University of Reading
(School of Agriculture, Policy and Development)

University of Nottingham
(School of Biosciences)

A multifaceted evaluation of the effects of heat stress on the pollen development of wheat

Peter J. L. Mark

March 2016

Thesis submitted for the degree of Doctor of Philosophy

Dedicated to

Peter Ross & Wilbert Steen

Declaration

I confirm that this is my own work, and the use of all material from other sources has been properly and fully acknowledged.

Peter J. L. Mark

Acknowledgements

Despite this Ph.D. being a largely individual exercise, I find myself so grateful for those who have, in one way or another, come alongside me over the last few years.

To the staff at both The University of Nottingham and the University of Reading, your help and advice was much appreciated. Dr. Jose Fernández Gómez, Dr. Alison Ferguson and Mr. Behzad Talle, you can rest assured that my numerous 'wee questions' will be no more. Your help and guidance through the world of molecular biology was given with such patience. I have no doubt great careers lie ahead for all three of you. Likewise, Ms. Caroline Hadley, Mr. Liam Doherty, Mr. Laurence Hansen, Mr. Richard Casebow, and Mr. Matthew Richardson, your patience and guidance was always most valued.

Many thanks go to Mr. Samuel Leigh and Ms. Erika Degani for letting me adopt a portion of their LIBERATION plots in the summer of 2015. The results proved of great interest.

To my friends David Bourne, Martin Winter, Gareth Leaney, and especially Jon Nurse, your comradeship over the years has meant a great deal. Thank you. In a world desperate for men of character, I am proud to know these four. Many thanks also go to Doug & Anna Lee, who provided me with accommodation when in Sutton Bonington. Thank-you for making your home my home for those eight months. You are a very special couple.

Over and above their input into my Ph.D., I am very thankful for my family. I am very blessed to have wonderful siblings. Jonathan (& Lucy), Catherine, Olwyn, Andrew (& Punita) and Ruth (& David), it has been a joy to be part of your lives over the past years. The opportunities of light relief from 'Ph.D. land' were always much needed also.

To my father and mother, whatever title follows, I will always be far prouder to be your son.

Rather than a number of years of research, in many ways this thesis is the culmination of a couple of decades' work. To all of those who gave me the support to deal with the challenges my dyslexia presented, I thank-you. May future generations of dyslexic children have the opportunity to realise their potential, where sadly past generations have not. On this note, many thanks go to my father who proof read this thesis for me.

Many thanks go to the University of Reading, The University of Nottingham and Rothamsted Research Ltd for providing the funding for me to do this Ph.D. I am also very grateful to The London Resin Co LTD, who kindly donated numerous bottles of LR White resin.

Finally, to my supervisors, Prof. Zoe Wilson and Dr. Hannah Jones, your patience, kindness and guidance over the past years has been a real blessing. When looking back, I will always appreciate the memories of two ladies whose primary concern was for me, rather than my work. A very profound “thank-you” goes to you both.

Abstract

Pollen formation is considered '*the Achilles tendon of reproductive development*'. Therefore, special attention must be directed towards making sure that pollen is sufficiently robust, in order to cope with future climatic changes. One such anticipated future change in climate is increasing global temperature. Wheat is a very important crop for global food security, but wheat pollen development has been shown particularly sensitive to temperature stress. Therefore, work is needed to increase the environmental resilience of wheat pollen.

In this thesis, attempts were made to not only find modern wheat varieties that had an increased tolerance to heat stress during pollen development, but also to clarify which stage(s) of wheat pollen development were the most sensitive to heat stress. Additionally, experimentation was conducted in order to assess the effect of heat stress on anther/pollen related gene expression, and the effect that inter-ear viable pollen movement had on yield restoration.

In spite of it being shown that there were varieties that sustained significantly less grain losses, due to a heat stress event, and that a reduced level of pollen damage played a key role in this, it was apparent that this was not an example of tolerance, but instead an example of avoidance. Additionally, unlike previous reports, the developmental stage around pollen mother cell meiosis was not found to be the most significantly affected by heat stress, either in relation to grain number or microspore/pollen wellbeing. Instead, this designation was given to the latter stages of pollen wall development. Heat stress, during pollen development, had a profound effect on the expression levels/patterns of six anther/pollen related genes.

This research has established a firm platform, in numerous different areas, for the future exploration of possibilities for reducing the effects of abiotic stress on wheat pollen development, and therefore directly increase yield resilience.

Contents

Chapter 1

General Introduction

1.1 Global cereal production in the presence of abiotic stress

1.1.1 Challenges to modern cereal husbandry

In the months following the sowing of cereals, and preceding their eventual harvest, it is undoubtedly of critical importance, for a grower, to provide, where possible, optimal conditions for their cereals to mature. Therefore, cereal husbandry has always been of the upmost importance, in order to optimise both harvest quality and quantity (Wibberley, 1989).

There are a number of factors, key to optimal harvest, which are not within the growers' control. This is particularly true in relation to climatic conditions, since cereals are grown in external environments, as opposed to within protected facilities (e.g. glasshouses). These factors include temperature, light and, to a lesser extent, hydration. It is well documented that departures from optimal levels of these factors, otherwise known as stresses, have a detrimental effect on cereal harvest quality/quantity (Barnabás *et al*., 2008) and inter-seasonal yield level stability (Kang & Banga, 2013). Additionally, extreme weather conditions are now occurring more frequently and, to a greater extent, than they have done in the past (IPCC, 2007; Bita & Gerats, 2013).

1.1.2 Patterns of climatic change, and its effects on food security

Average global temperatures are increasing. For example, May 2012 was not only the warmest (global average) May since records began in 1800, but also the 36th consecutive May, and 327th consecutive month, with a global temperature above the 20th century average (Kang & Banga, 2013).

Not only is there predicted to be widespread drought conditions, over the next decades, as a result of decreased precipitation and/or increased evaporation (Dai, 2013), it is also predicted that occurrences of extremely elevated temperature events will not only increase in frequency, but also in the amount of the earth's surface that they will effect (Hansen *et al*.,

2012). The reasons for these, and other, future projections (e.g. Sillmann & Roeckner, 2008) are very unlikely to be due to natural climatic cycling, but as a direct consequence of anthropogenic climate change, due to the accumulation of greenhouse gases (Coumou & Rahmstorf, 2012; Hansen *et al.*, 2012).

These current/future climatic changes are adding to past changes which have had '*a substantial impact on agricultural production worldwide*' (Bita & Gerats, 2013), and thus detrimentally affect a desire to increase food production, in order to attain food security. The Rome declaration on world food security defines food security as the situation were "*all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life*" (FAO, 1996).

Hansen *et al.* (2012), in stating that summer is '*when most biological productivity occurs*', subsequently acknowledge that it is during summer that changes in climatic conditions will have their greatest effect on humanity. These effects include those related to increases in the occurrences of seasonal temperature anomalies, towards higher values, which are now occurring over about 10% of global land area (Hansen *et al.*, 2012). Indeed, the timing and frequency of extreme temperature events could be more influential, in relation to yield loss, than increases in mean temperatures (White *et al.*, 2006).

1.1.3 Historic patterns of, and future challenges to, global cereal production

Beyond the individual grower's desire to achieve optimal harvests, by minimizing the negative effects that abiotic stresses have on their cereals, like those previously reported (e.g. Hedhly *et al.*, 2009), the global community is ever more aware that its population is continuing to grow (Figure 1.1). In addition, the global community has existing commitments to try and ensure adequate food and nutrition for such a population (e.g. Target 1.C of the UN's Millennium Development Goals), with global food supply needing to increase by about 70% by 2050 (FAO, 2009). However, despite there being enough food currently produced, per capita, to feed the global population (WFP, 2015), about 795 million people remain undernourished (FAO *et al.*, 2015), with sub-Saharan Africans having the highest proportions of food insecurity (FAO *et al.*, 2012). It is therefore clear that solutions to hunger and malnutrition are more than just agronomic.

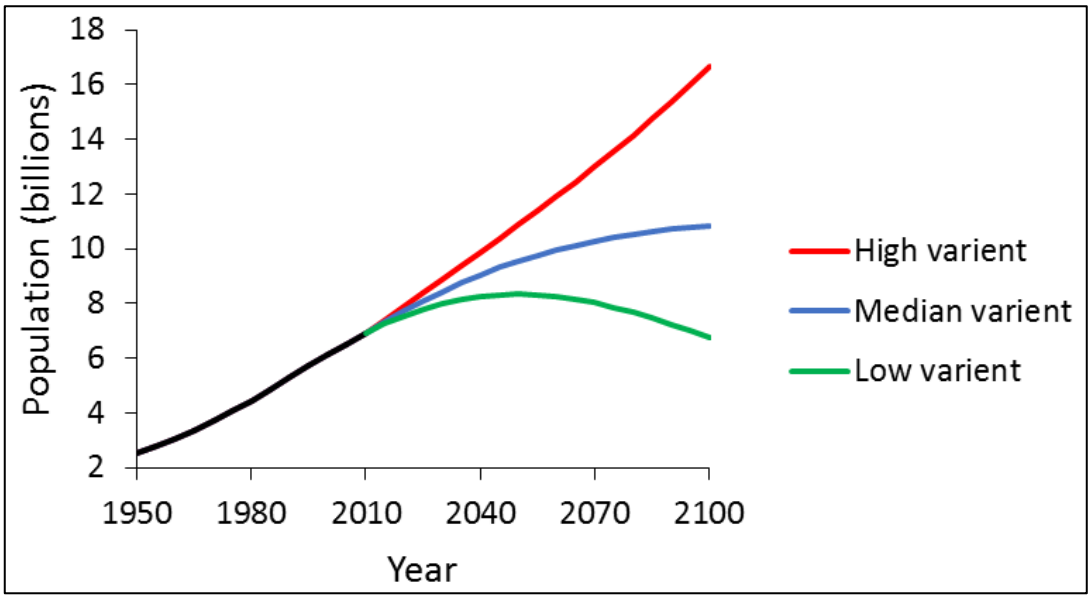


Figure 1.1: Actual, and projected, global, human population growth over a 150 year period (UN, 2013).

In light of commitments, such as those outlined in the Millennium Development Goals, changes in climatic conditions are likely to have the greatest effects on those living in developing countries, as nearly 50%, of these populations, rely entirely on agriculture (Bita & Gerats, 2013), with global levels of child malnutrition anticipated to increase by 20%, due to climate change, relative to a world in the absence of climate change (Nelson *et al.*, 2009).

Nevertheless, in a desire to increase future levels of cereal production amounts, it is worth noting that humanity has successfully increased the amounts of cereal production by 217%, between 1961 and 2013 (Figure 1.2a), outstripping the global population increase (129%), between 1960 and 2010 (UN, 2013). This increase in production, even in the presence of heightened risk from abiotic stress, is predominantly due to a 185% increase in the levels of average yield (Figure 1.2b), as opposed to greater amounts of land (11%) being given over to cultivation (Figure 1.2c), over the same time period. This therefore indicates that agronomic advance, over the past decades, was largely responsible for such increases in the amounts of cereal production.

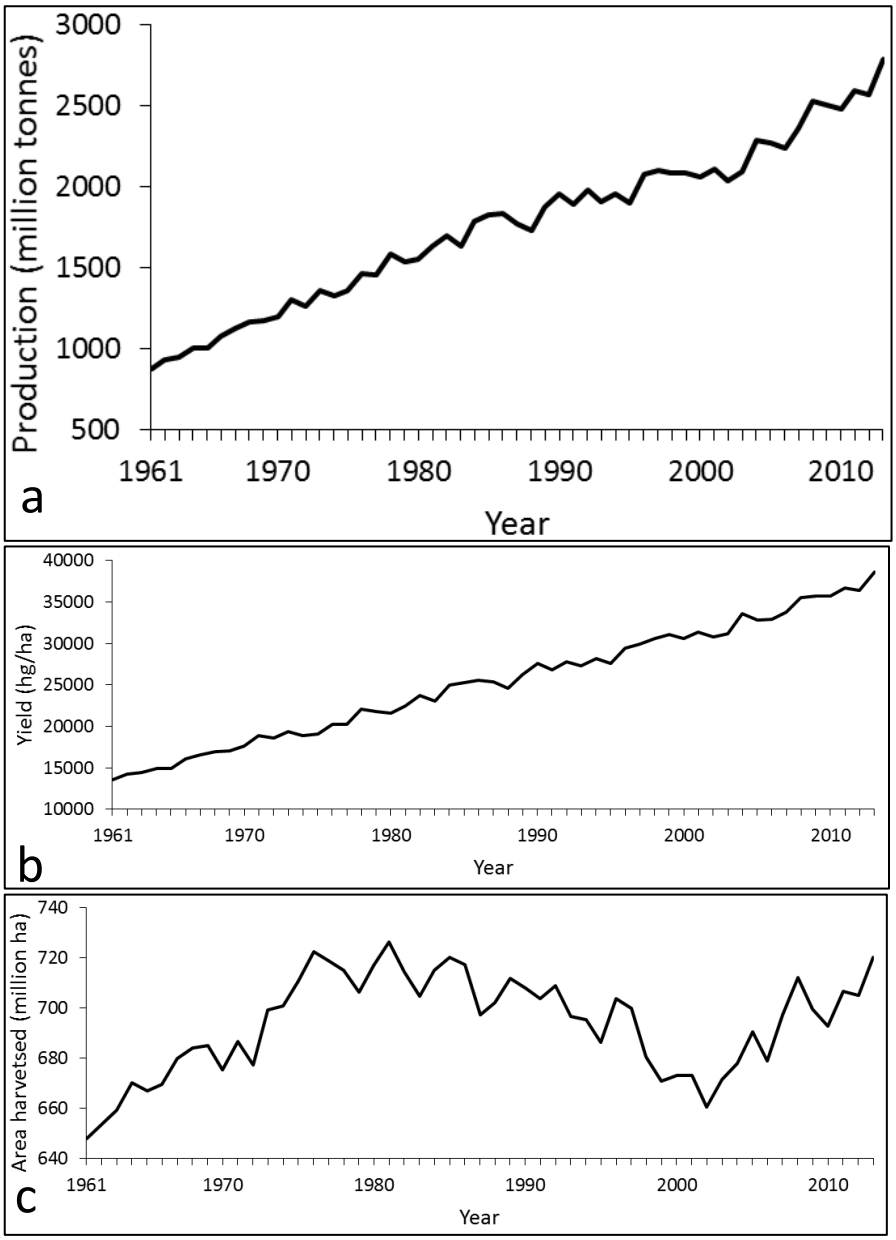


Figure 1.2: Changes in the levels of, (a) global cereal production amount, (b) average global cereal yield, and (c) global cereal area harvested, between 1961 and 2013 (FAOSTAT, 2015).

Conceivably, the aforementioned average yield increases, experienced over the past half century, were due to increases in each of three component parts of cereal yield. These yield components are:

1) Number of ears per unit of area
2) Number of grains per ear
3) Weight of individual grains

The first two yield components, which determine grain number per unit of area, are more instrumental in determining eventual yield than individual grain weight (Willey & Holliday, 1971; Ellen & Spiertz, 1980).

Due to each of the three yield components being determined at different times during wheat development, this means that cereals are often buffered against the possibility of very low yields, due to unforeseen circumstances (Evans *et al*., 1975). Ear number is determined well before anthesis, seed number just before, and during, anthesis, and grain weight right up until maturity (Evans *et al.*, 1975). Additionally, yield restorational compensation, between factors, has been reported in the past. For example, reduced grain set, due to drought stress, was partially compensated by an increase in the weight of the remaining individual grains (Saini & Aspinall, 1981).

