details on leaf protein extraction and immunoblotting.

Leaf protein extraction

The protein extraction protocol was modified from Knight (2010). Frozen leaf samples were ground to a fine power in tubes (Eppendorf™ tubes, Hamburg, Germany) with a 3 mm glass bead. Samples were placed in a tissue homogeniser (MM300, Retsch GmbH, Haan, Germany) for 45 s at 100 Hz, with samples being returned to liquid nitrogen after each round of beating (repeated 10x). A protein extraction buffer (100 mM Tris, 2.5% w/v SDS, 5 mM EDTA, with protease inhibitor cocktail (cOmplete™ ULTRA tablets; Merck, KGaA, Darmstadt, Germany)), was added (740 µL) and samples heated for 5 min before being rested for 1 h at room temperature. The supernatant was collected after centrifugation at 20 000 g for 10 min. The total amount of protein extracted from the samples was determined using BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) run in triplicate using BSA as a standard.

SDS-PAGE and immunoblotting

All gels were loaded with a molecular mass standard (Precision plus Kaleidoscope Standard) and three concentrations of positive control (30, 20 and 10 µg of *S. orbiculatum* leaves heated at 50°C for 3 h), for each sample 30 µg of protein was loaded. For each species, separate gels were run for each nutrient treatment (high and low) on each of the four replicate days. Gels were loaded with protein ladder, a positive control (*S. orbiculatum* leaves shock treated at 50°C for 3 h) and the three biological replicates (individual plants) each of the two temperature (ambient and heat stress) treatments. Due to storage/transport malfunction in spring, samples for the fourth heat stress event and third biological replicate of the high nutrient + heat stress treatment for both species were lost. In total, 31 gels were run (2 species x 2 seasons x 2 nutrient treatments x 4 replicate heat stresses, minus missing samples). Proteins were separated via gel electrophoresis using 4-15% Mini-PROTEAN TGX Stain-free gels before being transferred to PVDF membranes using standard procedures. Hsp70 was targeted first, followed by stripping with a mild stripping buffer ((1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% (v/v) Tween20, pH 2.2) for detection of chl-sHsp24. Primary antibodies used were mouse-raised anti-Hsp70 (1:2000 dilution; N27F34; ENZO Life Sciences Inc., Farmingdale, USA) and rabbit-raised anti-chlp sHsp (1:2000 dilution; using whole sera from rabbits (WEHI Antibody Facility, Melbourne, Australia), each inoculated with a KLH-conjugated synthetic peptide (Mimotopes, Melbourne, Australia) based on the sequence used by Downs *et al.* (1998). Proteins of interest were visualised using horseradish peroxidase-conjugated IgG secondary antibodies (1:2000 dilution anti-mouse; 9044 and 1:5000 dilution anti-rabbit;

A9169, both from Merck KGaA, Darmstadt, Germany) and Clarity™ Western ECL substrate captured on an Amersham 600 Imager (GE Healthcare, Little Chalfont, UK). For normalisation, total proteins on membranes were stained with Amido black stain solution (0.1% (w/v) naphthol blue black; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, 10% (v/v) methanol, 2% (v/v) acetic acid) for 3 min and then de-stained (50% (v/v) methanol, 7% acetic acid) for 15 min until bands were clear. SDS-PAGE and immunoblotting products were purchased from Bio-Rad (Hercules, CA, USA) unless otherwise stated.

HSP protein expression was semi-quantified using densitometry methods using ImageJ software (National Institutes of Health; Bethesda, MD). On each gel, proteins of interest were calculated relative to total protein. Due to the number of samples, it was necessary to produce multiple immunoblots; therefore, positive controls were used to standardise immunoblots.

Downs CA, Heckathorn S, Bryan JK, Coleman JS. 1998. The methionine-rich low-molecular-weight chloroplast heat-shock protein: Evolutionary conservation and accumulation in relation to thermotolerance. *American Journal of Botany* **85**(2): 175-183.

Table S3.1. Air temperature and VPD during heat stress treatments in spring and summer.

Season	HS treatment	Air temperature (°C)				VPD (kPa)			
		min	max	mean	heat sum	min	max	mean	deficit sum
Spring									
Overall mean	*Ambient*	30.5	32.5	31.4	5692.2	3.6	4	3.8	686.5
	HS	34.6	41.6	39.1	7083.7	4.6	7.2	6.2	1123.5
HS 2	*Ambient*	24.7	28.2	26.4	4781.2	2	2.6	2.3	411.1
	OTC1	32.7	41.1	38.5	6974.7	3.7	6.6	5.6	1010.6
	OTC2	31.1	37.6	34.7	6275.1	3.3	5.2	4.3	781.1
HS 3	*Ambient*	29.7	33.1	31.2	5654.6	3	4.1	3.6	657.6
	OTC1	32.1	38.6	36.2	6546.6	3.8	6	5.1	921.9
	OTC2	34.1	45.1	40.1	7262.1	4.2	8.6	6.5	1184.3
HS 4	*Ambient*	35.6	38.6	36.7	6640.8	5.1	6.1	5.5	991
	OTC1	38.6	46.6	42.8	7738.9	6.1	9.8	7.9	1429.6
	OTC2	37.6	47.1	42.6	7705.1	5.8	9.9	7.8	1413.6
Summer									
Overall mean	*Ambient*	34.5	38.2	36.7	6634.9	4.1	5.4	4.8	872.7
	HS	38.2	44.8	42.8	7746.5	5.3	8.1	7.1	1293.4
HS 1	*Ambient*	28.1	31.6	30.1	5440.1	2.6	6.1	4.8	869.4
	OTC1	30.1	44.1	38.3	6940.6	4.8	6.9	5.8	1047.4
	OTC2	29.7	40.6	37.1	6709.4	5.8	9.2	7.8	1417.3
HS 2	*Ambient*	36.6	41.6	39.2	7095.9	6.2	10.2	8.7	1581.5
	OTC1	40.1	46.6	44	7972	5.5	8.3	6.8	1232.4
	OTC2	41.1	48.6	46.1	8341.4	7.2	11.2	9.6	1737.1
HS 3	*Ambient*	37.6	44.6	41.2	7449.2	6.6	10.4	9	1635.5
	OTC1	42.1	50	47.2	8548.6	3.3	4.7	3.9	705.9
	OTC2	40.6	48.6	46.1	8347.1	4.5	6.9	5.8	1048.3
HS 4	*Ambient*	34.6	38.6	36.2	6554.1	4.6	7	6.1	1099.9
	OTC1	38.1	44.1	41.4	7493.4	2.3	3.2	2.8	505.1
	OTC2	38.1	44.1	42.1	7619.7	2.8	7.5	5.3	958.4

Heat stresses were imposed in open top chambers using infrared lamps. Ambient conditions were measured adjacent to chambers. Minimum, maximum and mean are given for air temperature and VPD. Heat sum and deficit sum are the sum of all readings logged at one min intervals for the 180 min duration of the experiment. No data collected for HS 1 in spring due to non-functional data loggers.