In light of these past achievements in cereal production, a proverbial gauntlet has now been thrown down for current, and future generations of agronomists. Future crop yield increases do not need to be as rapid as in the past (Alexandratos & Bruinsma, 2012), where global supplies of rice, wheat and maize rose so dramatically, that their prices fell by approximately 60%, between 1960 and 2000 (FAO, 2002). However, in order to meet future demands, the global agronomic research community must not be found complacent in its endeavours in trying to minimise the extent that abiotic stress effects cereal yields. In the face of yield declines already linked to climatic change, it is estimated that grain production per unit of land will need to more than double to address rising demand over the course of the 21st century (Lobell & Asner, 2003; Long & Ort, 2010).

In addition, for the major crops (wheat, maize and rice), in both tropical and temperate regions, climate change, in the absence of adequate agronomic adaptation, will negatively impact production levels, even if mean temperatures rise as little as 2^{o}C above late 20th century levels (IPCC, 2014$_{c}$). Due to changing climatic conditions potentially having more of a negative effect on tropical regions, as opposed to temperate regions, and most developing countries being located within the tropics, it is likely that climate driven yield reductions will have the greatest effect on those who can least afford it and/or be able to adapt to the associated challenges (Dar & Gowda, 2013).

1.2 Wheat

1.2.1 Introduction

Wheat is the generic name given to species found within the genus *Triticum* L. (Poaceae). Even though not in large scale commercial agronomic cultivation anymore, the ancestors of modern cultivated species (e.g. Emmer & Einkorn wheat) originated, via domestication, from those geographical regions around the foothills of the Zagros Mountains in Iraq-Iran, the Taurus Mountains in Turkey, and the coastal mountains along the eastern end of the Mediterranean Sea (Peterson, 1965). The earliest signs of such wheat domestication in this region appeared about 10,000 years ago, and was a key point in the development of human civilization, with such domestication enabling the transition from a nomadic hunter-gatherer society to a more sedentary agrarian one (Eckardt, 2010). There were two important traits to evolve, which allowed such crop domestication; the increase in grain size, and the development of seeds that did not naturally disperse, but instead allowed humans to harvest them (Eckardt, 2010).

Despite there being approximately 25,000 different cultivars, modern wheat cultivars belong primarily to two polyploid species, hexaploid bread wheat (*T. aestivum* L.) and tetraploid durum-type wheat (*T. turgidum* L. (Thell.)) (Gustafson *et al*., 2009).

Being a grass, each plant has multiple pseudostems, known as tillers, which originate from approximately soil level (Figure 1.3a). The majority of these tillers will, in time, produce a spike (a.k.a. ear or head), composed of approximately twenty spikelets, which are arranged alternately along the length of the spike (Figure 1.3b). These spikelets are, in turn, composed of numerous florets, which are arranged alternately (Figure 1.3c).

The majority of these florets will eventually produce one grain. Therefore, one plant can produce hundreds of grains.

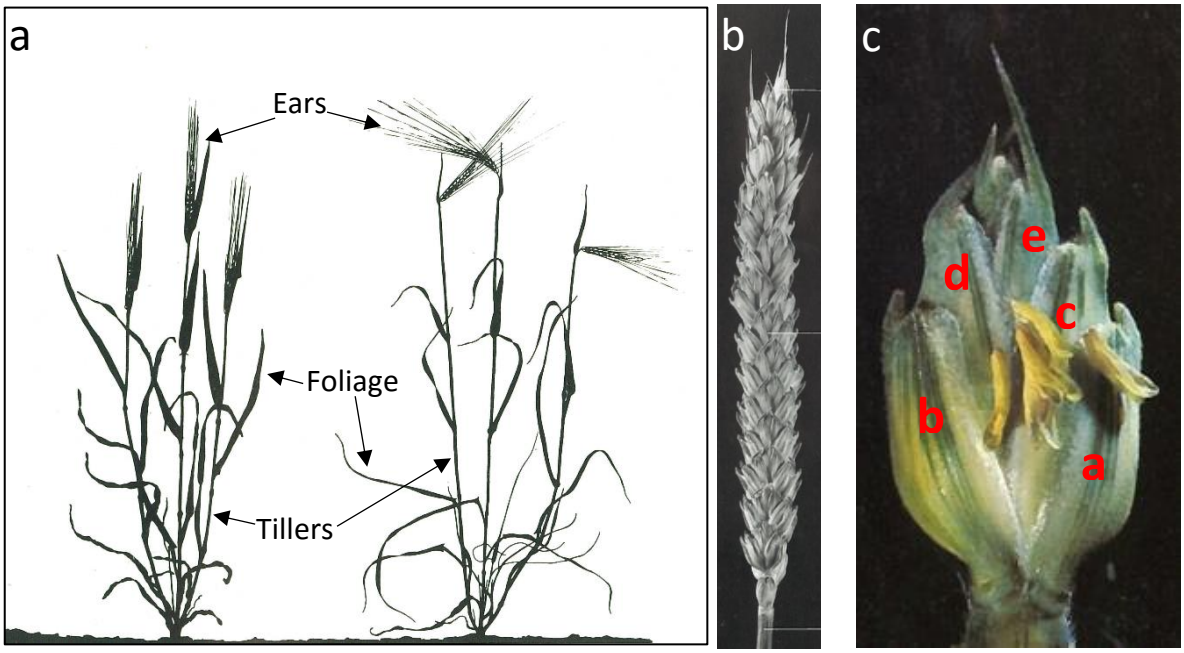


Figure 1.3: Wheat plant morphology. (a) The latter stages of wheat plant development, (b) composition of a wheat ear, and (c) composition of a wheat spikelet, with the first five florets labelled sequentially (Kirby & Appleyard, 1984).

Each viable floret contains three anthers and one superior ovary, found below a two lobed stigma (Figure 1.4). Self-pollination tends to happen within each floret of a wheat ear (De Vries, 1971).

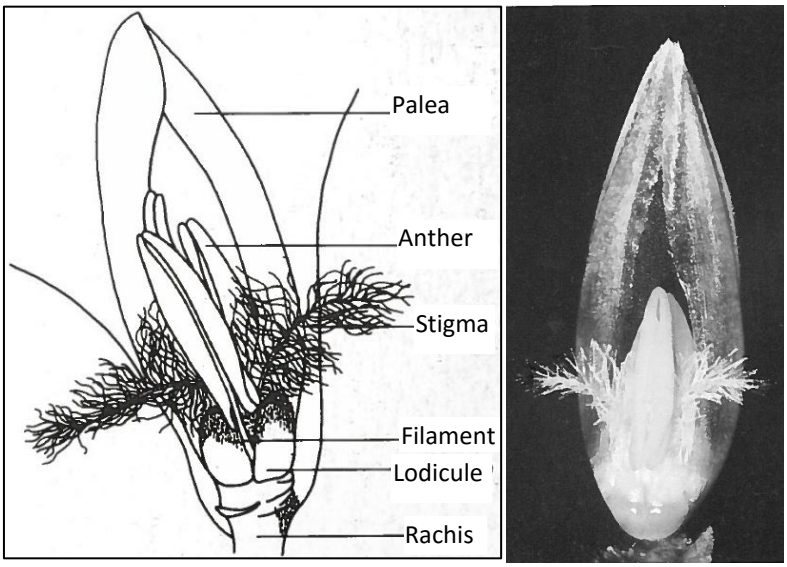


Figure 1.4: Composition of a wheat floret prior to anthesis. The lemma is removed for visualisation of interior anatomy (Kirby & Appleyard, 1984).

Due to the morphological and anatomical changes occuring during the season long life cycle of wheat, it is possible to determine the extent of plant maturity at any particular occasion

within a growth season (Leather, 2010). To date, there have been a number of 'scales' to aid with this assessment of maturity. However, perhaps the most widley used of these scales, amongst the agronomic community, is the Zadoks scale (Zadoks *et al.* 1974). This is also one of the more detailed scales available to agronomists (Figure 1.5).

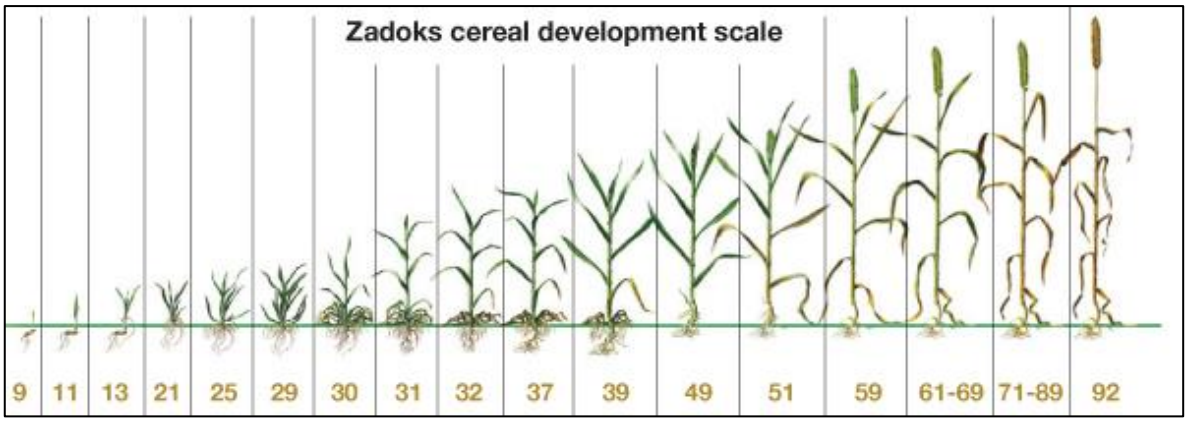


Figure 1.5: Seventeen stages within Zadoks cereal development scale (CerealCentral, 2015).

With the seasonal patterns of growth corresponding with changes in plant physiology, including plant chemistry (Kohler, 1944), the timing of stressful events, including heat stress (Saini & Aspinall, 1982), results in a varying degree of yield loss, depending on the stage of exposure (Leather, 2010). Therefore, a comprehensive understanding of, and ability to, stage wheat plants, throughout the growth season, is essential to those studying the effects of stress on yield.

1.2.2 Global wheat production

Wheat, after undergoing the first steps of evolution as a crop plant almost 10,000 years ago (Harlan & Zohary, 1966), is now a staple crop for about 40% of the world's population (Eastham & Sweet, 2002), and contributes about 20% of the total, worldwide, dietary calories and proteins (Shiferaw *et al.*, 2013). Wheat is the third most produced cereal in the world, behind only rice and maize (Figure 1.6a), and is, by some distance, the cereal occupying the most hectares (Figure 1.6c), possibly due to it being relatively low yielding (Figure 1.6b). There may also be the possibility that wheat occupies more hectares because it is more adapted to growing in a greater range of global environments, when compared to the other two cereals.

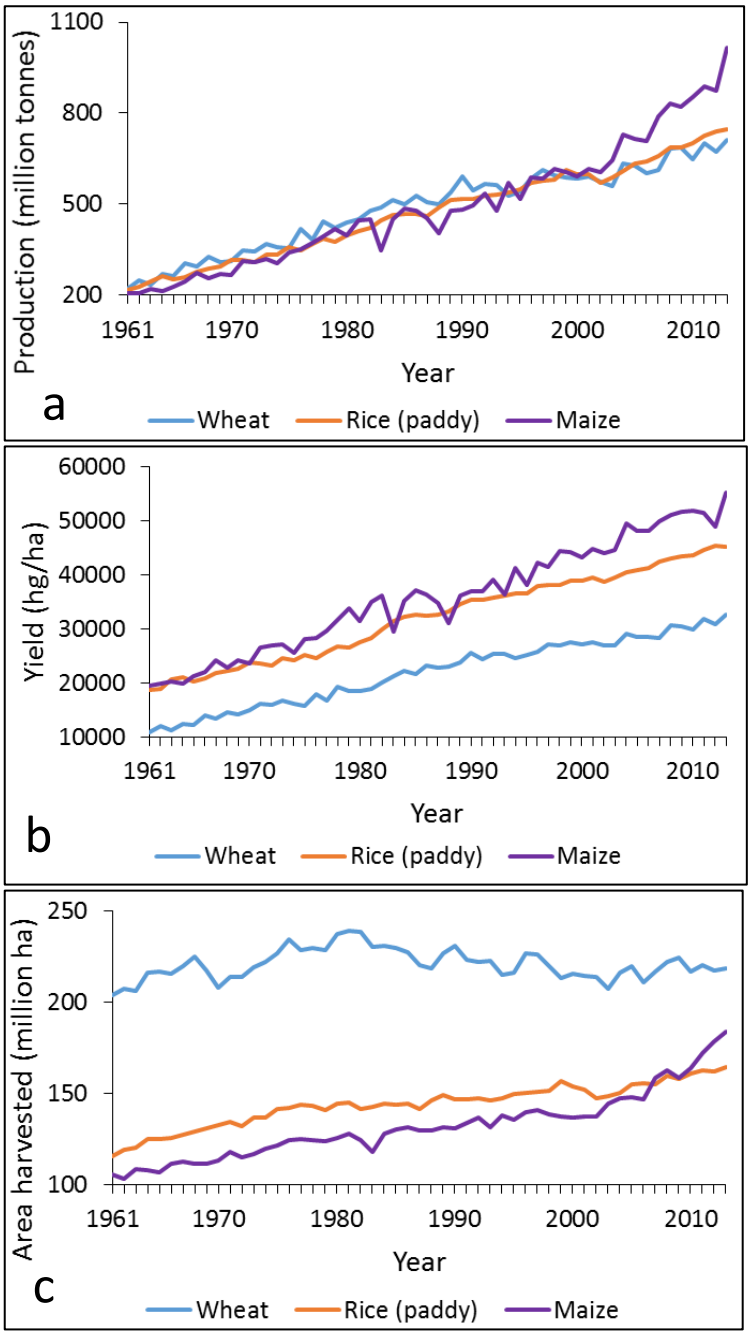


Figure 1.6: Changes in the production levels of the three principal cereals', (a) global production amount, (b) average global yield, and (c) global area harvested, between 1961 and 2013 (FAOSTAT, 2015).

Much like cereals in general, the increase in production amounts of wheat, between 1961 and 2010 (221%) had more to do with an increase in average yield (200%), than increases in land devoted to growth (7%) (FAOSTAT, 2015). However, the percentage change in regional levels of area harvested, average yield, and production amounts differ greatly (Figure 1.7).

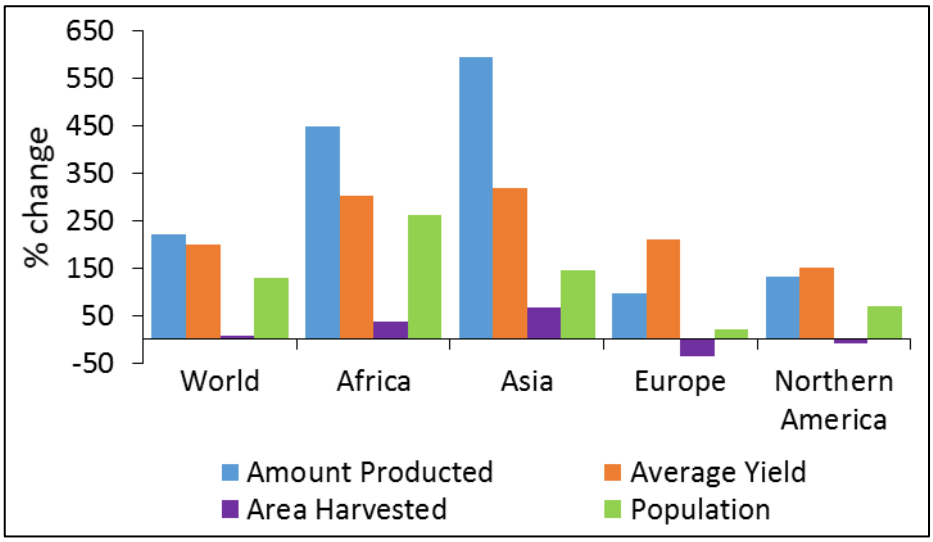


Figure 1.7: Percentage changes in the levels of wheat production amount, average yield, and area harvested, between 1961 and 2013 (FAOSTAT, 2015), and percentage population change, between 1960 and 2010 (UN, 2013).

It is apparent that both Africa and Asia, whose constituent countries are largely within the economic category of 'developing', have had higher levels of percentage increase in production, average yield and area harvested than the global average (Figure 1.7). In contrast, Europe and North America, whose constituent countries are largely within the economic category of 'developed', have had lower levels of increase in these three areas, even to the point of decreasing the amount of land they have dedicated to wheat production over this period. Encouragingly, like cereals in general, it is evident that, across these regions, percentage increases in both wheat yield and production amount, even though slowing, have outstripped population growth (Figure 1.7).

1.2.3 Reasons behind historic wheat yield increases

When considering changes in historic levels of wheat yield, England proves typical of most industrialised countries. Where it took nearly 1,000 years for yields to increase from 0.5 to 2 tonnes per hectare, yield levels increased to 7 tonnes per hectare, during the 20th century alone (Hazell, 2009). Through advances, in not only plant (including wheat) breeding techniques, but also the development and application of inorganic fertilizers, and improved agronomic practices, the second half of the 20th century effectively saw the eradication of occurrences of food shortages in industrialised countries (Hazell, 2009).

In the past, a yield level of approximately 1.7 tonnes per hectare was considered somewhat of a yield 'take off' threshold, in that it signified the transition point from traditional

agricultural practices, with little outside inputs, to modern agriculture, with the considerable inputting of outside resources (Tribe, 1994). Therefore, by the beginning of the 1970s, the implementation of a yield beneficial global agricultural research framework, especially for wheat and rice, commonly known as the 'Green Revolution', was well established in developing countries.

The term 'Green Revolution' refers to a period of time, around the middle of the twentieth century, where primarily through the uptake of technological advances in a number of agronomic disciplines, crop yields rose dramatically. This increase in yields was primarily experienced by farmers in the developing world, with the 'Father of the Green Revolution', Norman Borlaug, widely credited with saving over a billion people from starvation. He was thus awarded the Noble Peace Prize in 1970.

In the developed world, especially in traditionally wheat producing regions (e.g. North America & Europe), such yield increases, associated with the Green Revolution, had happened more gradually over the previous decades (Hazell, 2009). This gradual increase is perhaps one reason why, over the latter stages of the twentieth century, yield increases in these developed regions were not as marked as in the developing world. However, it would be remiss to say that the Green Revolution did not benefit developed countries as well. For example, in 1994, of the total area of wheat sown in Australia, 87% was sown with CIMMYT based varieties, despite CIMMYT's primary purpose being to serve the developing world (Tribe, 1994). Additionally, between 1973 and the mid-1990's, New Zealand farmers had reportedly benefited by $0.5 million annually, as they increasingly used CIMMYT lines as parents in local crosses (Tribe, 1994).

With one of the principal factors behind historic global wheat yield increases being the improvement of germplasm, Pfeiffer (2003) states that the genetic improvement of wheat was characterised in a number of phenotypic ways, including:

- Faster maturation – This allows for more crops per year, compared to conventional lines.
- Disease resistance – This is due to the integration of alleles, into cultivated lines, conferring resistance to certain diseases.
- Semi-dwarf growth habit – Semi-dwarf varieties (90cm) are less likely to topple over under the weight of grain than their full size (120cm) counterparts. This not only

prevents the growth of fungal spores on the ear, due to soil moisture, but also makes harvesting easier. This was particularly true when in the presence of high levels of nitrogen fertilization, another facet of the Green Revolution (discussed later).

Often through integrating these three characteristics, by crossing, into existing locally adapted germplasm, wheat yields rose exponentially in countries such as India and Mexico (Figure 1.8) during the 1960's to early 1970's.

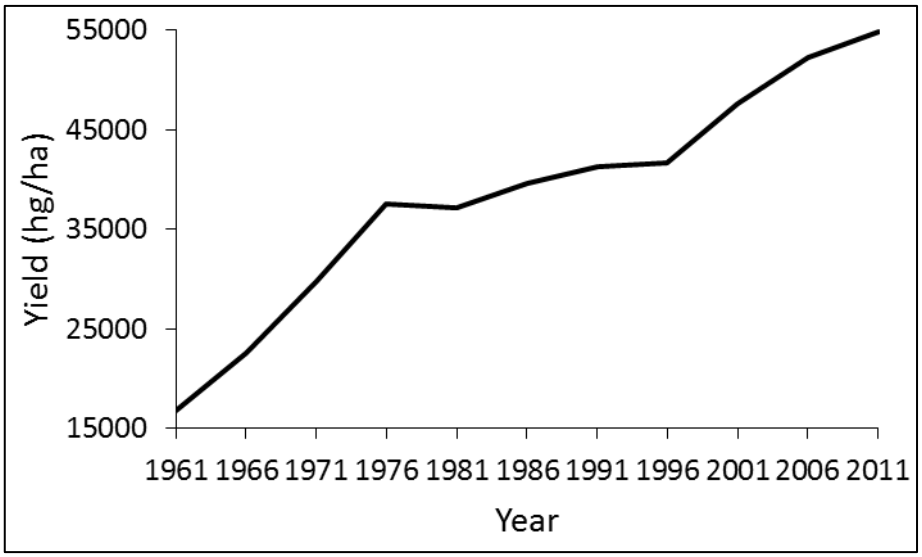


Figure 1.8: Changes in the average wheat yield levels of Mexico, between 1961 and 2011 (FAOSTAT, 2015).

However, as detailed by other sources, the Green Revolution in wheat, as in other crops, was not solely due to farmers adopting new germplasm, but also by adopting new practices (e.g. use of fertilizers, use of insecticides, use of herbicides, use of equipment for irrigation, adaptation of cropping cycles and increased employment levels) (Chrispeels & Sadava, 2003). Examples of this expansion of non-biological resources, in order to increase yields, were apparent in the latter part of the twentieth century in Asia, where fertilizer use and irrigation rose dramatically (Table 1.1).

Table 1.1: Input use change in Asian agriculture, during the Green Revolution (Hazell, 2009).

	Irrigated Area (% of agricultural area)			Fertilizer Application (kg/ha)		
	1970	1995	% change	1970	1995	% change
Bangladesh	11.6	37.6	224	15.7	135.5	763
China	37.2	37	-0.5	43	346.1	705
India	18.4	31.8	72	13.7	81.9	498
Indonesia	15	15.2	1	9.2	84.7	821
Malaysia	5.9	4.5	-24	43.6	148.6	241
Myanmar	8	15.4	93	2.1	16.9	705
Nepal	5.9	29.8	405	2.7	31.6	1070
Pakistan	67	79.6	19	14.6	116.1	695
Philippines	11	16.6	51	28.9	63.4	119
South Korea	51.5	60.8	18	251.7	486.7	93
Sri Lanka	24.6	29.2	19	55.5	106	91
Thailand	14.2	22.7	60	5.9	76.5	1197
Vietnam	16	29.6	85	50.7	214.3	323

In light of the many benefits which resulted from those practices associated with the Green Revolution, and which continue, there are sufficiently strong arguments to suggest that it was not an all-encompassing solution to global challenges. Three areas in which this is so are poverty alleviation, nutritional improvement and environmental preservation.

- Poverty alleviation – Transnational econometric evidence indicates that the agricultural sector is significantly more effective in reducing poverty amongst the poorest of the poor ($1-per-day), when compared to the non-agricultural sector (Christiaensen *et al*., 2011). However, because Green Revolution strategies were fundamentally designed for intensification within pre-existing agronomically favourable areas, its contributions to poverty alleviation within marginal production environments, such as those which are purely rain-fed, is relatively limited (Fan & Hazell, 2001).
- Nutritional improvements – Due to a fall in the price of staple food prices, as a result of the Green Revolution, money was able to be spent on non-staple micro-nutrient-

dense foods (Torlesse *et al.*, 2003). However, at times, dietary diversity, and therefore micronutrient acquisition, actually decreased. An example of this was reported in the Philippines, where the development of intensive rice monocultural systems led to the loss of fish and wild leafy vegetables that had been previously harvested by the poor (Cagauan, 1995).

- Environmental preservation – Despite not being a principal impetus for its development, one of the major global benefits of the Green Revolution is that associated intensification has prevented the need to expand into new areas of previously uncultivated land (Figure 1.7). However, such intensification can have consequences on both the cultivated land and surrounding areas, as a result of factors like chemical runoff and soil degradation (Burney *et al.*, 2010). These detrimental, environmental effects are thought to be a long-term threat to the sustainability of the successes of the Green Revolution (Pingali & Rosegrant, 1994).

1.2.4 The future of global wheat production

When examining the future global production projections of wheat, and cereals in general, it is apparent that many of the trends, seen over the last number of decades, are set to continue further into the 21st century. For example, even though decreasing in rate of growth (Bita & Gerats, 2013), the amount of yield (Langer *et al.*, 2014), production amount and area harvested are likely to all increase over the coming years (Figure 1.9). In addition, the amount of resources dedicated to such production, including fertilizer use (Figure 1.10), will likely increase, especially within the developing world. The majority of fertilizer use, on a global level, is directed towards cereal production and, in particular, wheat, rice and maize (Alexandratos & Bruinsma, 2012).

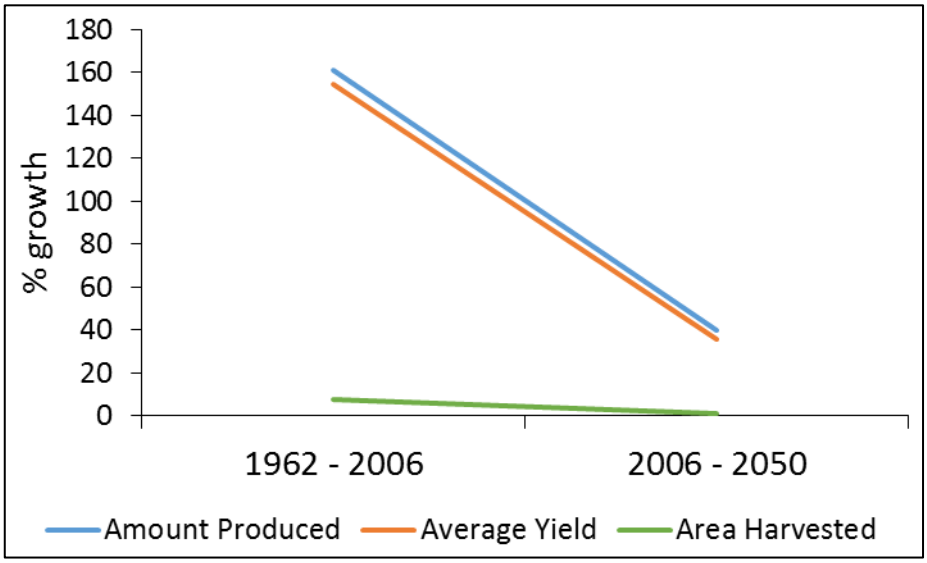


Figure 1.9: Percentage growth in global wheat production amount, average yield, and area harvested, over two 44 year periods, historic and projected (Alexandratos & Bruinsma, 2012).

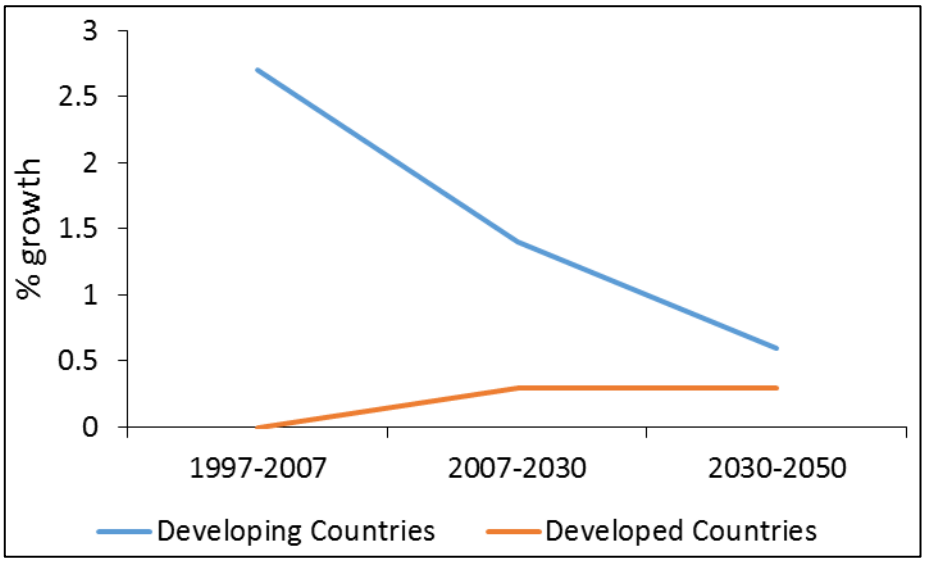


Figure 1.10: Percentage growth in average fertilizer usage, per annum, over three time periods, historic and projected (Alexandratos & Bruinsma, 2012).

Continuing investment in technological input will, as in the past, achieve higher yields. For example, at the global level, wheat grown on irrigated land, where there are most likely to also be higher amounts of other inputs (e.g. pesticides, herbicides & fertilizers) will, between 2006 and 2050, probably experience a higher level of yield increase (43%) than their purely rain fed counterparts (34%) (Alexandratos & Bruinsma, 2012).

In contrast to developing countries tending to be net exporters of rice, they are, on the most part, net importers of wheat (Figure 1.11), with exports from developed countries mirroring this continuing trend (Alexandratos & Bruinsma, 2012). Alongside this increase in the levels of

wheat imported, it is estimated that some 80 percent of future increases in crop production (including wheat) in developing countries will have to come from intensification (e.g. higher yields, increased multiple cropping and shorter fallow periods) (FAO, 2002).

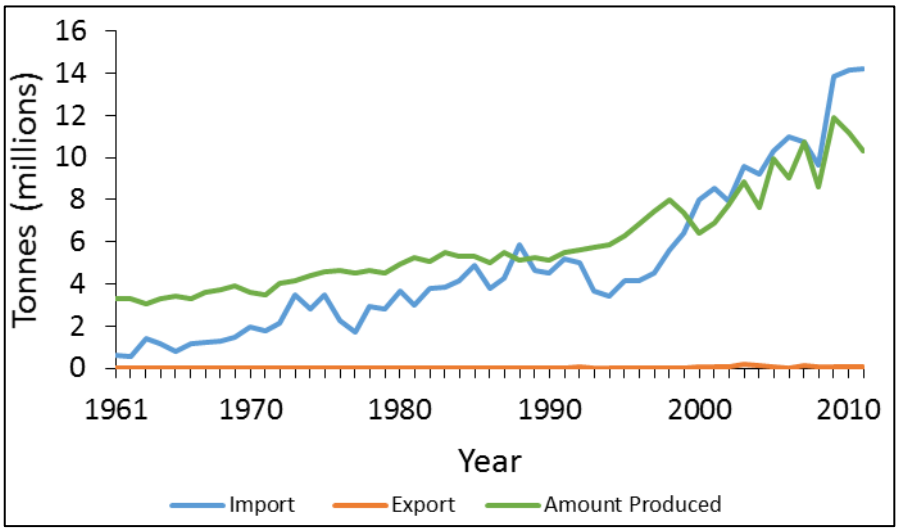


Figure 1.11: Quantity of wheat imports, exports, and production amounts, in '*least developed countries*', between 1961 and 2011 (FAOSTAT, 2015).