Table S3.2. Main factor means (± SE) short- and long-term responses to heat stress experiment during spring *versus* summer.

	Species		**Season**		**Nutrient**		**Heat stress treatment**	
	S. oligacanthum	*S. orbiculatum*	Spring	Summer	High	Low	Ambient	Heat stress
D_{PSII}	0.05 ± 0.02	0.06 ± 0.02	0.08 ± 0.02	0.03 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.02 ± 0.01	0.09 ± 0.02
MSI	0.95 ± 0.01	0.98 ± 0	0.98 ± 0	0.95 ± 0.01	0.95 ± 0.01	0.97 ± 0	0.97 ± 0.01	0.95 ± 0.01
Hsp70 (rel. exp.)	26442.33 ± 5675.42	45921.82 ± 5216.37	19074.87 ± 3205.88	50517.15 ± 5649.6	30912.42 ± 4714.12	41771.02 ± 6360.94	30657.67 ± 4605.58	42427.94 ± 6522.23
chl-sHsp24 (rel. exp.)	17694.77 ± 5611.93	22384.17 ± 3859.98	4597.35 ± 1679.53	32549.48 ± 4801.42	20870.59 ± 4044.2	19435.2 ± 5317	12796.45 ± 3411.77	27456.18 ± 5464.04
Stem:leaf (g/g)	3.3 ± 0.74	1.58 ± 0.31	1.45 ± 0.18	3.36 ± 0.75	1.45 ± 0.18	3.43 ± 0.77	2.14 ± 0.51	2.79 ± 0.67
RGR_{leaf} (g/day)	0.01 ± 0.01	0.01 ± 0	0.03 ± 0	-0.01 ± 0	0.02 ± 0.01	0 ± 0	0.02 ± 0.01	0.01 ± 0.01
LMA (g/m^2)	90.37 ± 5.01	134.19 ± 7.44	109.06 ± 6.12	115.29 ± 8.6	108.9 ± 6.1	115.66 ± 8.76	118.58 ± 7.55	105.04 ± 7.32
Flower/day	2.81 ± 0.48	0.9 ± 0.18	2.12 ± 0.46	1.51 ± 0.29	3.09 ± 0.44	0.65 ± 0.16	2.06 ± 0.4	1.59 ± 0.39
Fruit/day	0.1 ± 0.02	0.15 ± 0.03	0.16 ± 0.03	0.1 ± 0.03	0.22 ± 0.03	0.04 ± 0.01	0.15 ± 0.03	0.11 ± 0.02
Flower:AG (g/g)	0.09 ± 0.02	0.03 ± 0	0.09 ± 0.02	0.03 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Fruit:AG (g/g)	0.01 ± 0	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Survival (prop.)	0.94 ± 0.03	0.82 ± 0.05	0.91 ± 0.04	0.86 ± 0.04	0.89 ± 0.04	0.88 ± 0.04	0.96 ± 0.02	0.81 ± 0.05
Damage (prop.)	0.31 ± 0.07	0.23 ± 0.05	0.24 ± 0.07	0.3 ± 0.06	0.28 ± 0.06	0.26 ± 0.07	0.1 ± 0.04	0.44 ± 0.07
AG biomass (g)	8.98 ± 1.98	12.98 ± 2.15	8.53 ± 1.4	13.43 ± 2.54	18.64 ± 2.13	3.31 ± 0.34	11.46 ± 2.09	10.43 ± 2.09
LA (m^2)	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.01 ± 0	0.05 ± 0.01	0.04 ± 0.01
Pre-Root mass (g)	8.69 ± 3.93	11.28 ± 4.36	0.8 ± 0.08	19.17 ± 4.41	15.85 ± 5.19	4.13 ± 1.24		
Pre-Root:shoot (g/g)	1.24 ± 0.16	1.34 ± 0.19	0.97 ± 0.08	1.61 ± 0.19	1.16 ± 0.18	1.43 ± 0.16		
Pre- AG (g)	5.65 ± 1.94	8.3 ± 3.55	0.85 ± 0.07	13.1 ± 3.14	11.47 ± 3.55	2.48 ± 0.61		
Seed output	*S. oligacanthum*		0.11 ± 0.02	0.09 ± 0.02	0.17 ± 0.02	0.04 ± 0.01	0.10 ± 0.02	0.10 ± 0.02
	S. orbiculatum		11.97 ± 2.22	6.28 ± 1.87	15.39 ± 2.62	2.866 ± 0.02	11.63 ± 2.25	6.62 ± 1.84

Parameters are explained in Table 1 with the exception of aboveground (AG) biomass and Pre-heat stress harvest AG biomass (pre-AG); Leaf area (LA, m^2), total LA of plant; Pre-heat stress harvest Root biomass (Pre-Root mass); and Pre-heat stress harvest root:shoot ratio (Pre-Root:shoot), ratio belowground to

aboveground biomass seed output, the number of seeds fruit^{-1} normalised to day. Seed output has not been analysed (see Methods) and data are shown for means of *Solanum oligacanthum* and *S. orbiculatum* calcuatedseparately.

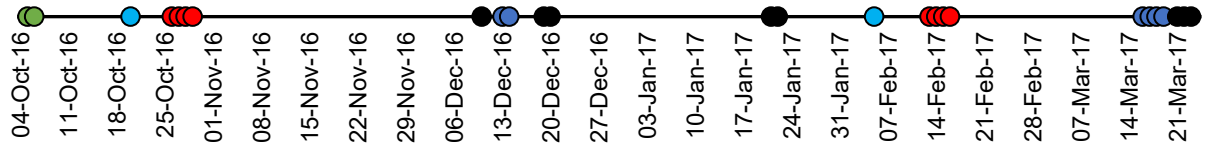


Figure S3.1. Timeline of seasonal heat stress experiment. Plants were grown from cuttings and allocated to nutrient treatments (green points); a sub-set of plants were harvested prior to the heat stress treatments (pre-harvest; pale blue points); heat stress treatments were imposed on four consecutive days (red points) in Austral spring (October) and summer (February). After the heat stress treatments, plants were left to grow and a sub-sample was destructively harvested for biomass and fitness (post-harvest; black points). Non-destructive sampling for visible damage, survival and numbers of flowers and fruit of all remaining plants were counted (dark blue points).