Within developing sub-Saharan African, wheat production falls a long way short of its potential (Coghlan & Marchall, 2012). Even in areas, with conditions conducive to growing wheat, yields are only 10-25% of their potential, thus leaving the populations overly reliant on foreign markets, where prices can rise by '*50% in a few months*' (Coghlan & Marchall, 2012). An example of fluctuating global wheat price is that of 2010, where a heat wave in Russia led to a Russian export ban, resulting in global prices doubling by the end of the year (IPCC, 2014$_c$). Therefore, it is apparent that the principles behind the Green Revolution still have yet to be fully implemented within regions such as developing sub-Saharan Africa. This continued need to embrace these Green Revolution practices, in developing countries' wheat production, is critical as, unlike crops such as maize, which is the major cereal crop in Sub-Saharan Africa and Latin America, the bulk of wheat is produced in the land scarce regions of Asia, Near East and North Africa (Alexandratos & Bruinsma, 2012). Therefore, the large scale expansion of land devoted to wheat production, in order to increase the levels of production, is not a widespread option. Instead, even in the face of the majority of African countries, over half their crop area is going to experience climates outside their current range by 2050 (Burke *et al.*, 2009), increasing productivity, in these areas, will need to come from yield increases. In addition, historic approaches to increasing cereal yields over the last decades, such as increasing the

proportion of plant biomass (at harvest), comprised of grain (currently approx. 60%), and increasing the interception of seasonal solar radiation by the crop canopy (currently approx. 90%), leave little room for dramatic improvement (Zhu *et al*., 2010). Instead, emerging technologies and understandings, such as a greater understanding of gene networks controlling yield components, may hold the key to increasing yields (Long & Ort, 2010).

Despite, based on numerous studies, there being a '*medium confidence*' that future climate trends will have a negative effect on wheat production in many global regions, wheat based systems are more adaptable to future climatic challenges than, say maize, especially in temperate regions (IPCC, 2014_c). This presence of adaptability, through management options (e.g. cultivar and planting date adjustment), may be a key reason for a lesser projected increase in wheat prices (67%) by 2050, without action on climate change, when compared to rice (78%), and especially maize (131%) (Dar & Gowda, 2013).

Ways in which future climatic change is expected to affect future wheat production levels include:

1) Changes in atmospheric gases – Despite increases in levels of carbon dioxide (CO_2), 100ppm since pre-industrial times, enhancing water use efficiency and yields, especially in C_3 crops such as wheat, increases in other, accompanied, atmospheric component, such as ozone (O_3), has very likely suppressed global yield levels (IPCC, 2014_c). Past losses of approximately 10%, for wheat, due to gaseous changes (Van Dingenen *et al.*, 2009), are expected to continue into the future (Van Dingenen *et al.*, 2009; Long & Ort, 2010; Avnery *et al.*, 2011).
2) Temperature increases - Yields of wheat can begin to decline with as little as a 1-2^oC rise in local temperatures (IPCC, 2014_c). Every 1^oC rise in mean temperature is expected to result in a 6 million tonne reduction in Indian wheat productivity (Kang & Banga, 2013). At least half of the Indo-Gangetic Plains (IPG), which is a heavily populated region, comprising 13 million hectares that extends from Pakistan to Bangladesh, and produces 15% of the world's wheat, may, in the future, be too heat stressed to grow wheat (Banga & Kang, 2013). Indications, based upon stochastic simulations of wheat growth, indicate that a greater interannual variation of temperature reduces average wheat yields more than a simple change in mean temperature (Mearns *et al.,* 1997; Easterling 2005).

3) Changes in rainfall patterns - Progressively decreased rainfalls detrimentally affect achievable cereal sowing dates (IPCC, 2014_c). Drought will also have a directly negative effect on eventual wheat yields (Foulkes *et al*., 2007).
4) Combinations of effects – Elevated levels of O_3, combined with higher temperatures, will possibly cause greater losses in wheat, when compared to either in isolation (Long, 2012). Additionally, in light of the response of photosynthesis to higher temperatures being a major driver in any effects temperature has on yield, increased temperatures are in fact associated with lower CO_2 assimilation by decreased stomatal conductance (Long & Ort, 2010). Future, hotter and drier, climatic conditions, in southern and western regions of Australia, are predicted to reduce wheat yields by up to 15% (Kokic *et al*., 2005).

1.3 Case study: Past, current and future wheat production in China

1.3.1 Background

On a global level, the variation in the yield discrepancy between the top and bottom 10 countries, in relation to levels of yield, have stayed relatively stable over the past decades (FAOSTAT, 2015). In contrast, despite the levels of production amounts of the top 10 producers always being markedly higher than those of the bottom ten, the discrepancies, between the two, have fluctuated greatly (Table 1.2).

Table 1.2: Percentage yield of the bottom ten countries, when compared to the top ten countries, when ranked in relation to production amount and yield (FAOSTAT, 2015).

	1970	**1980**	**1990**	**2000**	**2010**
Production amount	71%	56%	44%	68%	46%
Yield	12%	11%	9%	7%	10%

At the time increments displayed within Table 1.2, only two countries (France and Germany) ever appear in both the global top tens of wheat yield, and wheat production amount, and they do so consistently. Therefore, in order to attain greater global food security, the optimization of these larger producers' yields may be prudent, as relatively small increases in yield may have great effects on global amounts of wheat production. One of these high producing/relatively low yielding countries is China, a country in which future food demand is expected to '*surge*' (Bita & Gerats, 2013).

Since 1991, China, based on annual reporting (FAOSTAT, 2015), has been the largest wheat producer in the world. However, it has never been in the top ten countries in relation to yield (FAOSTAT, 2015), despite making marked improvements (Figure 1.12), primarily through the adoption of practices associated with the Green Revolution (Liu et al., 2010; He *et al*., 2014).

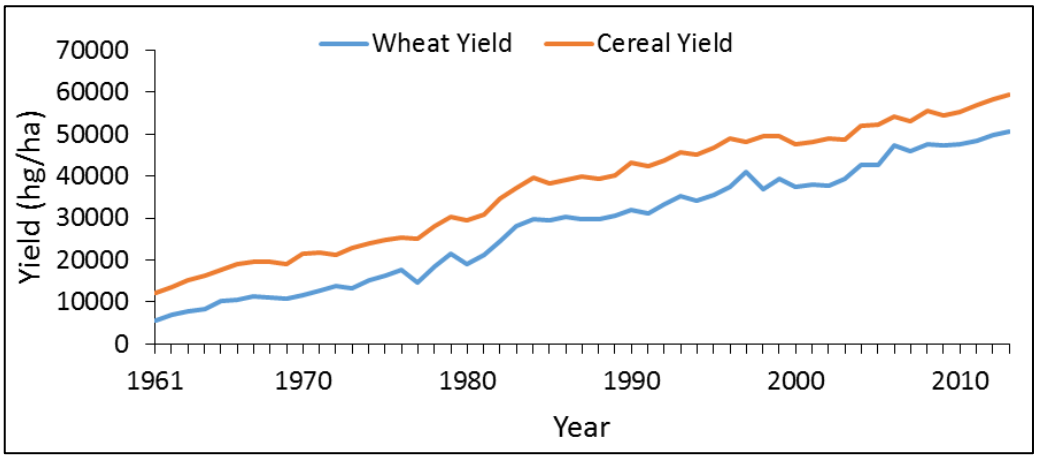


Figure 1.12: Average cereal and wheat yields in China, between 1961 and 2013 (FAOSTAT, 2015).

Therefore, it is unsurprising to find that, over the last decades, it has also consistently been one of the countries which devoted the highest amounts of land to wheat growth (FAOSTAT, 2015). However, when espousing the significant role that Chinese wheat plays in global wheat production (Figure 1.13), it is important to note that it is still, after rice and maize, arguably the tertiary cereal in Chinese agronomy (Figure 1.14), despite it being the dominant staple food in the northern part of the country (CIMMYT, 2015).

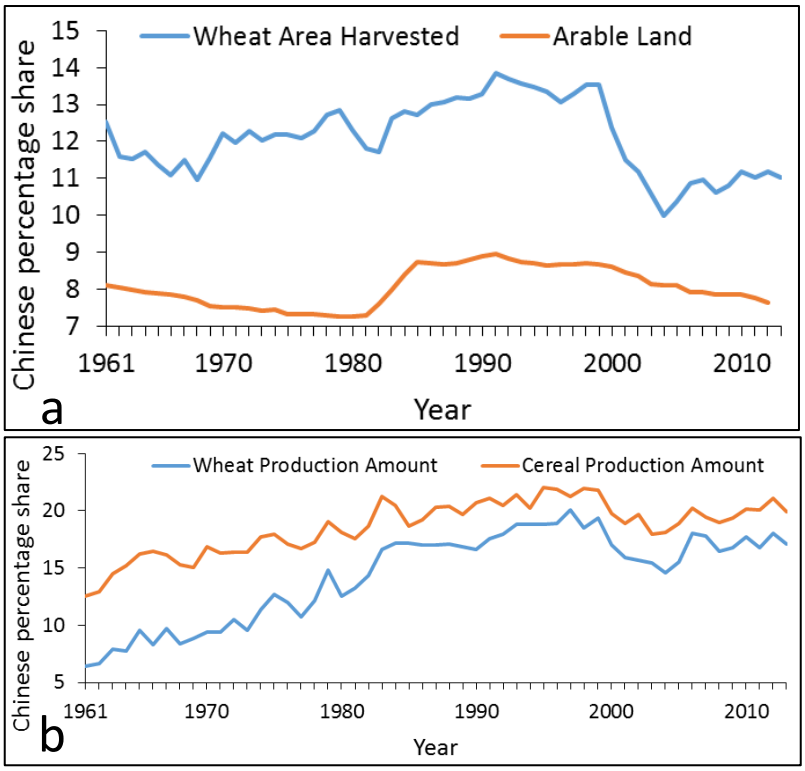


Figure 1.13: The percentage share China has of (a) global wheat land use, and (b) global wheat production amounts, between 1961 and 2013 (FAOSTAT, 2015).

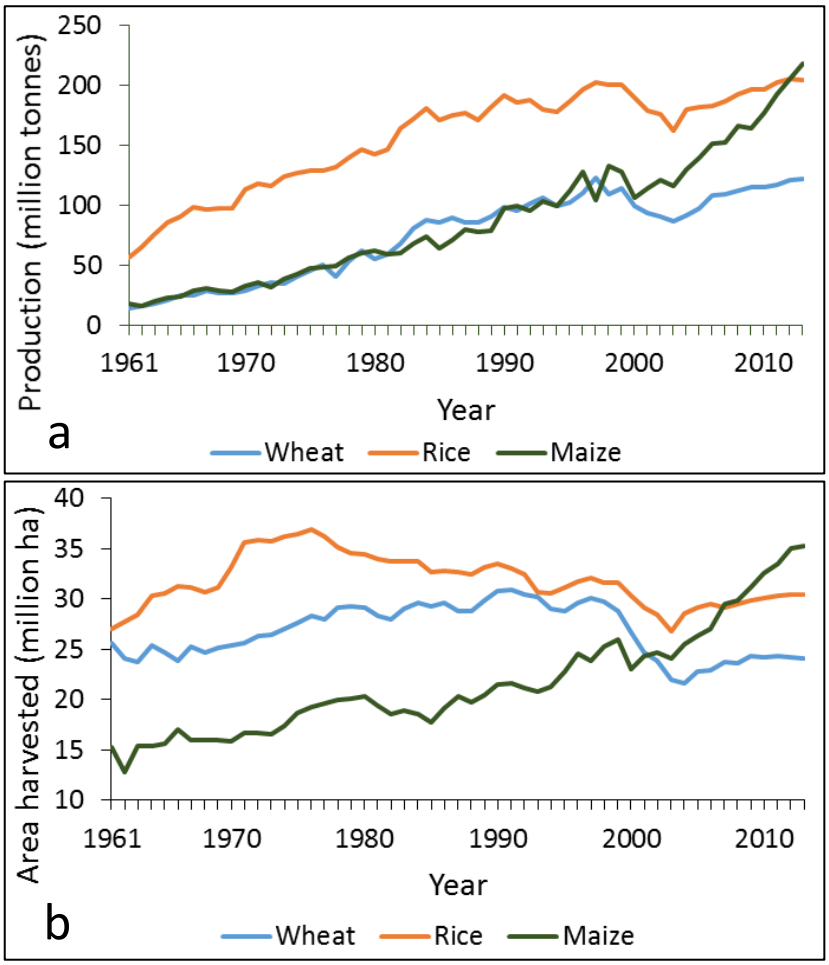


Figure 1.14: The 'big three' cereals in China. (a) Production amounts, and (b) extent of area harvested, between 1961 and 2013 (FAOSTAT, 2015).

Despite wheat making up 40 percent of Chinese grain consumption, and about 60 percent of the population consuming it daily (CIMMYT, 2015), per capita consumption is likely to decrease, if only slightly, over the coming decades (Alexandratos & Bruinsma, 2012). In spite of there being no consumption projections for wheat, over the next decades, overall cereal consumption is expected to increase significantly in the future (He *et al.*, 2014). Therefore, a reduction in per capita consumption will most likely do little to offset this consumption trend.

1.3.2 Future of Chinese wheat production

With China producing about 17% of global wheat (Figure 1.13), it is undoubtedly a major force in international wheat markets. This influence is not so much enacted by their actions in the markets, but by their comparative lack of participation. With the majority of the wheat produced being consumed within the country (lack of export) and little foreign wheat being consumed (lack of import) (Figure 1.15).

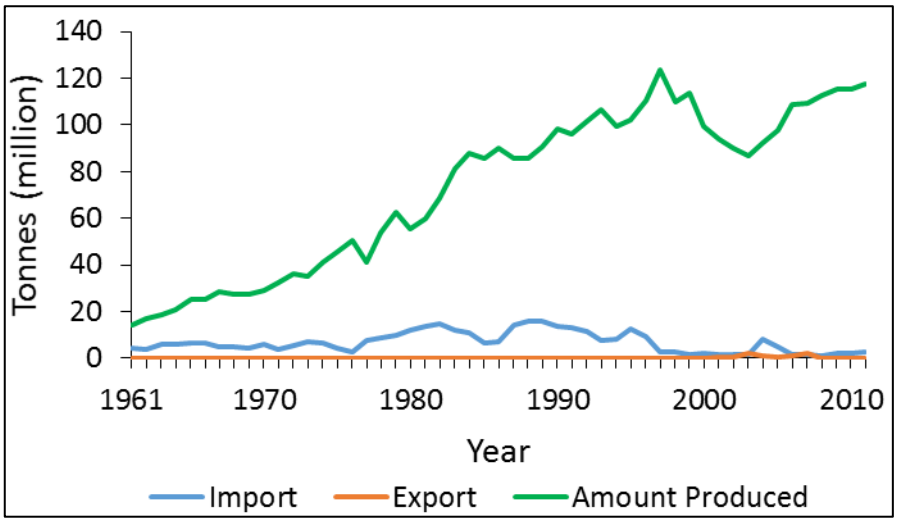


Figure 1.15: Chinese global trade in, and production amounts of, wheat, between 1961 and 2011. (FAOSTAT, 2015).

In China, internally driven food security has, for some time, been a national priority (Liu *et al.*, 2010), and an increasingly important international issue, with the total amount of agricultural products imported expected to rise from the relatively low levels of 11% in 2012 (He *et al.*, 2014). From an international perspective, the less China can access external markets the better, as even a small percentage increase in imports could increase global prices and affect the cost of living in many developing countries, who are increasingly reliant on the importing of wheat (Alexandratos & Bruinsma, 2012).

At the present time two principal issues are claimed to affect Chinese wheat production, if it were not to import from external markets. Both of these issues are greatly exacerbated by China only having 0.1 hectares of arable land per capita, a figure well below the global average (He *et al.*, 2014).