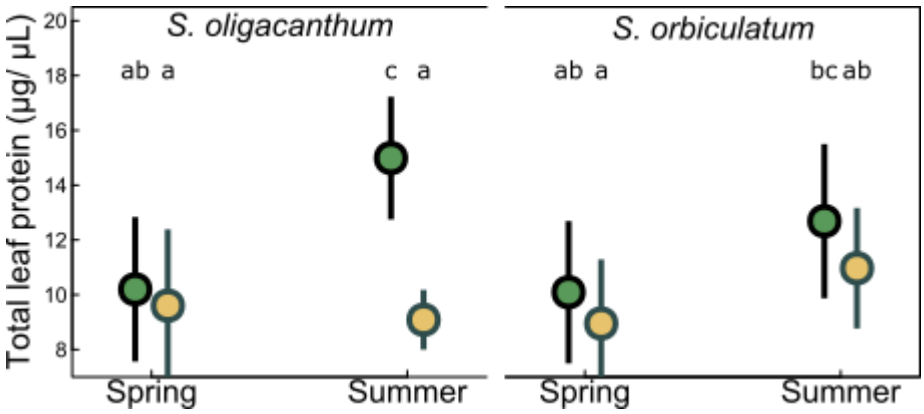


Figure S3.2. Nitrogen status of *Solanum oligacanthum* (left) and *Solanum orbiculatum* (right). Total leaf protein concentration (mean ± SD, n = 15) in plants following application of fertiliser (green points) or growth in sand and potting mix alone (yellow poings). Nutrient status was influeced by species and time in a three-way interaction (ANOVA $F_{1,112}$ = 7.31, p = 0.007). Different letters indicate significant (P < 0.05) differences between the means of treatments.

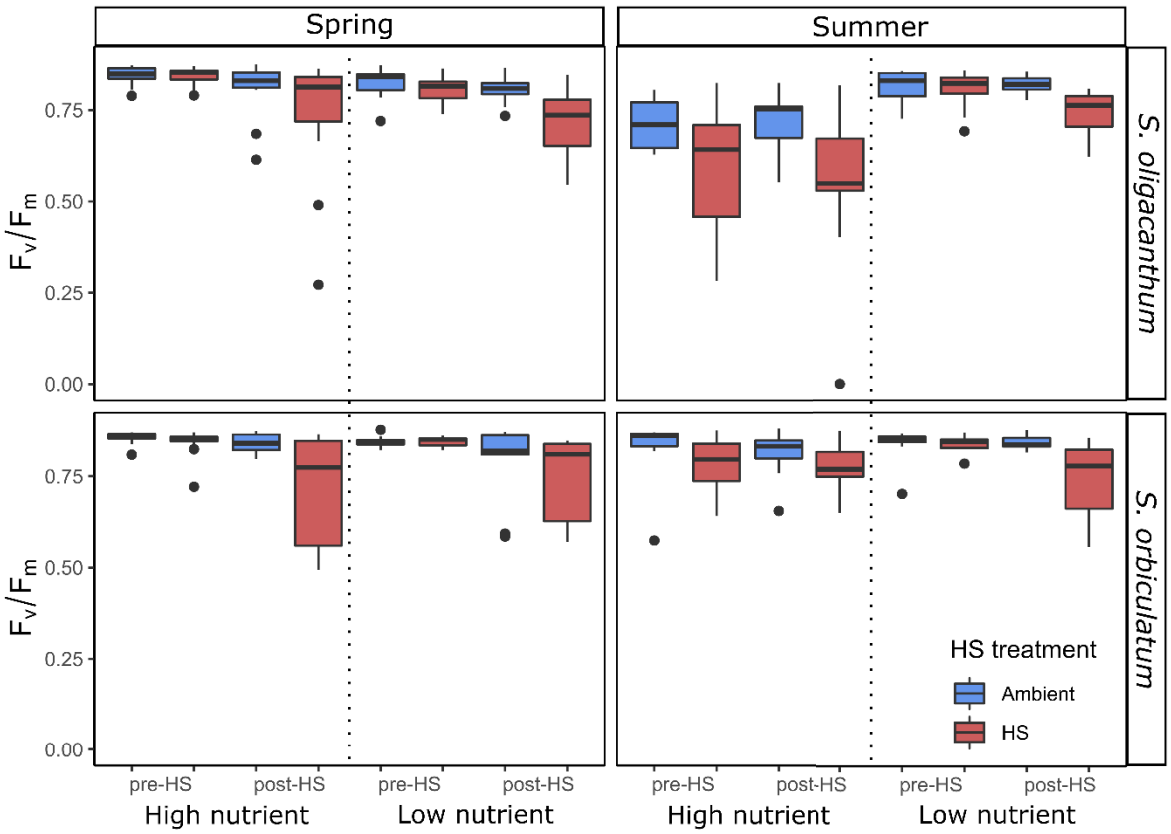


Figure S3.3. Maximum quantum yield (F_v/F_m) of *Solanum* plants pre- and post-heat stress. *Solanum oligacanthum* (top panels) and *Solanum orbiculatum* (bottom) plants were grown in high or low nutrients. In spring (left panels) or summer (right) plants were water stressed before exposure to heat stress (red) or ambient conditions (blue). F_v/F_m was measured pre-dawn on the mornings pre- and post-heat stress. Boxplots include all individual plants (n = 24, except *S. oligacanthum* high nutrient summer = 18). Box and whisker plots (in the style of Tukey: interquartiles with whiskers extending to lowest and highest datum within 1.5*IQR of lower and upper quartiles respectively).

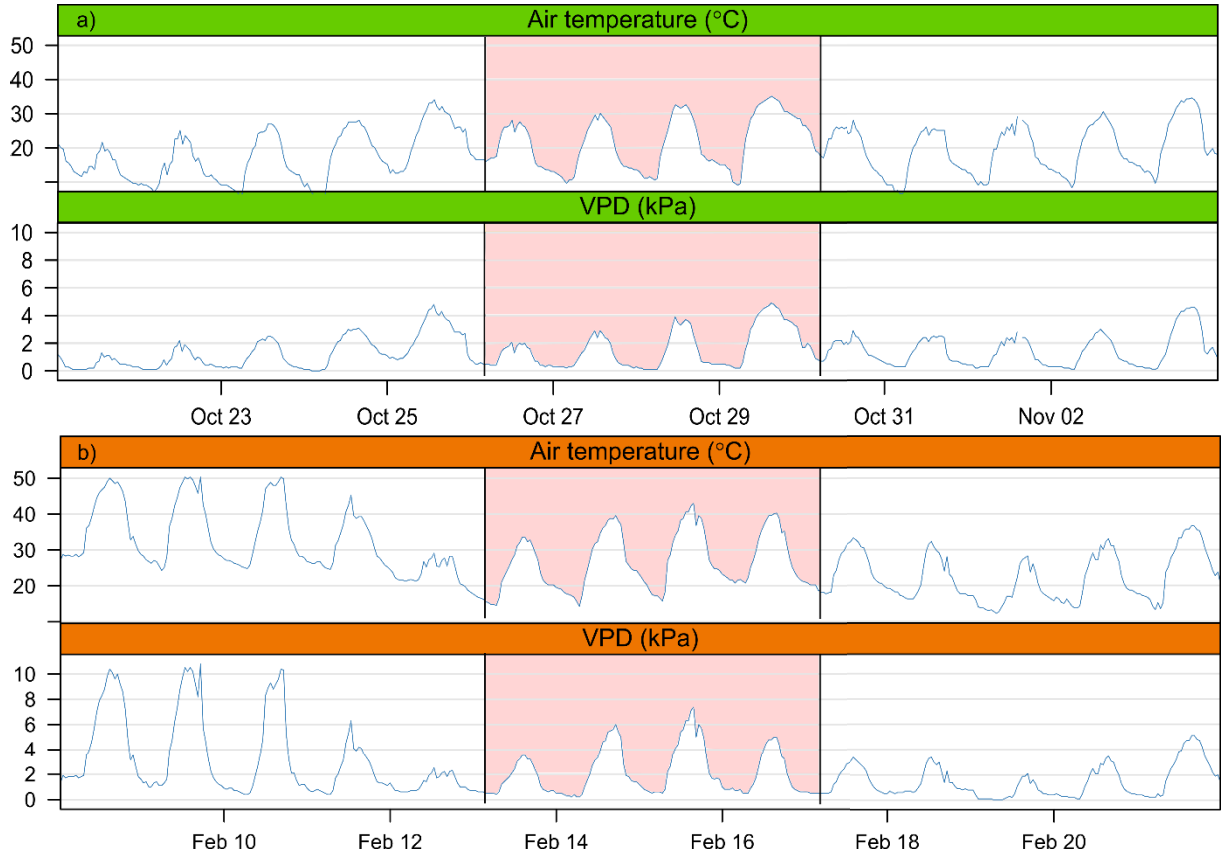


Figure S3.4. Ambient air temperature and VPD at Australian Arid Lands Botanic Gardens, Port Augusta, South Australia. Data for the five days preceding, four days during (shaded area) and five days following heat stresses in spring (a) and summer (b).

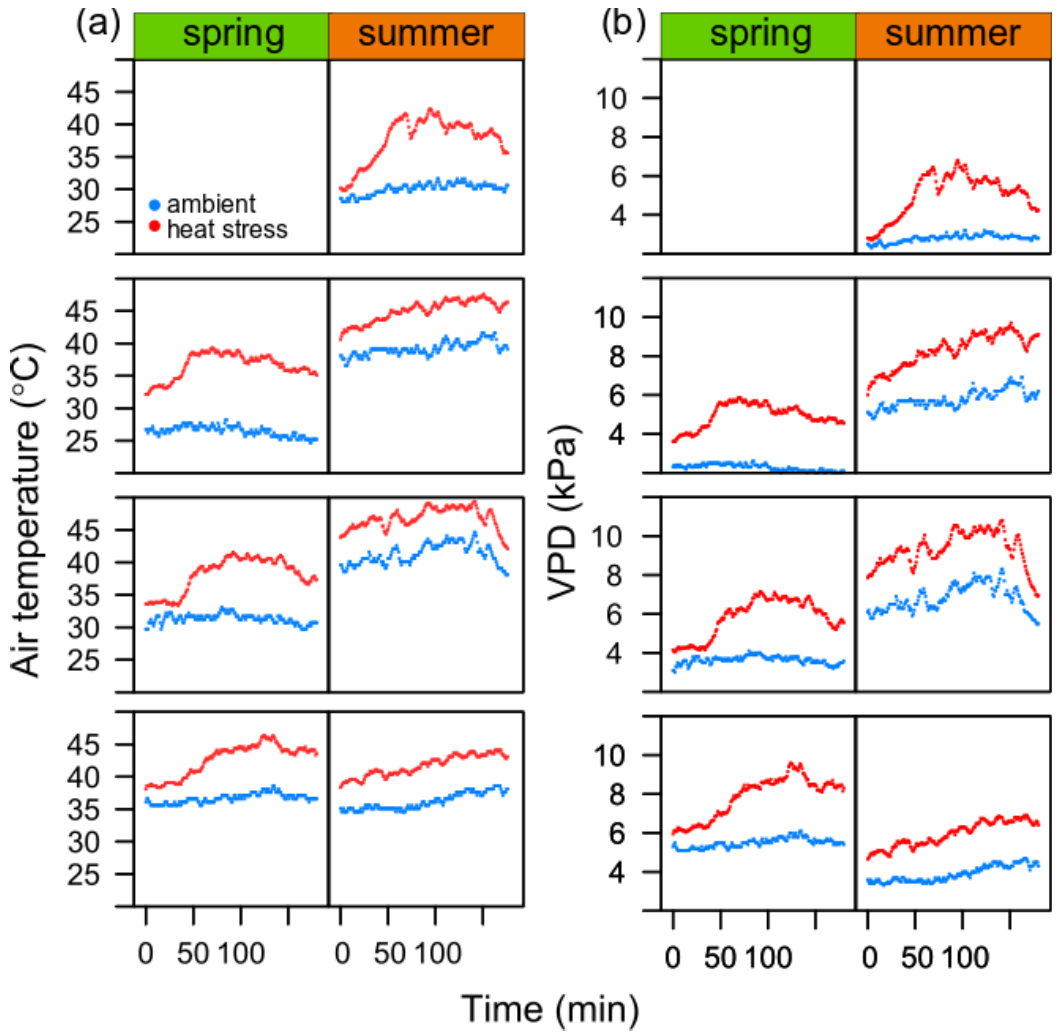


Figure S3.5. Air temperature (°C) and VPD (kPa) during four replicate heat stress treatments (one replicate per row) imposed in spring (a, c) and summer (b, d). Heat stress conditions are shown within open top chambers (red lines) and ambient conditions adjacent to chambers (blue lines). No data were collected for the first replicate treatment in spring due to non-functional data loggers.