The first of these issues is an increase in the amount produced for animal feed, with an increasing amount of Chinese arable land being dedicated to the production of maize (Figure 1.14b) for the feeding of livestock (He *et al.*, 2014), which is a growing market (Figure 1.16). In addition to the levels of maize production overtaking both wheat and rice within the last decades (Figure 1.14a), the proportion of wheat being given over to livestock feed has increased from 5% in 2000 to 15% in 2014 (He *et al.*, 2014).

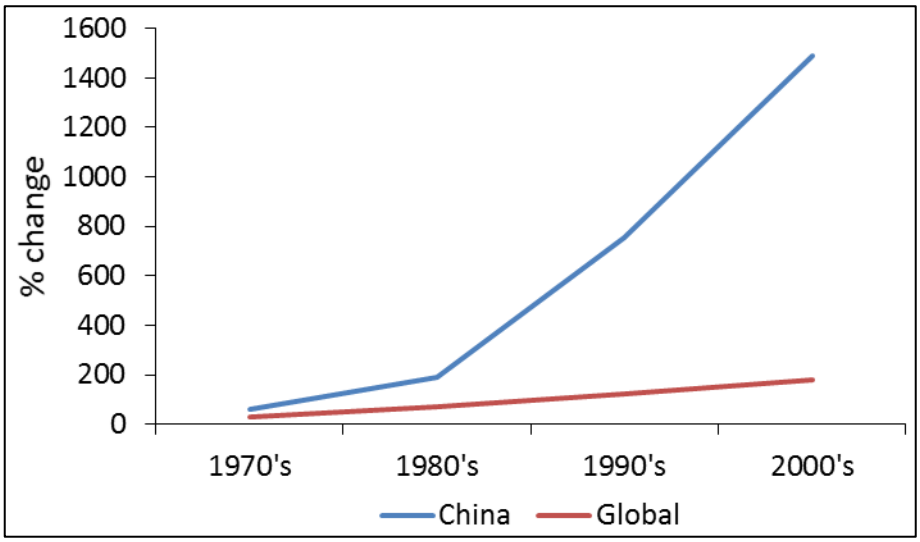


Figure 1.16: Levels of Chinese and global production of animals (Cattle, Chickens, Sheep & Pigs) for slaughter, based on the levels of the 1960's (FAOSTAT, 2015).

The second of these issues, and more in keeping with the rest of this work, is changing environmental conditions. For example, despite wheat yields in the North China Plain (NCP), one of China's largest agricultural regions of production, increasing over the last decades (Liu *et al*., 2010), there are potential risks to future production (Kendy *et al.,* 2003). Whilst supplying 50% of China's wheat, the NCP possesses potential evapotranspiration greatly exceeding its annual precipitation, therefore increasing the chances of wheat experiencing drought stress (He *et al.*, 2014). In addition, Tao *et al.*, (2006) show yield reductions associated with other aspects of climatic change. Due to '*significant warming trends*' (Table 1.3), Tao *et al.* (2006) document the shifting of wheat crop phenology, and the associated reduction in wheat yields during the 1980's and 1990's (Table 1.4). Warming occurs mainly during the vegetative (pre-flowering) growth stages of wheat, whilst there was either a cooling trend, or no significant change in temperatures, during the post-flowering stages (Liu *et al*., 2010).

Table 1.3: Trends in seasonal climate at two field stations in eastern China, during the period 1981-2000 (* trends are significant *p*=<0.05, ** trends significant *p*=<0.01) (Tao *et al.*, 2006).

Field Station	Season	Maximum Temperature		Minimum Temperature		Precipitation	
		Trend (days/decade)	R^2	Trend (days/decade)	R^2	Trend (days/decade)	R^2
Zhengzhou	Winter	0.9*	0.22	0.9**	0.44	1.5	0.007
	Spring	0.2	0.006	0.7	0.31	-1.8	0.002
Tianshui	Summer	0.4	0.11	0.5*	0.23	-2.1	0.001
	Winter	1	0.16	0.7*	0.27	0.1	0
	Sping	1	0.24	1.1**	0.65	7.1	0.11

Table 1.4: Trends in phenology and wheat yields at two field stations in eastern China, during the period 1981–2000 (* trends are significant *p*=<0.05, ** trends significant *p*=<0.01) (Tao *et al.*, 2006).

Field Station	Planting Date		Anthesis Date		Maturity Date		Yield	
	Trend (days/decade)	R^2	Trend (days/decade)	R^2	Trend (days/decade)	R^2	Trend (kg/ha/yr)	R^2
Zhengzhou	-3.4	0.09	-3.0	0.21	0	0	-112.8	0.15
Tianshui	-2.9	0.18	-2.7	0.11	-3.3*	0.28	-7.1	0.007

1.3.3 Principles for promoting future Chinese wheat security

Looking into the future, and seeking to learn from the lessons of the past, it is clear that, in a desire to establish internally driven wheat security for the Chinese population, two possible areas of focus are possibly prudent.

1) The governmental level - considering that only 40% of the total amount of Chinese wheat production, in 2007, was for supplying the market, as opposed to personal consumption or trading with neighbours (Lohmar *et al.*, 2009), the more the Chinese authorities can do to encourage the expansion, from this relatively low level, is to be encouraged. From encouraging individual farm size increases, to maximise economies of scale, to providing subsidies and price support that encourages farmers to keep producing wheat, even though it consistently brings the lowest profits among major crops in China (Lohmar *et al.,* 2009), the government undoubtedly has a role to play in these developments.

   As well as setting production aiding economic policies, the creation of new, expansion of existing, and large scale implementation of environmental policies, may be crucial. For example, legislation to reduce aerosol pollution in the Yellow and Huai River Valleys, which produce around 70% of the Chinese wheat crop, to levels that would not, as in the past (He *et al*., 2014), cause a significant decrease in sunshine hours and solar radiation, and therefore impact upon yields, would be advisable. However, pragmatically, the overall economic development of China, which may likely be somewhat based upon high aerosol emitting industry, may override this comparatively minor economic concern.

   On the non-legislative front, the Chinese government should, especially in light of previously discussed land scarcity (He *et al*., 2014), continue to increase their investment in both national and international breeding programmes, and other agronomic technological collaborations (e.g. synthetic fertiliser creation).

2) The grower level – through the 'on the ground' adoption, of not only new, but existing cultivation practices, Chinese wheat growers will hopefully be able to match their leading role in harvested area and amount produced, on the world stage, with their levels of yields. As in the past, the adoption of, and more efficient use of both improved

wheat germplasm and technological advances, will undoubtedly be the driving forces behind this. Past instances of successes in these areas include the development of new varieties, with reduced vernalisation requirements and lower photoperiod sensitivity, to compensate for the negative impact of climate change (He *et al.*, 2014), and the advent of mechanised pumping wells which allows the production of two crops per year in the NCP, instead of the historic levels of 2-3 crops every two years (Kendy *et al.,* 2003).

In light of a global population projected to continue to grow until the middle of the 21st century (UN, 2013), and an ever changing climate, where China's food security could be even more challenged, due to increased occurrence of biotic stresses (He *et al.*, 2014), the rest of the world will look on in the hope that China will not have to significantly enter the global markets to obtain wheat.

Can technological advance sustain the required yield increases in the face of, for example, total water resources, per capita, being about one quarter of the world average (Liu *et al*., 2010; He *et al.*, 2014)? The answer to this remains unclear. However, through both internal and international collaboration, and the creation of new, well suited, germplasm, via both conventional breeding and the increased integration of biotechnology into conventional breeding programmes, germplasm improvement is one of China's best hopes.

1.4 Conclusions

With global agronomy seeking to efficiently breed/find wheat varieties able to cope with the challenges facing global production, especially since those management opinions including '*cultivar adjustment*', having the greatest projected benefits (IPCC, 2014_c), there is importance in identifying which detrimental phenomenon will be most prevalent. However, since potential to address such issues is exacerbated by relatively limited time and resources, it is more important to identify which phenomenon, even at the regional level, will have the most detrimental effect on production levels. Therefore, despite drought stress being the most significant environmental stress affecting global agriculture (Cattivellie *et al.*, 2008; Jäger *et al.*, 2008), it is predicted that in Europe heat stress will have a greater effect on wheat production levels (Semenov & Shewry, 2011), with warming indeed expected to be most prevalent in the northern latitudes (IPCC, 2007).

Therefore, a special focus on breeding wheat varieties able to withstand such heat stresses is of the upmost importance to European agronomy, and perhaps beyond. However, it would be remiss to disregard an appreciation of the effects of drought stress. The greatest practical implications of increased changes in global temperature are not those directly associated with temperature stress, but those related to the associated detrimental effects that these will have on the water cycle (Hansen *et al.*, 2012). Larkindale (2005) also highlights the accompaniment of heat stress by high irradiance stress.

However, I concur with the sentiments of Bita & Gerats (2013), in that they state that despite heat stress being compounded by other additional abiotic stresses, such as drought, it is important to determine the independently arrived upon biological consequences of periods of high temperature, in order to mitigate the effects of combined abiotic stresses. This is why the research contained within this thesis will concentrate upon the effect that heat stress has on an aspect of wheat development, even in the presence of adequate hydration.

Due to the complexity of the heat stress syndrome (Bita & Gerats, 2013), and the resulting need for holistic approaches to any future investigations, within this work an array of different methods will be used, from field scale assessments to molecular genetics. However, as temperatures cannot be controlled in the field, a large amount of this work will be conducted within controlled environment facilities.

In addition to acknowledging heat stress as the predominating challenge facing future levels of wheat production in Europe, and perhaps beyond, there must be an appreciation of which, if any, yield related biological processes elevated temperature effects the most. Therefore, the remainder of this thesis will explore, through both the reviewing of current literature and conducting of primary research, the negative effects heat stress has on several aspects of the process of pollen development, which has been shown to be disproportionally affected by heat stress (Saini & Aspinall, 1982).

Chapter 2

Analysis of the Sensitivity of Wheat Pollen to Heat Stress at Different Developmental Stages

2.1 Introduction

2.1.1 Heat stress during wheat development

Plants experience a wide fluctuation of ambient temperature on both a seasonal and daily basis (Larkindale, 2005), with the optimal thermal range of most plants being approximately 10^{o}C (Mahan *et al.*, 1995). Numerous mechanisms have evolved (e.g. changing leaf orientation, transpirational cooling & alterations in the membrane lipid composition) to mitigate the negative, but not necessarily lethal, effects of elevated temperature (Bita & Garats, 2013). Nevertheless, Bita & Garats (2013) also report that elevated temperatures frequently and negatively affect plant wellbeing. This is largely due to heat affecting a wide range of structures and functions at the cellular level, not least limiting photosynthetic output (Larkindale, 2005). However, heat stress will negatively affect plants, including wheat, significantly more at certain points in their development, especially during the early stages of reproductive development (Hedhly *et al*., 2009; Dolferus *et al*., 2011).

Despite potentially falling into the development period in which any temperature increase will occur in China, the world's largest global wheat producer (Liu *et al*., 2010), no particular stages of wheat floral development, between the appearance of double ridges on the shoot apex (approx. 14-18 Zadoks Scale (ZS)) and emergence of the flag leaf (37ZS), appear to be significantly more sensitive to high temperature stress, in relation to impact on eventual yield (Barnabas *et al.*, 2008; Craufurd *et al*., 2013). This is despite these stages encompassing the developmental point(s) when the number of florets reaching anthesis, which will have a direct influence on eventual grain number, will be partially determined (Rawson & Bagga, 1979).

However, Saini & Aspinall (1982) showed, by exposing wheat to heat stress (30 day/30^{o}C night) for three consecutive days, at six different, post-37ZS, developmental stages (Figure 2.1), that there are specific developmental periods, which have significantly greater sensitivity to

elevated temperatures, as measured by reductions in grain set (Figure 2.2). Interestingly, using the same wheat cultivar, these periods of increased sensitivity to heat stress correspond to the developmental periods of sensitivity to water deficiency stress (Saini & Aspinall, 1981).

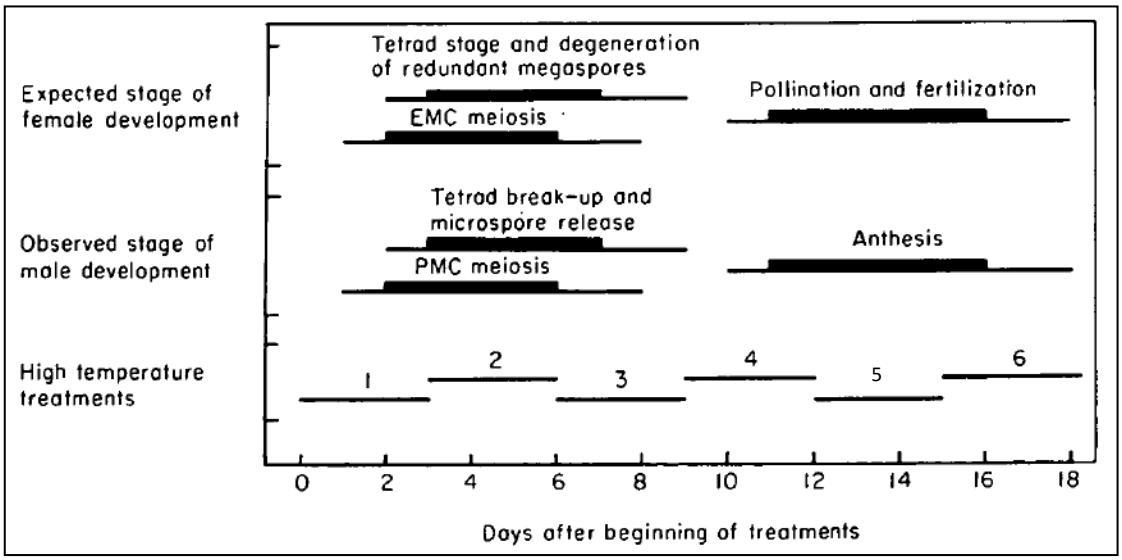


Figure 2.1: Relationship between periods of exposure to heat stress (30/30°C), and the observed development of male reproductive tissues, and expected development of female reproductive tissue. EMC = embryo sac mother cell, PMC = pollen mother cells (Saini & Aspinall, 1982).

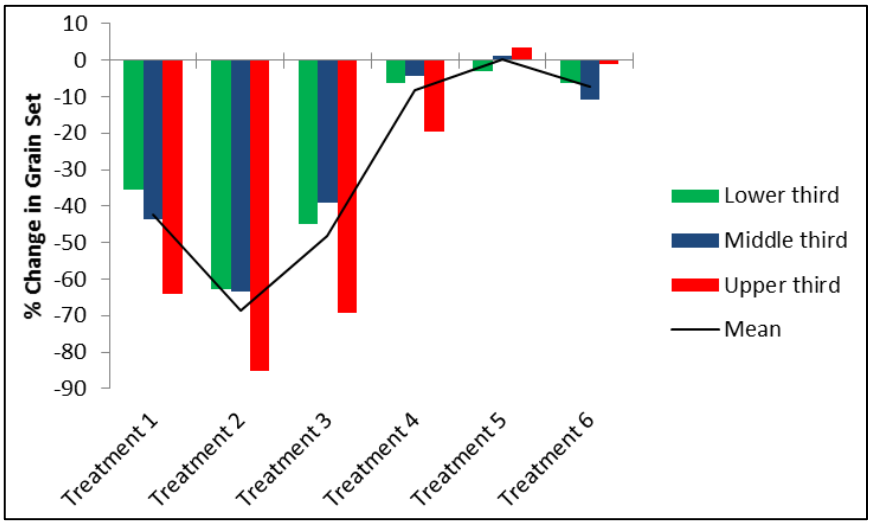


Figure 2.2: The effect of 3 days exposure to 30/30°C, at six stages of ear development, on the eventual grain set, in different spatial regions of wheat ears (adapted from Saini & Aspinall, 1982).