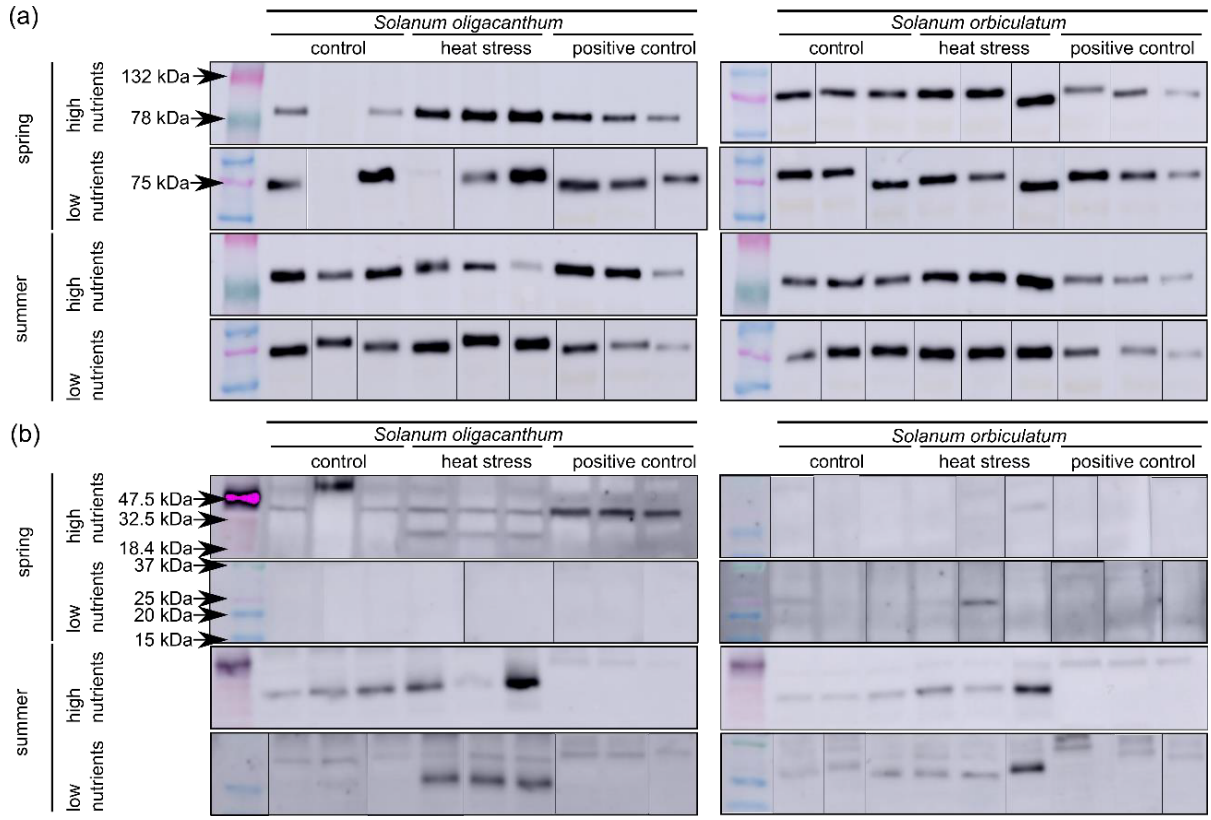


Figure S3.6. Example immunoblots of HSP expression in Solanums. Hsp70 (a) and chlp-sHsp24 (b) expression are shown for *S. oligacanthum* (left) and *S. orbiculatum* (right). Immunoblots shown are representative of all blots, each row is from a single membrane. Some lanes have been reordered for ease of interpretation (borders show where image was spliced).

Figure S3.7. Resprouting *Solanum oligacanthum* following heat stress.

Supporting information for Chapter 4

Table S4.1. F- and p-values of species and seasonal comparisons of thermal thresholds of membrane (MSI) and PSII (F_v/F_m) thresholds.

Comparison	among	MSI F-value	MSI p value		F_v/F_m F-value	F_v/F_m p value	
species	winter	3.8	0.0528		0.46	0.643	
	spring	28.2	<0.001	***	0.92	0.424	
	summer	4.42	0.036	*	17.42	<0.001	***
season	*A. ligulata*	1.61	0.241		7.00	0.011	*
	M. montanum	5.12	0.025	*	43.57	<0.001	***
	S. oligacanthum	26.59	<0.001	***	6.88	0.01	*

Table S4.2. F- and p-values of amounts and percentages of leaf proteins. Proteomes of *Acacia ligulata*, *Myoporum montanum* and *Solanum oligacanthum* were measured in winter, spring and summer and amounts of total proteins and Rubisco and percentages of photosynthetic proteins and Rubisco to total proteins compared.

		Total proteins (mg m^{-2})		% Photosynthetic proteins		Rubisco (mg m^{-2})		% Rubisco	
	df	F-value	P value	F-value	P value	F-value	P value	F-value	P value
species	2	3.56	**0.046**	22.13	**<0.001**	5.73	**0.010**	11.01	**<0.001**
season	2	1.83	0.184	2.65	0.093	1.11	0.347	0.05	0.952
residuals	22								

Table S4.3. Leaf proteins of *Acacia ligulata, Myoporum montanum* and *Solanum oligacanthum* arranged at two levels of hierarchy according to functional protein BINs in MapMan.

Mercator BIN no.	Level 1	Level 2	*A. lig*	*M. mon*	*S. oli*
1.1	Photosynthesis	photophosphorylation			
1.2		calvin cycle			
1.3		photorespiration			
1.4		CAM/C4 photosynthesis			
2.1	Cellular respiration	glycolysis			
2.2		pyruvate oxidation			
2.3		tricarboxylic acid cycle			
2.4		oxidative phosphorylation			
3.1	Carbohydrate metabolism	sucrose metabolism			
3.2		starch metabolism			
3.5		sorbitol metabolism			
3.6		mannose metabolism			
3.8		nucleotide sugar biosynthesis			
3.9		fermentation		ND	ND
3.10		oxidative pentose phosphate pathway			
3.11		gluconeogenesis			
4.1	Amino acid metabolism	biosynthesis			
4.2		degradation			
5.1	Lipid metabolism	fatty acid synthesis			
5.3		galactolipid and sulfolipid synthesis			
5.4		sphingolipid metabolism	ND	ND	
5.5		phytosterols		ND	
5.7		lipid degradation			
5.8		lipid transport			