As can be seen from Figure 2.2, in light of Figure 2.1, the developmental periods, when heat stress had its most negative effect on eventual grain set, are those at the beginning of macro/microspore development (treatments 1, 2 & 3), with treatment 2 producing the lowest

eventual grain set. During these times the developing ear is still enclosed within one or more leaf sheaths of the pseudostem (also known as 'booting'). This phenomenon, of the greatest damage being inflicted at the earlier periods of gamete development, has been previously documented, with heat associated abnormalities in the meiotic progression of *Zea* L. (Randolf, 1932) and *Tradescantia* L. (Sax*,* 1937) having been reported.

From Figure 2.2, it is apparent that the upper third of the ear, during the first four treatments, is more susceptible to heat stress, when compared to the middle or lower third, a phenomenon also noted by Jäger *et al.* (2008). As well as the different impact that heat stress has on different regions of the ear, Saini & Aspinall (1982) also present data suggesting that the position of a floret, within a spikelet, influences the response to heat stress, in relation to eventual grain set. In treatment 2, grain set in primary, most proximal, florets is reduced by 58%, when compared to the control, and 41% in secondary florets. However, the eventual grain set in tertiary, more distal, florets remained '*unaffected*' by the elevated temperatures of the same treatment (Saini & Aspinall, 1982).

When viewing these regional and intra-spikelet discrepancies, in the light of Lukac *et al.* (2012), who extensively documented intra-ear floral developmental synchronicity, and assuming the cultivars used by Saini & Aspinall and Lukac *et al.* were relatively similar in their patterns of floral development, it is likely that other factors, underlying yield loss, other than floral developmental stage, may have an influence.

When considering that a wheat ear is a complex inflorescence, made up of dozens of individual flowers, which are not developmentally synchronous, the reason for the consistent, comparative, vulnerability of the upper third, for example, is as yet unclear. This is because the levels of regional yield depletion (Figure 2.2) do not suggest that the inherent developmental asynchronicity between the florets of the ear, up to 3 days (Saini & Aspinall, 1982), has a major, if any, role in determining the extent of regional yield loss due to sequential periods of heat stress.

2.1.2 Heat stress during wheat's reproductive development

Grains are the end result of sexual reproduction in wheat, a process disrupted by heat stress (Saini & Aspinall, 1982).

Saini & Aspinall (1982) ascribed responsibility for the heat associated decrease in grain set to abnormalities in both the male and female reproductive anatomy. This is unsurprising, as not

only are both male and female reproductive organs damaged by high temperatures, but their thresholds of damage are considerably lower than that in non-reproductive tissues (Barnabás *et al*., 2008). Even mild abiotic stresses, whilst not affecting the survival of vegetative plant parts, can irreversibly affect grain yield (Larkindale, 2005; Dolferus *et al*., 2011). This is particularly the case if heat stress occurs in conjunction with water deficiency as, under the outlined experimental conditions, the critical temperature for wheat yield loss during reproductive development is 32.4^{o}C for irrigated soils, as opposed to 24.5^{o}C in non-irrigated soils (Semenov *et al*., 2014).

Heat stress, during reproductive development/function also detrimentally effects yield in other crops, with the best single variable for estimating yield loss being the extent to which the daily maximum temperature exceeds 32^{o}C during pollination (Dale, 1983). Additionally, the highest yields of soybean have been correlated with cool temperatures during reproductive development (Martineau *et al*., 1979).

2.1.2.1 The effect of heat stress on female reproductive tissues

Female reproductive tissue is impacted by heat stress. Saini & Aspinall (1982) showed that when the pollen from control plants (20^{o}C day/20^{o}C night), was used to pollinate stigmas of other control plants, the grain set was 73.3%. However, when the pollen from control plants was used to pollinate the stigmas of heat stressed plant (30^{o}C day/30^{o}C night) the grain set was 57.9%, a 21% decrease. This indicates that some of the responsibility for decreased grain set is due to heat-associated failures in female reproductive development.

2.1.2.2 The effect of heat stress on male reproductive tissues

Data from Saini & Aspinall (1982) confirms that there are heat-associated failures in male reproductive tissue. Control plants had a grain set of 89.3%, compared to heat stressed plants, which were also allowed to self-pollinate, which had a grain set of only 42.2%, a decrease of 53%. This is a greater percentage decrease that when just the female's reproductive tissue is found damaged, thus suggesting that male reproductive tissue is also sensitive.

2.1.2.3 Proportional responsibility between the effect of heat stress on female and male reproductive tissue

Having confirmed that heat-associated abnormalities in both the female and male's reproductive function contributed to the decreased eventual grain set, the data produced by Saini & Aspinall (1982) gives insights into the proportion of responsibility that should be allocated to each. It seems that decreased pollen viability, through a combination of heat

stress retarding anthers, to the point of sterility, and decreasing the pollen viability within those anthers that were apparently un-retarded, had a greater proportional responsibility for the eventual decrease in grain set than decreased ovule viability did. Dolferus *et al.* (2011) call pollen formation '*the Achilles tendon of reproductive development*', and Bita & Gerats (2013) documented a strong correlation of pollen production, pollen viability, and anther dehiscence, with grain set. In rice, cold stress, inflicted at meiosis, whilst affecting male fertility, left the female tissue '*virtually not injured*' (Hayase *et al*., 1969).

Saini and Aspinall (1981) suggest that, unlike heat stress, water deficiency, at least within certain temporal and temperature thresholds, does not affect female fertility. Instead, they attribute reductions in eventual grain set solely to the negative effect water stress has on male fertility.

Due to a comparison between heat and water stress resulting in morphologically similar anthers, containing sterile pollen grains, with depleted cytoplasmic contents, and both stresses having similar microspore developmental stages of particularly sensitivity, Saini *et al*. (1984) suggests that the two types of stress may cause male sterility through similar mechanisms.

2.1.3 Pollen and anther development

An understanding of the underlying processes, involved in pollen and anther development, has increased over the past decades. This is partially due to the disproportionate effect abiotic stress has on pollen development (e.g. Saini & Aspinall, 1982), but also the fundamental importance of pollen developmental pathways for breeding and crop yields. Perhaps foremost amongst such studies of pollen development is Sanders *et al.* (1999), who categorised the anther development of *Arabidopsis* into 14 separate stages. Even though many of the fine scale intricacies of pollen development differ between species, the general sequence of events, and component parts, appear conserved (Wilson & Zhang, 2009).

2.1.3.1 Pollen development

Pollen development comprises two principal stages. Firstly microsporogenesis, which is characterised by the progression of meiotic division of diploid pollen mother cells to produce tetrads of haploid microspores. The individual microspores are then released from tetrads, due to secretions of callase(s) (β-1,3 glucanases) from the tapetum, which break down the callose walls that are holding them together (Scott et al., 2004; Giorno *et al.*, 2013; Lu *et al*.,

2014). The release of the microspores from the tetrads indicates the end of microsporogenesis and the commencement of the next stage, microgametogenesis. In this latter stage of pollen development much more inter-specific variation occurs. Microgametogenesis is characterised by a number of events. These stages include the development of a large vacuole within the microspore, two rounds of mitosis, which produces both vegetative and germ cells, and the formation of pollen grain wall (Scott *et al*., 2004; Giorno *et al.*, 2013).

2.1.3.2 Anther development

Pollen is formed within specialised organs, called stamens (Figure 2.3), whose development involves a both complex, and co-ordinated, interactions between sporophytic and gametophytic tissue (Wilson & Zhang, 2009). Stamens, which are contained within the perianth until the latter stages of their development, are comprised of two principal component parts, the filament and the anther.

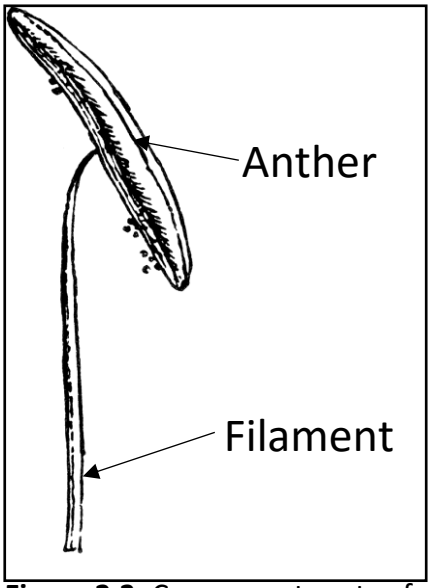


Figure 2.3: Component parts of a stamen.

The filament, which is often long and thin, and is a key feature, due to its increasing length, in allowing pollen to be eventually released into the external atmosphere, contains vascular tissue which supplies the needs of the anther (Wilson & Zhang, 2009; Bita & Gerats, 2013).

The anther originates from periclinal divisions of cells, which in turn form four clusters of archesporial cells (Figure 2.4a). These archesporial cells subsequently divide mitotically to form both sporogenous cells and primary parietal cells. The sporogenous cells form pollen mother cells, after a small number of divisions. The primary parietal cells undergo numerous

divisions, to eventually form the innermost three, of four maternal layers of the anther which surround the sporogonous cells (Figure 2.4b), with the epidermis being derived from a non-archesporial cell layer (Wilson & Zhang, 2009; Wilson *et al*., 2011).

The anther is relatively complex, in both its development and its structure. Table 2.1 outlines the names, and functions, of the principle component parts of an anther.

Table 2.1: anatomical features found within an anther, and a summary of their purpose.

Anatomical feature	Definition
Epidermis	The one cell think, outermost, layer of the anther.
Endothecium	The layer of cells lying directly beneath the epidermis. As the anther matures, fibrous bands often develop in the cell walls of the endothecium. These fibrous bands aid dehiscence.
Middle layer	A transient layer of cells found between the endothecium and the tapetum.
Tapetum	The transitory, specialised, layer of cells found directly adjacent to the microspores. It provides nutrition to developing microspores.
Stomium	An area of thin-walled cells in the anther that breaks, at dehiscence, to release pollen grains.
Pollen (microspore) mother cell	The diploid microsporocyte which undergoes meiotic division to produce four haploid microspores.

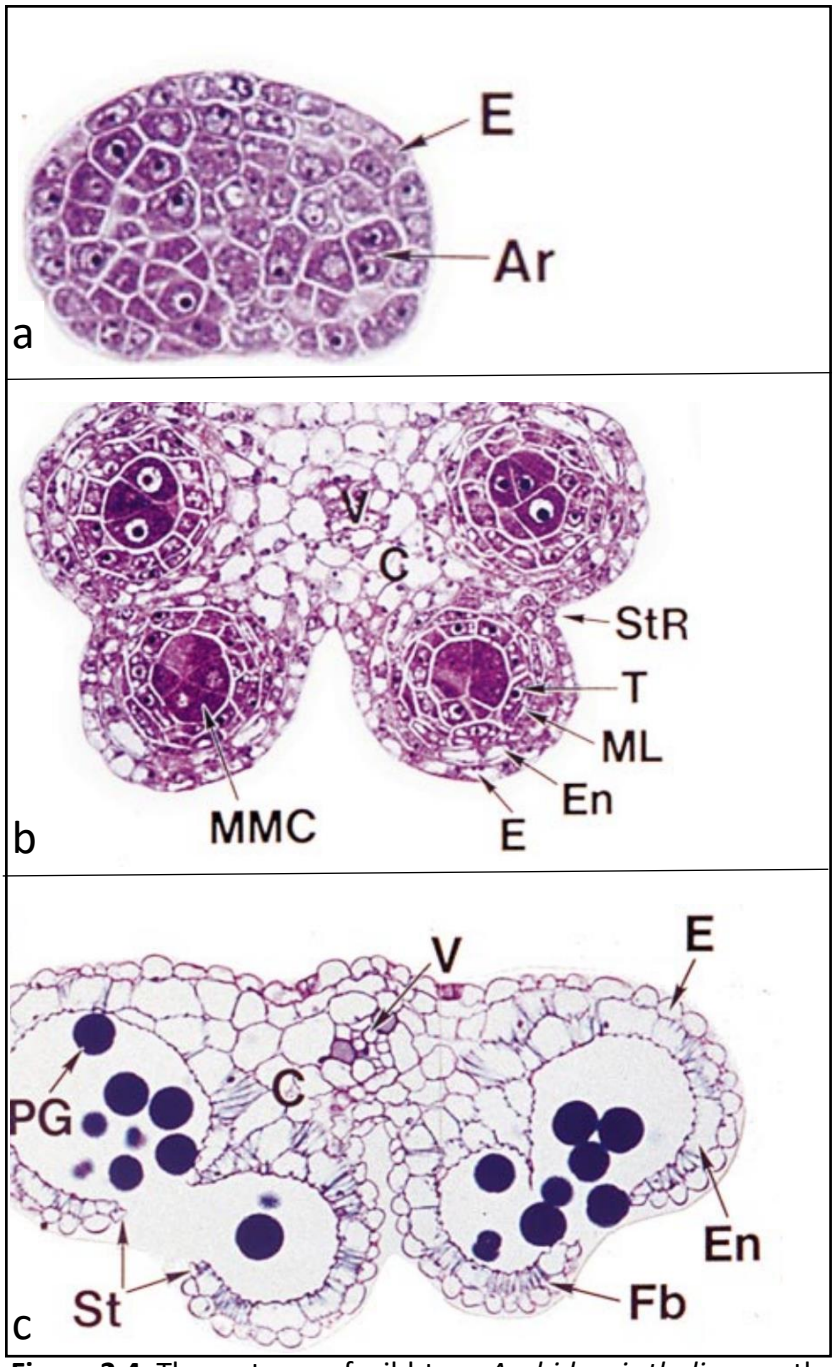


Figure 2.4: Three stages of wild-type *Arabidopsis thaliana* anther development. (a) Immature, (b) semi-mature, (c) mature. Ar= archesporial cell, C= connective, E= epidermis, En= endothecium, Fb= fibrous bands ML= middle layer, MMC= microspore mother cells, PG= pollen grains, St= stomium, StR= stomium region, T= tapetum, V= vascular region (Sanders *et al*., 1999).

Even though somewhat dependent upon the species and environment, the endothecium, middle layer and tapetum undergo large changes between their first appearance (Figure 2.4b), and final dehiscence of pollen (Figure 2.4c). The tapetum naturally degrades at approximately mitosis in both *Arabidopsis* (Sanders *et al*., 1999) and wheat (Saini *et al.*, 1984), whereas the

middle layer degrades earlier, at meiosis (Sanders *et al*., 1999). The endothecium starts to expand in size during the pollen mitosis and continues up until anther dehiscence. This coincides with the progressive deposition of fibrous bands in this layer (a.k.a. secondary thickening) (Sanders *et al*., 1999).

In the past it has been widely reported that abiotic stress can disrupt the timing, and even the extent of anther related development, with Sato *et al.* (2002) documenting, in tomatoes, not only shrunken pollen grains but also a failure of anthers to dehisce, partially due to the irregular arrangement of both the endothecium and epidermis. Porch & Jahn (2001) also document, in string beans, the failure of the interlocular septa to fully degrade and the lack of development of secondary thickening in the endothecium. In addition, Porch & Jahn (2001) also describe premature degradation of the tapetum, due to heat stress.

2.1.4 The effect of heat stress on wheat pollen development.

In wheat, Saini *et al.* (1984) did not observe any particular developmental period(s), between pollen mother cell (PMC) meiosis and microspore release, which had a hypersensitivity to heat stress. However, by using microscopic techniques, and successive harvesting of anthers over their development, they were able to observe two separate consequences of imposing heat stress for 3 days, starting from the onset of PMC meiosis. They classified these as 'type 1' and 'type 3'.