Mercator BIN no.	Level 1	Level 2	*A. lig*	*M. mon*	*S. oli*
6.1	Nucleotide metabolism	purines			
6.2		pyrimidines			ND
6.3		deoxynucleotide metabolism			
7.2	Coenzyme metabolism	thiamine pyrophosphate synthesis			
7.3		S-adenosyl methionine (SAM) cycle			
7.5		tetrahydrofolate synthesis			
7.7		pyridoxalphosphate synthesis			
7.8		prenylquinone synthesis			
7.9		NAD/NADP biosynthesis	ND	ND	
7.10		FMN/FAD biosynthesis	ND		
7.11		iron-sulfur cluster assembly machineries			
7.12		tetrapyrrol biosynthesis			
8.1	Polyamine metabolism	putrescine			ND
8.2		spermidine/spermine			
9.1	Secondary metabolism	terpenoids			
9.2		phenolics		ND	ND
9.3		nitrogen-containing secondary compounds			
10.2	Redox homeostasis	enzymatic reactive oxygen species scavengers			
10.3		low-molecular-weight scavengers			
10.4		hydrogen peroxide removal			
10.5		chloroplast redox homeostasis			
10.6		cytosol/mitochondrion/nucleus redox homeostasis		ND	
11.1	Phytohormones	abscisic acid		ND	
11.3		brassinosteroid			ND
11.4		cytokinin	ND	ND	
11.5		ethylene			ND

Mercator BIN no.	Level 1	Level 2	*A. lig*	*M. mon*	*S. oli*
11.7		jasmonic acid			
11.10		signalling peptides		ND	ND
12.1	Chromatin organisation	histones			
12.3		histone modifications	ND		ND
12.5		DNA methylation			
13.1	Cell cycle	regulation		ND	
13.3		mitosis and meiosis	ND	ND	
13.4		cytokinesis	ND	ND	
13.5		organelle machineries			
15.3	RNA biosynthesis	RNA polymerase II-dependent transcription		ND	
15.7		transcriptional activation		ND	
15.9		organelle machineries			
16.4	RNA processing	RNA splicing			
16.7		RNA modification	ND		
16.9		messenger ribonucleoprotein particle (mRNP)			
16.10		organelle machineries			
17.1	Protein biosynthesis	cytosolic ribosome			
17.2		aminoacyl-tRNA synthetase activities			
17.3		translation initiation			
17.4		translation elongation			
17.5		translation termination		ND	
17.6		organelle translation machineries			
18.1	Protein modification	N-linked glycosylation			ND
18.8		phosphorylation			
18.10		dephosphorylation			
18.11		S-nitrosylation and denitrosylation			

Mercator BIN no.	Level 1	Level 2	*A. lig*	*M. mon*	*S. oli*
18.12		S-glutathionylation and deglutathionylation			
18.13		protein folding and quality control			
18.14		peptide maturation			
19.1	Protein degradation	ER-associated protein degradation (ERAD) machinery	ND		ND
19.2		26S proteasome			
19.4		peptide tagging			
19.5		peptidase families			
20.1	Cytoskeleton	microtubular network			
20.2		microfilament network			
20.3		actin and tubulin folding			
20.5		cp-actin-dependent plastid movement	ND	ND	
21.2	Cell wall	hemicellulose	ND	ND	
21.3		pectin			
21.4		cell wall proteins		ND	
21.6		lignin		ND	
21.9		cutin and suberin		ND	ND
22.1	Vesicle trafficking	clathrin coated vesicle (CCV) machinery			
22.3		Coat protein I (COPI) coatomer machinery			
22.4		Coat protein II (COPII) coatomer machinery			
22.8		SNARE target membrane recognition and fusion complexes	ND	ND	
22.9		regulation of membrane tethering and fusion			
23.1	Protein translocation	chloroplast			
23.2		mitochondrion		ND	ND
23.5		nucleus			
24.1	Solute transport	primary active transport			
24.2		carrier-mediated transport			

Mercator BIN no.	Level 1	Level 2	*A. lig*	*M. mon*	*S. oli*
24.3		channels			
24.4		porins			
25.1	Nutrient uptake	nitrogen assimilation			
25.2		sulfur assimilation			
25.4		iron uptake			
25.5		copper uptake			ND
26.1	External stimuli response	light		ND	ND
26.3		temperature			
26.4		drought			
26.6		biotic stress		ND	
26.35		defence response		ND	
27.2	Multi-process regulation	TOR signalling pathway	ND	ND	
27.4		Rop GTPase regulatory system	ND	ND	
35.1	Unassigned				
50.1	Enzyme classification	EC_1 oxidoreductases			
50.2		EC_2 transferases			
50.3		EC_3 hydrolases			
50.4		EC_4 lyases			
50.5		EC_5 isomerases			
50.6		EC_6 ligases	ND		

Table S4.4. Top three most influential proteins in each functional protein group contributing to dissimilarities amongst seasons in Australian arid zone plants using SIMPER analysis.

Protein group	Seasonal comparison	Av. diss. (1 v 2)[#]	Protein BIN no.	Protein function			Av. abund.[^] 1	Av. abund.[^] 2	Diss/ SD[*]	% contr.
Photosynthesis	winter (1) v spring (2)	8.42	1.2.1.3	Calvin cycle	regulation	Rubisco activase	3.83	4.87	1.39	11.13
			1.2.11	Calvin cycle	phosphoribulokinase		2.42	2.56	1.02	7.34
			1.2.7	Calvin cycle	transketolase		2.29	2.35	0.95	6.56
Lipid metabolism	winter (1) v spring (2)	18.18	5.5.1	phytosterols	campesterol synthesis		0.81	0.68	0.95	13.79
			5.1.3	fatty acid synthesis	acetyl-CoA generation		1.24	1.03	1.33	13.45
			5.7.2	lipid degradation	phospholipase activities		2.22	1.98	1.21	10.28
Redox homeostasis	winter (1) v spring (2)	15.76	10.5.6	chloroplast	X-type thioredoxin		1.37	1.41	1.2	8.99
			10.5.35	chloroplast	Thioredoxin-like protein CDSP32		0.76	0.83	0.93	7.97
			10.5.2	chloroplast	NADPH-dependent thioredoxin reductase		1.02	1.01	1.26	7.69
	winter (1) v summer (2)	15.88	10.6.1	cytosol/mitochondrion/nucleus	H-type thioredoxin		1.05	1.49	1.29	8.79
			10.5.6	chloroplast	X-type thioredoxin		1.37	1.12	1.29	8.53
			10.5.35	chloroplast	Thioredoxin-like protein CDSP32		0.76	0.88	0.93	8.41
	spring (1) v summer (2)	14.81	10.5.35	chloroplast	Thioredoxin-like protein CDSP32		0.83	0.88	0.94	9.17
			10.5.6	chloroplast	X-type thioredoxin		1.41	1.12	1.27	9.05
			10.6.1	cytosol/mitochondrion/nucleus	H-type thioredoxin		1.24	1.49	1.18	8.95
External stimuli response	winter (1) v spring (2)	7.06	26.3.2.5	temperature	HSP	sHsp families	1.88	2.38	1.54	29.92
			26.3.2.2	temperature	HSP	Hsp90 family	3.99	4.17	1.49	26.32
			26.3.2.3	temperature	HSP	Hsp70 family	4.68	4.76	1.45	19.86
	winter (1) v summer (2)	9.02	26.3.2.5	temperature	HSP	sHsp families	1.88	3.32	1.78	43.9
			26.3.2.2	temperature	HSP	Hsp90 family	3.99	4.13	1.42	18.06

Protein group	Seasonal comparison	Av. diss. (1 v 2)[#]	Protein BIN no.	Protein function		Av. abund.[^] 1	Av. abund.[^] 2	Diss/ SD[*]	% contr.
External stimuli response	spring (1) v summer (2)	8.33	26.3.2.3	temperature	HSP	Hsp70 family	4.68	4.68	1.33	15.79
			26.3.2.5	temperature	HSP	sHsp families	2.38	3.32	1.59	36.26
			26.3.2.2	temperature	HSP	Hsp90 family	4.17	4.13	1.33	23.61
			26.3.2.3	temperature	HSP	Hsp70 family	4.76	4.68	1.38	16.22

[#] The average dissimiliarty between seasons.
[^] The average abundance of protein within a season is presented with the season appearing first and second in the seasonal comparision column shown in the first and second average abundance columns, respectively.
[*] High Diss/SD values identify proteins that are consistently more likely to differ amongst any pair of seasons.
Protein BIN number from MapMan (see Table S4.6 for list of proteins).

Table S4.5. 4 Pseudo-F and p-values (Monte Carlo; MC) of PERMANOVA analysis of species and seasonal expression of entire leaf proteome. Pair-wise t-tests identified where the species*season interaction lay.

Main test				Pair-wise test							
						A. ligulata		*M. montanum*		*S. oligacanthum*	
	df	F	P(MC)			t test	P(MC)	t test	P(MC)	t test	P(MC)
species	2	57.28	**0.001**	winter	spring	1.25	0.232	1.93	**0.047**	1.19	0.263
season	2	2.21	**0.003**	winter	summer	1.45	0.117	2.49	**0.023**	1.56	0.076
spp.* season	4	1.86	**0.006**	spring	summer	1.27	0.202	2.13	**0.029**	1.22	0.248
total	18										

Table S4.6. Leaf proteins of interest detected in *Acacia ligulata, Myoporum montanum* and *Solanum oligacanthum* grouped into complexes or functional groups, up to four levels of hierarchy according to functional protein BINs in MapMan.

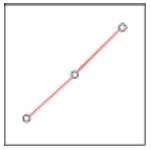
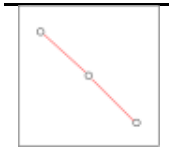
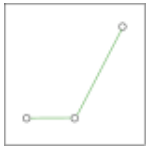
Mercator BIN no.	Level 1	Level 2	Level 3	Level 4	*A. lig*	*M. mon*	*S. oli*
1.1.1.1	Photosynthesis	photophosphorylation	photosystem II	LHC-II complex			
1.1.1.2				PS-II complex assembly and maintenance			
1.1.1.3				photosynthetic acclimation			
1.1.1.4				photoprotection			
1.1.1.5				LHC-related protein groups		ND	ND
1.1.1.6							
1.1.2			cytochrome b6/f complex				
1.1.3			Cytb6/f to PS-I electron carriers				
1.1.4.1			photosystem I	LHC-I complex			
1.1.4.2				PS-I complex assembly and maintenance			
1.1.4.3				ferredoxin electron carrier			
1.1.5.1			linear electron flow				
1.1.5.2				FNR activity			
1.1.6.1			cyclic electron flow	PGR5/PGRL1 complex			
1.1.8.1			chlororespiration	NDH complex			
1.1.9			ATP synthase complex				
1.2.1.1		Calvin cycle	RuBisCo activity	RuBisCo dimer			
1.2.1.2				RuBisCo assembly			
1.2.1.3				regulation			
1.2.2			phosphoglycerate kinase				
1.2.3			glyceraldehyde 3-phosphate dehydrogenase				

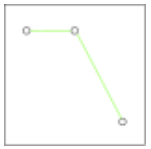
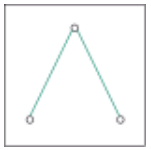
Mercator BIN no.	Level 1	Level 2	Level 3	Level 4	*A. lig*	*M. mon*	*S. oli*
1.2.4			triosephosphate isomerase				
1.2.5			fructose 1,6-bisphosphate aldolase				
1.2.6			fructose-1,6-bisphosphatase				
1.2.7			transketolase				
1.2.8			sedoheptulose-1,7-bisphosphatase				
1.2.9			phosphopentose isomerase				
1.2.10			phosphopentose epimerase				
1.2.11			phosphoribulokinase				ND
1.2.12			NADPH-dependent malate dehydrogenase				
1.3.1		photorespiration	phosphoglycolate phosphatase				
1.3.2			glycolate oxidase				
1.3.3			aminotransferases				
1.3.4			glycine cleavage system				
1.3.5			serine hydroxymethyltransferase				
1.3.6			hydroxypyruvate reductase				
1.3.7			glycerate kinase				
1.3.8			glycerate:glycolate transporter			ND	ND
1.4.1		CAM/C4 photosynthesis	phosphoenolpyruvate (PEP) carboxylase activity				
1.4.2			NAD-dependent malate dehydrogenase				
5.1.1	Lipid metabolism	fatty acid synthesis	citrate shuttle				
5.1.3			acetyl-CoA generation				ND
5.1.4			acetyl-CoA carboxylation				
5.1.5			plastidial Type II fatty acid synthase (ptFAS) system				
5.1.6			mitochondrial Type II fatty acid synthase (mtFAS) system				