Type 1 was characterised by the pre-mature degeneration of the tapetum during meiosis, thus meaning that the microspores failed to orient along the periphery of the anther lumen and undergo mitosis; this led to all the locules of type 1 anthers being sterile. This sterility is unsurprising, in the light of the tapetum being a highly specialised sporophytic secretion cell layer dedicated to feeding developing microspores (Dolferus *et al.*, 2011). A similar phenomenon was observed, due to water deficiency during the same stage of pollen development in wheat (Lalonde *et al*., 1997). This further supports the conclusion of Saini *et al*. (1984) that the two types of stress possibly cause male sterility through similar mechanisms.

In addition to Saini *et al.* (1984) describing 'type 1' anthers having degraded outer anther layers, they also describe pollen grains being negatively affected. Despite having apparently normal levels of exine, pollen grains had little to no intine or cytoplasm at anthesis. Sakata *et al.* (2000) reported a similar phenomenon in barley pollen, when heat stressed at a similar, if

not slightly earlier, developmental stage. Exine formation is largely due to the role of the tapetum and the intine's formation is due to the role of the microspores/pollen grains (Shi *et al.*, 2015). This therefore would explain the past observed absence of one pollen coat component, and not the other, in both wheat and barley.

Type 3 was characterised by the initially normal development of the anther, and its contents, up until the end of the pollen grains first mitosis, at which point a proportion became disorientated from their peripheral location in the anther. In contrast to 'type 1', Saini *et al.* (1984) state that tapetal degeneration was '*normal*' and that, the microspores not disoriented, completed the second pollen grain mitosis, and subsequently went on to produce normal pollen grains. Therefore, type 3 anthers contained a mixture of fertile and sterile pollen grains.

Despite similarly sensitive pollen developmental stages within numerous species, including wheat and barley, there seems to be some variation in stages of developmental sensitivity amongst plant species (Sakata *et al.*, 2000). In Cowpea (Fabaceae), for example, anthers developing under both optimal and high night temperatures developed normally through meiosis. However, after tetrad release, the tapetal layer degenerated prematurely under high night temperatures (Ahmed *et al.*, 1992). This raises the question whether, within the plant family of Poaceae, there is uniformity in stages of pollen development sensitive to stress? If this is indeed the case, this characteristic would not be unique to Poaceae. Tomatoes, which are in the plant family of Solanaceae, are also sensitive to heat stress around PMC meiosis (Iwahori, 1966; Peet *et al*., 1998).

With grain number being the primary yield component associated with yield increase/decrease in wheat (Dolferus *et al*., 2011), and wheat pollen development being shown to be detrimentally affected by pre-anthesis abiotic stress, there is a strong case for the continued study. This is ever more validated by models which predicts that an increased frequency of heat stress, during wheat pre-anthesis in Europe, is likely over the coming years (Semenov *et al.*, 2014).

2.2 Objectives

1) To assess whether there is a specific temporal period, during booting, that is particularly sensitive to heat stress, in relation to both anther/pollen development, and eventual grain set.
2) To assess the relationship between anther/pollen damage, and grain number reduction, due to heat stress.
3) To assess the effect heat stress has on the grain set levels of different spatial regions of wheat ears.
4) To assess the possibility of assessing pollen developmental stage, by means of assessing the temporal/gross developmental progression of wheat.

2.3 Methods

2.3.1 Pot experiment

2.3.1.1 Plant growth and stress implementation

Pots (12.5cm) were filled with a 4:4:2:1 mixture of steam sterilised 6mm gravel, steam sterilised 3mm sharp sand, medium grade vermiculite and peat based potting compost, respectively. 1kg of Osmocote Pro 3–4 months (Scotts, UK) per 0.55m^3 of planting mixture was added. This mix is known as 'PEL mix'.

320 pots were sown, each with 3 untreated seeds of the spring wheat variety Paragon (Plant Breeding International Cambridge Ltd). All pots were initially placed in an unheated polytunnel at the Plant Environment Laboratory, University of Reading, UK (51.413349, -0.93749225).

When these plants reached approximately 23ZS, 200 pots were moved to the controlled environmental facilities of the Harborne Building, University of Reading, UK (51.437551, -0.94186842) where they were randomly placed into two walk-in growth chambers. Within this facility they continued their growth at 20±1°C (day & night), 16 h photoperiod, and an average irradiance, to booting canopy level, of 330 µmol/m^2/sec, fluorescent and incandescent illumination. At approximately 31ZS the plants were thinned down to one plant per pot, and three tillers per plant. Tiller thinning was repeated approximately every 10 days throughout the rest of the growing season.

Plants, based on developmental stage (Table 2.2), were then transferred into a 'heat stress' growth cabinet at 35±1°C (day & night), with otherwise identical climatic conditions, before being returned to their original location within the 20°C environment. A control growth temperature of 20°C was chosen, based upon Bennett *et al.* (1973).

During the high temperature treatments (35/35°C) and control temperature (20/20°C), the atmospheric relative humidity was kept above 60% (v/v). In addition, through regular physical examination, the water content of the medium was kept relatively stable, at approximately field capacity.

Table 2.2: Details on heat stress treatments during intra-varietal experiment.

Number of days after 39ZS plants were exposed to an episode of heat stress	Duration of heat stress episode (days/hours)	Fate of plant
0	3/72	5 plants went for sectioning 24 plants went to grain set
3	3/72	5 plants went for sectioning 24 plants went to grain set
6	3/72	5 plants went for sectioning 24 plants went to grain set
9	3/72	5 plants went for sectioning 24 plants went to grain set
12	3/72	5 plants went for sectioning 24 plants went to grain set

2.3.1.2 Microscopy analysis

At regular intervals (Table 2.3), anthers, from both stressed and control, main tiller, ears, were collected and prepared for microscopy.

Table 2.3: Details on stages of anther collection. 'x' equals five plants.

	Days post 39ZS (of the main tiller)					
	0	**3**	**6**	**9**	**12**	**15**
Control (20/20°C)	x	x	x	x	x	
Stressed (35/35°C)		x	x	x	x	x

After removing, where necessary, the ear from surrounding leaf sheaths spikelets/anthers from the lower, middle and uppermost third of the ears (Appendix 1), were removed and placed directly into Karnovsky's fixative (2% paraformaldehyde (v/v), 2.5% glutaraldehyde (v/v), in 0.05m phosphate buffer), for approximately 24 hours, before being stored in 0.1m phosphate buffer at 4^{o}C. Where only anthers were collected, due to increased spikelet maturity, these were from floret 'a', as defined by Lukac *et al.* (2012).

Samples were removed from the buffer and dehydrated through an ethanol series ((10%, 30%, 50%, 70%, 90%, 100% & 100% (v/v)) 1 hour per solution). The samples were then gradually infiltrated with medium grade LR White resin (London Resin Company) through a sequential

gradation of ethanol to resin ((3:1, 1:1, 1:3 (v/v)) 1.5 hours per solution). Samples were then placed in 100% resin and left for 2 hours, before the resin was replaced and left for another 2 hours. Samples were placed in gelatine capsules (size 00) (Agar Scientific) and polymerised at 58^{o}C for 24 hours.

From the addition of fixative to the infiltration with resin, during working hours, samples were kept moving in solution using a rotating platform. During non-working hours the samples were stored at 4^{o}C.

Approximately 1.2µm thick transverse sections, of the anthers of florets at the bases of spikelets, florets 'a' or 'b', as defined by Lukac *et al*. (2012), were cut, using a glass knife, and a Leica EM UC6 microtome, and then stained with 0.5% (w/v) Toluidine Blue O. Images were taken with a Leica DM5000 B light microscope.

2.3.1.3 Post-treatment plant growth

Plants intended for grain set, both stressed and unstressed, had 55mm x 190mm cellophane crossing bags (Focus Packaging & Design LTD) sealed over their ears, after their emergence from the flag leaf sheath, and before anthesis, in order to prevent inter-ear cross pollination. After pollination was complete the crossing bags were removed and the plants were randomly arranged within an outside area at the Harborne Building and surrounded by bird proof netting.

2.3.1.4 Ear collection and analysis

After grain development was complete the ears were collected, individually stored in labelled paper bags and dried at room temperature. Grain presence/absence was recorded for each of the main ears' florets. Ear third allocations were in line with parameters set forth in 'Appendix 2' and floret labelling followed the scheme of Lukac *et al.* (2012), with the first floret from the lower glume labelled as 'a', and subsequent florets labelled sequentially.

Whole ear, along with regional grain set levels, were analysed via ANOVA. Regional grain set percentage analysis was performed after transforming the initial percentages to empirical logit. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, *p*-values were drawn from permutation tests (4999 random permutations). Maximum least significant differences (LSDs) were used in order to most conservatively determine significance. Discrete data (e.g. spikelet numbers) was analysed via a Generalised Linear Mixed Model, using a Poisson distribution.

All analysis was done using GenStat 16. Differences with p=<0.05 were deemed 'significant'. Models can be seen within 'Appendix 3'.

In order for the accurate analysis of regional grain set levels, those spikelets not possessing at least five florets, most often those at the base and top of an ear, were standardised. This standardisation took the form of 'topping-up' those spikelets with less than 5 florets, with supplementary, non-seed bearing florets. This prevented a loss in floret presence, due to heat stress, distorting the percentages, and instead gave better appreciation for regional sensitivity.

2.3.2 Field experiment

Within a crop of field grown Paragon, when at approximately 33ZS, approximately 250 main tillers of wheat plants, growing in the centre of 7 rows, were labelled sequentially with six eventual collection times. This crop was grown at Sonning Farm, University of Reading, UK (51.472935, -0.90414518).

At six developmental points, ears were collected, dried (80^{o}C for 48 hours), and weighed. These developmental points were:

- 1st day 39ZS was present (0 days after the start of booting)
- 1 day after the start of booting
- 2 days after the start of booting
- 3 days after the start of booting
- 1st day 47ZS was present (0 days after the end of booting)
- 1st day anthers extruded from the ear (for the purposes of this investigation this was considered 'anthesis')

Average spikelet dry weight was calculated via the below formula. Viable spikelets were those that visibly looked like they had the potential to possess grains. This often excluded the bottom few spikelets of each ear.

- Ear dry weight
  (Total number of spikelets x % viable spikelets)

Resulting data was analysed via ANOVA. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, p-values were drawn from permutation tests (4999 random permutations), and maximum least significant differences (LSDs) were used in order to most conservatively

determine significance. All analysis was done using GenStat 16. Differences with p=<0.05 were deemed ‘significant’. Models can be seen within ‘Appendix 4’.

2.4 Results

2.4.1 Pot experiment

2.4.1.1 Grain number

There was no significant difference (p=0.963) between the spikelet numbers possessed by the wheat ears of each of the six temperature treatments.

In this experiment, devised to find the temporal region of pollen development most susceptible to heat stress, it is important to note that the timing of the increased temperature treatment had a highly significant (p=<0.001) effect on grain set, with each of the five stress treatments applied resulting in a significant reduction in grain set, when compared to the control (Figure 2.5a). The lowest number of grains were recorded when the stress took place between 6 and 12 days after the start of booting. The stress treatments at 0 to 3 days, and from 9 to 12 days, after the start of booting, had a significantly greater grain set than those stresses in-between (Figure 2.5a).

With the retrospective ability to assess yield sensitivity, in relation to a stresses distance from the end of booting (47ZS), it is apparent that the timing had a highly significant (p=<0.001) effect. Stresses one to two days before the end of booting resulted in significantly lower grain sets.

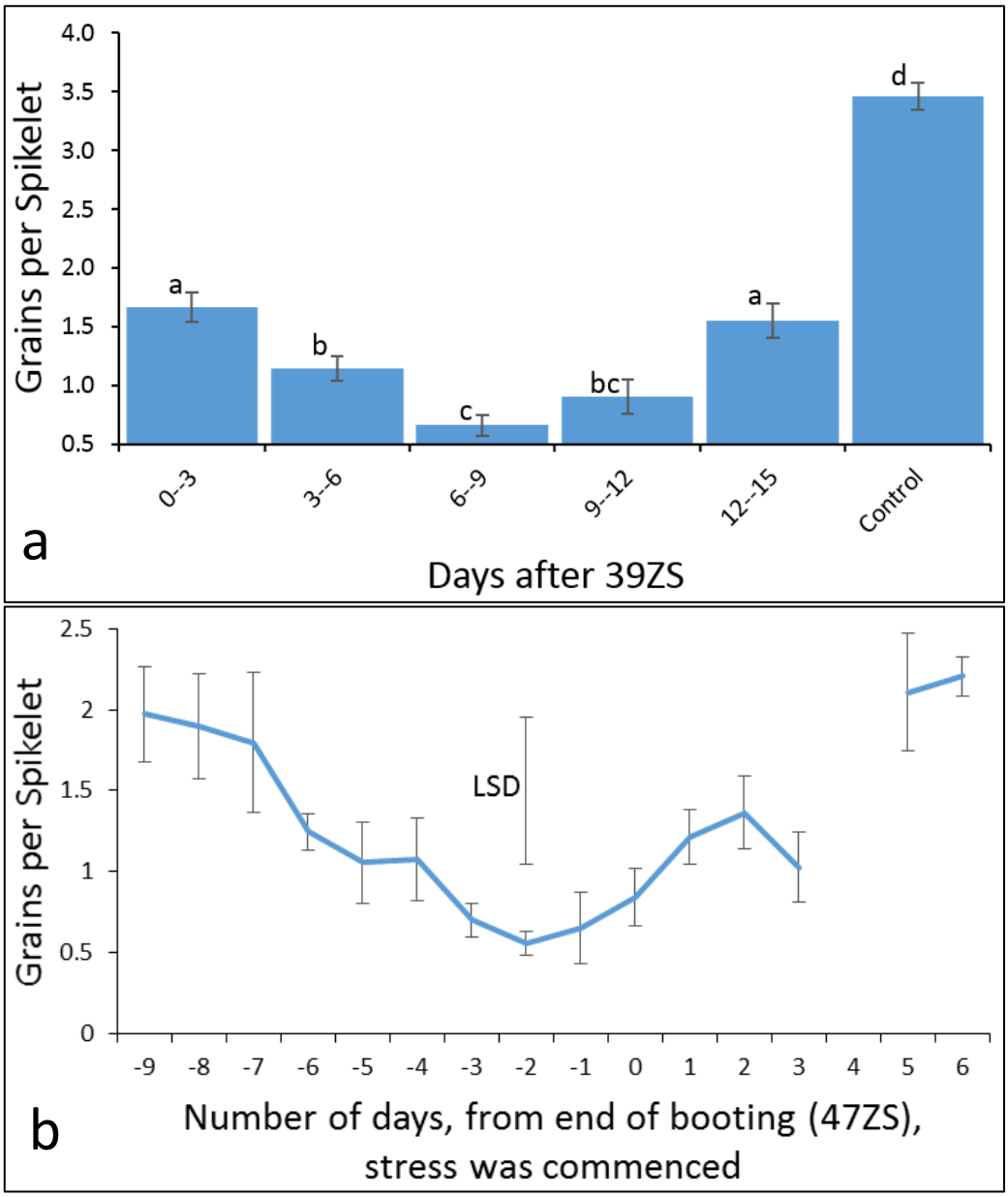


Figure 2.5: The effect that heat stress (35/35°C; 3 days), given at five different developmental stages, had on main tiller grain number per spikelet, based on the distance from the (a) start and (b) end of booting. Identical letters indicate non-significant difference (*p*=>0.05). Error bars indicate standard error. *n*= 21-24 & 3-13, respectively. *df*= 135 & 108, respectively.