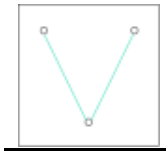
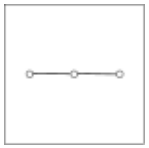
Mercator BIN no.	Level 1	Level 2	Level 3	Level 4	*A. lig*	*M. mon*	*S. oli*
5.3.3		galactolipid and sulfolipid synthesis	galactolipid galactosyltransferase (SFR2)		ND		ND
5.3.5			UDP-sulfoquinovose synthase				
5.4.12		sphingolipid metabolism	ceramidase activities		ND	ND	
5.5.1		phytosterols	campesterol synthesis		ND	ND	
5.5.2			phytosterol conjugation			ND	ND
5.7.2		lipid degradation	phospholipase activities				
5.7.3			fatty acid degradation				
5.8.2		lipid transport	FAX fatty acid export protein				
9.1.1	Secondary metabolism	terpenoids	mevalonate pathway			ND	
9.1.2			methylerythritol phosphate pathway				
9.1.3			terpenoid synthesis			ND	
9.2.1		phenolics	p-coumaroyl-CoA synthesis			ND	ND
9.2.2			flavonoid synthesis and modification			ND	ND
9.3		N-containing secondary compounds				ND	
10.2.1	Redox homeostasis	enzymatic ROS scavengers	catalase				
10.2.2			superoxide dismutase activities				
10.3.1		low-molecular-weight scavengers	ascorbate biosynthesis				
10.3.2			tocopherol biosynthesis				
10.3.3			glutathione metabolism				ND
10.4.1		hydrogen peroxide removal	ascorbate-glutathione cycle				
10.4.2			glutathione peroxidase				
10.4.3			peroxiredoxin activities				
10.5.1		chloroplast redox homeostasis	ferredoxin-dependent thioredoxin reductase (FTR) complex				
10.5.2			NADPH-dependent thioredoxin reductase			ND	
10.5.3			2-Cys peroxiredoxin activities				

Mercator BIN no.	Level 1	Level 2	Level 3	Level 4	*A. lig*	*M. mon*	*S. oli*
10.5.4			F-type thioredoxin				
10.5.5			M-type thioredoxin				
10.5.6			X-type thioredoxin			ND	
10.5.7			ACHT atypical thioredoxin			ND	
10.5.8			Y-type thioredoxin				
10.5.35			Thioredoxin-like protein CDSP32		ND	ND	
10.6.1		cytosol/mitochondrion/nucleus redox homeostasis	H-type thioredoxin			ND	
10.6.2			O-type thioredoxin			ND	
26.1.3	External stimuli response	light	UV-B light			ND	ND
26.3.2.1		temperature	Hsp (heat-shock-responsive protein) families	Hsp100 protein			
26.3.2.2				Hsp90 family			
26.3.2.3				Hsp70 family			
26.3.2.4				Hsp60 family			
26.3.2.5				sHsp families			
26.3.3			Csd (cold-shock-domain) protein		ND	ND	
26.4.1		drought	stomatal closure signalling				
26.6.2		biotic stress	pathogen effector			ND	
26.35		defense response				ND	

Table S4.7. Protein correlation with expression patterns across season for *Acacia ligulata*, *Myoporum montanum* and *Solanum oligacanthum*

Seasonal expression pattern	*A. ligulata*	*M. montanum*	*S. oligacanthum*
	low-MW scavenger (10.3.1) fatty acid synthesis (5.1.6)	calvin cycle (1.2.5) Photorespiration (1.3.6) low-molecular-weight scavengers (10.3.2) chloroplast redox homeostasis (10.5.3) Hsp100 protein(26.3.2.1) Hsp60 family (26.3.2.4) sHsp (26.3.2.5) galacto- & sulfo-lipid synth (5.3.5) lipid degradation (5.7.3)	cytosol/mitochondrion/ nucleus redox homeostasis (10.6.1)
	low-MW scavenger (10.3.3)	terpenoids (9.1.2)	enzymatic ROS scavengers (10.2.1)
	calvin cycle (1.2.5)	PSII (1.1.1.3) calvin cycle(1.2.10)(1.2.2)(1.2.3)(1.2.4) photorespiration(1.3.2)(1.3.4)(1.3.5) hydrogen peroxide removal (10.4.3) fatty acid synthesis (5.1.3)	

Seasonal expression pattern	*A. ligulata*	*M. montanum*	*S. oligacanthum*
	calvin cycle (1.2.6)	lipid degradation (5.7.2)	Calvin cycle (1.2.7)(1.2.8) photorespiration (1.3.1) PEP carboxylase activity (1.4.2) hydrogen peroxide removal (10.4.2) chloroplast redox homeostasis (10.5.2/6) fatty acid synthesis (5.1.4) terpenoids(9.1.2)
	chlororespiration (1.1.8.1) RuBisCo activity (1.2.1.3) Photorespiration (1.3.2)(1.3.6) enzymatic ROS scavengers (10.2.1) chloroplast redox homeostasis (10.5.3/5)	PSII (1.1.1.5) calvin cycle (1.2.11) low-MW scavenger (10.3.1) hydrogen peroxide removal (10.4.1) Hsp70 family (26.3.2.3) Hsp90 family (26.3.2.2) Lipid transport (5.8.2)	
	fatty acid synthesis (5.1.3)	fatty acid synthesis (5.1.1)	PEP carboxylase activity (1.4.1) cold-shock-domain protein (26.3.3)
	calvin cycle (1.2.7) Photorespiration (1.3.4) hydrogen peroxide removal (10.4.2) chloroplast redox homeostasis (10.5.4)		PSII complex (1.1.1.2) PSII(1.1.1.4) PSI (1.1.4.2) calvin cycle(1.2.3) photorespiration(1.3.6)

Seasonal expression pattern	*A. ligulata*	*M. montanum*	*S. oligacanthum*
	N-containing 2° compounds (9.3) lipid degradation (5.7.2)	PSII (1.1.1.4) PEP carboxylase activity (1.4.1) chloroplast redox homeostasis(10.5.1)	low-MW scavenger (10.3.1) galacto- & sulfo-lipid synth (5.3.5)
	PSII.LHC-II complex (1.1.1.1) cytochrome b6/f complex (1.1.2) RuBisCo activity (1.2.1.2) photorespiration(1.3.1) PEP carboxylase activity (1.4.1/2) galacto- & sulfo-lipid synth (5.3.5) phytosterols (5.5.2) Hsp70 family (26.3.2.3)	PSI (1.1.4.1/2) calvin cycle(1.2.8)(1.2.9) chloroplast redox homeostasis (10.5.4)	calvin cycle(1.2.4)(1.2.10) photorespiration(1.3.5)(1.3.7) low-molecular-weight scavengers (10.3.2) chloroplast redox homeostasis (10.5.5)

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