As outlined further below, the differences in grain number per spikelet (Figure 2.5) are in keeping with the apparent effects that heat stress has on anther/pollen development. Those stressed anthers, whose phenotypes are most deviant from their respective control (Figure 2.6), resulted in the lowest grain set.

- Figure 2.6 (a-d) shows both control and stressed anthers from two time points in wheat's pollen development during booting. Figure 2.6a,b show anthers at approximately meiosis. The stressed anther (b) had relatively little tapetum and

distorted meiotic cells, when compared to its control (a). The stressed anther also had reduced amounts of endothecium and middle layer, when compared to the control.

- Figure 2.6c,d shows anthers at a later stages of pollen development. The most observable difference between the control (c) and stressed (d) anther, apart from the stressed anther having already begun dehiscence, is that the pollen grains/microspores have collapsed in upon themselves and detached from their peripheral location within the stressed anther's lumen. In addition, even though very little is left within the control, there is no tapetum present with the stressed anther.
- Figure 2.6e,f shows an anther and pollen grain 15 days (post 39ZS), and after three days of heat stress. Even though the pollen grains of Figure 2.6e,f do not look as functionally competent as those of Figure 2.6c, in that they are apparently missing vacuoles and equivalent levels of starch, they do not look nearly as damaged as those grains of Figure 2.6d. This may explain the paralleled increase in grain set when the stress was imposed between 12-15 days (post 39ZS). Figure 2.6e shows that heat stress imposed over this time did not affect the anthers' ability to dehisce.

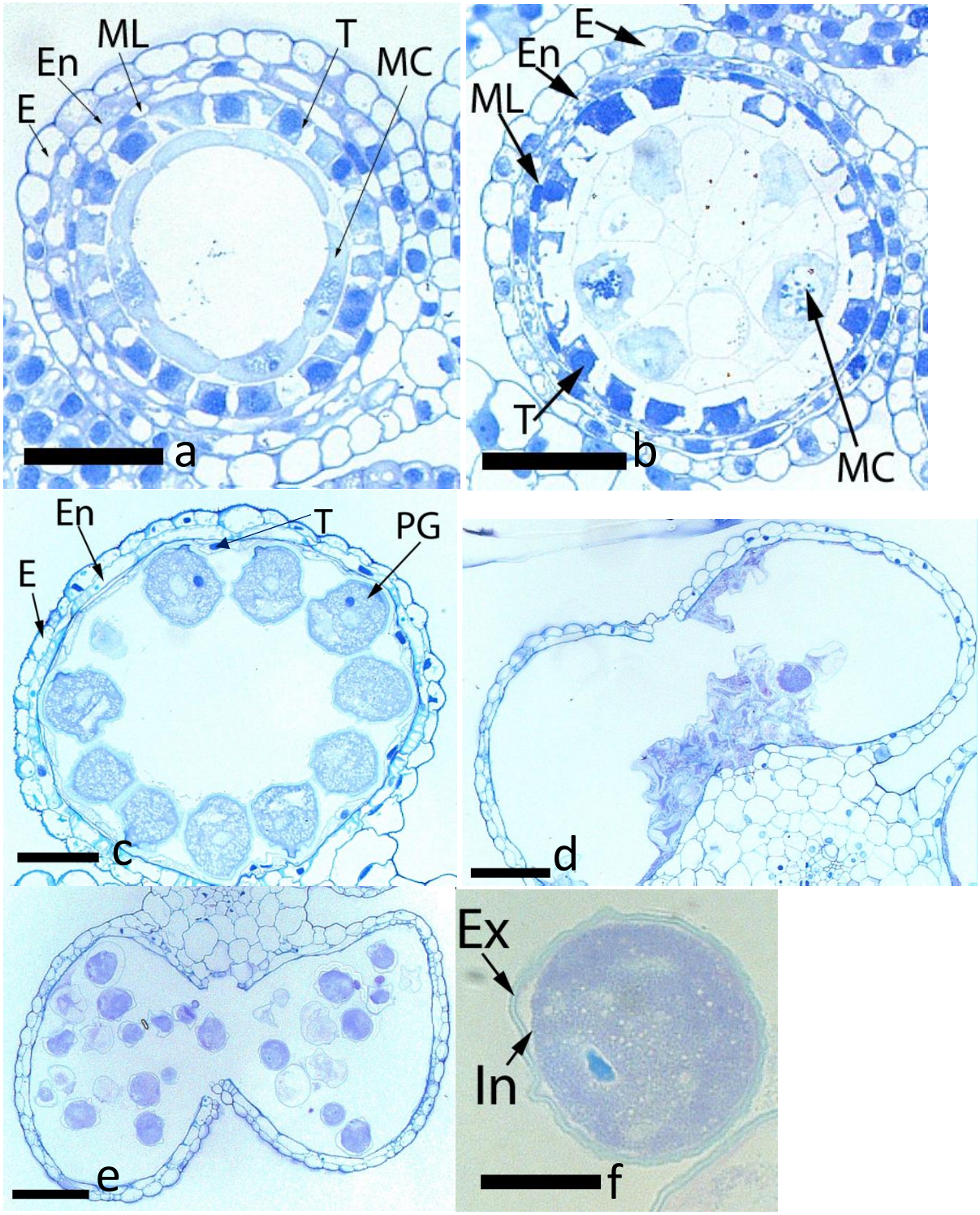


Figure 2.6: Transversely sectioned anthers of the main tiller (middle spikelets) from control (20/20°C) and heat stressed (35/35°C) plants. (a) 3 days post 39ZS, control, (b) 3 day post 39ZS, after 3 days of stress, (c) 12 days post 39ZS, control, (d) 12 days post 39ZS, after 3 days of stress, (e,f) 15 days post 39ZS, after 3 days of stress. E= epidermis, En= endothecium, Ex= exine, In= intine, MC= meiotic cell, ML= middle layer, PG= pollen grain, T= tapetum. Scale bars 50, 50, 50, 25, 25, 20µm (respectively).

2.4.2.2 Regional losses due to heat stress

Despite, at times, in light of LSDs, there being non-significant differences in levels of grain set within a position, over each of the floret and third positions there were significant (p=<0.001 (floret 'c' p=0.003)) effects of the different heat stress treatments (Figures 2.7f & 2.8f).

As well as the inclination of lines, within both Figure 2.7a-e & Figure 2.8a-e, being different from one another, when viewed in sequence, a progressive change in these inclinations can be seen. With the earlier stresses (0-3 & 3-6 days after 39ZS) causing the greatest regional losses in the distal thirds and proximal florets of ears, progressive changes meant that, when stressed at the later stages (12-15 days after 39ZS) the proximal thirds and distal florets of ears bore the greatest losses.

When Paragon was heat stressed upon the first three days of booting, the percentage decline in the grains found in florets 'a', 'b', 'c' & 'd', which are the more proximal florets within a spikelet, was slightly offset by an increase in the number of grains found in position 'e' (Figure 2.7a), a more distal floret within the spikelet. This phenomenon was not seen throughout the other four temporal intervals at which stress were inflicted.

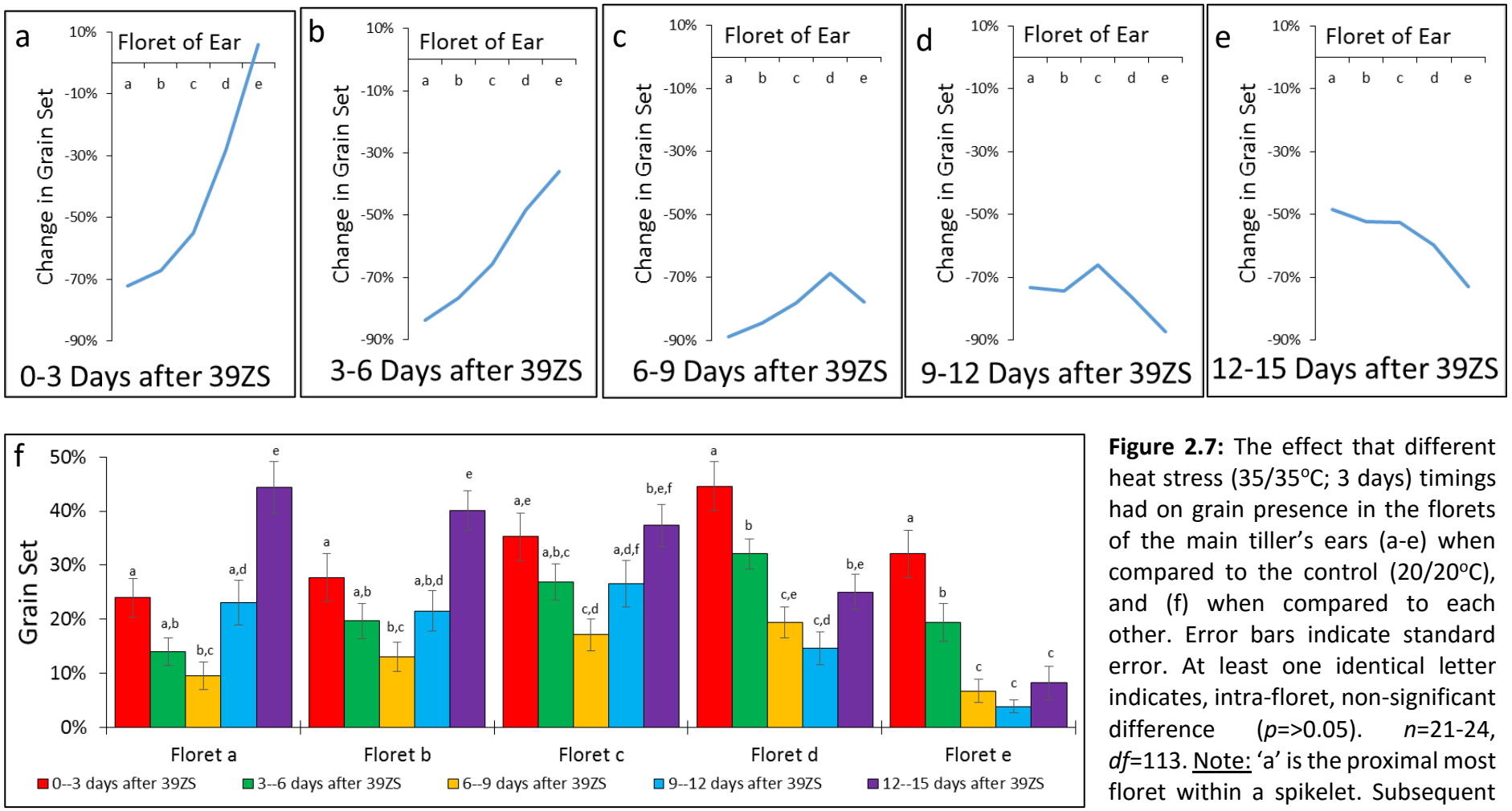


Figure 2.7: The effect that different heat stress (35/35°C; 3 days) timings had on grain presence in the florets of the main tiller's ears (a-e) when compared to the control (20/20°C), and (f) when compared to each other. Error bars indicate standard error. At least one identical letter indicates, intra-floret, non-significant difference (p=>0.05). n=21-24, df=113. Note: 'a' is the proximal most floret within a spikelet. Subsequent florets are labelled sequentially.

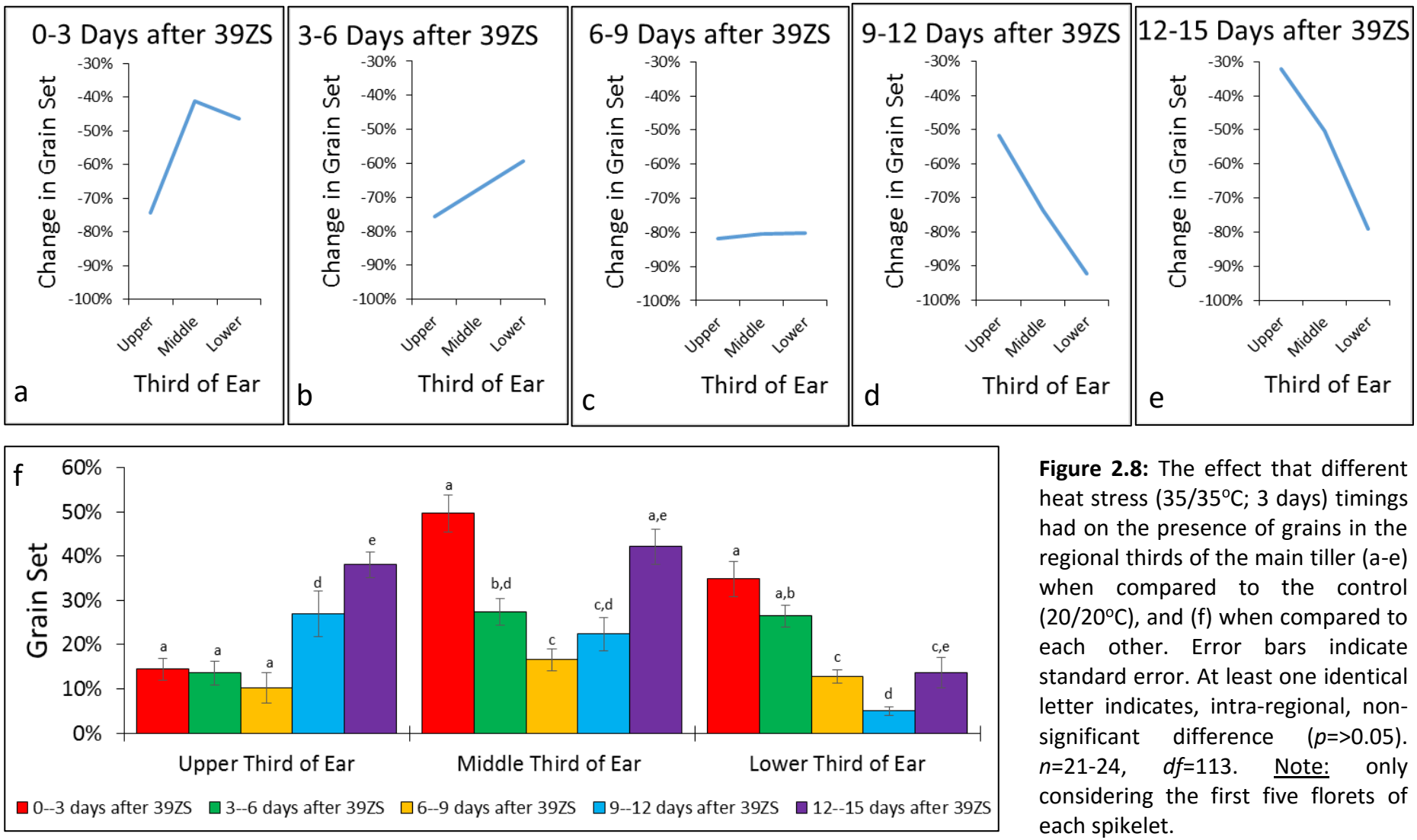


Figure 2.8: The effect that different heat stress (35/35°C; 3 days) timings had on the presence of grains in the regional thirds of the main tiller (a-e) when compared to the control (20/20°C), and (f) when compared to each other. Error bars indicate standard error. At least one identical letter indicates, intra-regional, non-significant difference (p=>0.05). n=21-24, df=113. Note: only considering the first five florets of each spikelet.

Figure 2.9 shows the pollen developmental asynchronicity (difference in stage) within an individual wheat ear which has been stressed. Not only are those anthers at the proximal (a) and distal (c) regions of the ear at approximately the same stage of development, but they are also less damaged by the heat stress episode, when compared to the anthers in the middle of the ear, whose pollen grains/microspores have collapsed in upon themselves. From the examination of a range of material from across a time course, it can be determined that the anthers in the middle of the ear are more mature than those in the centre of the ear by approximately three days.

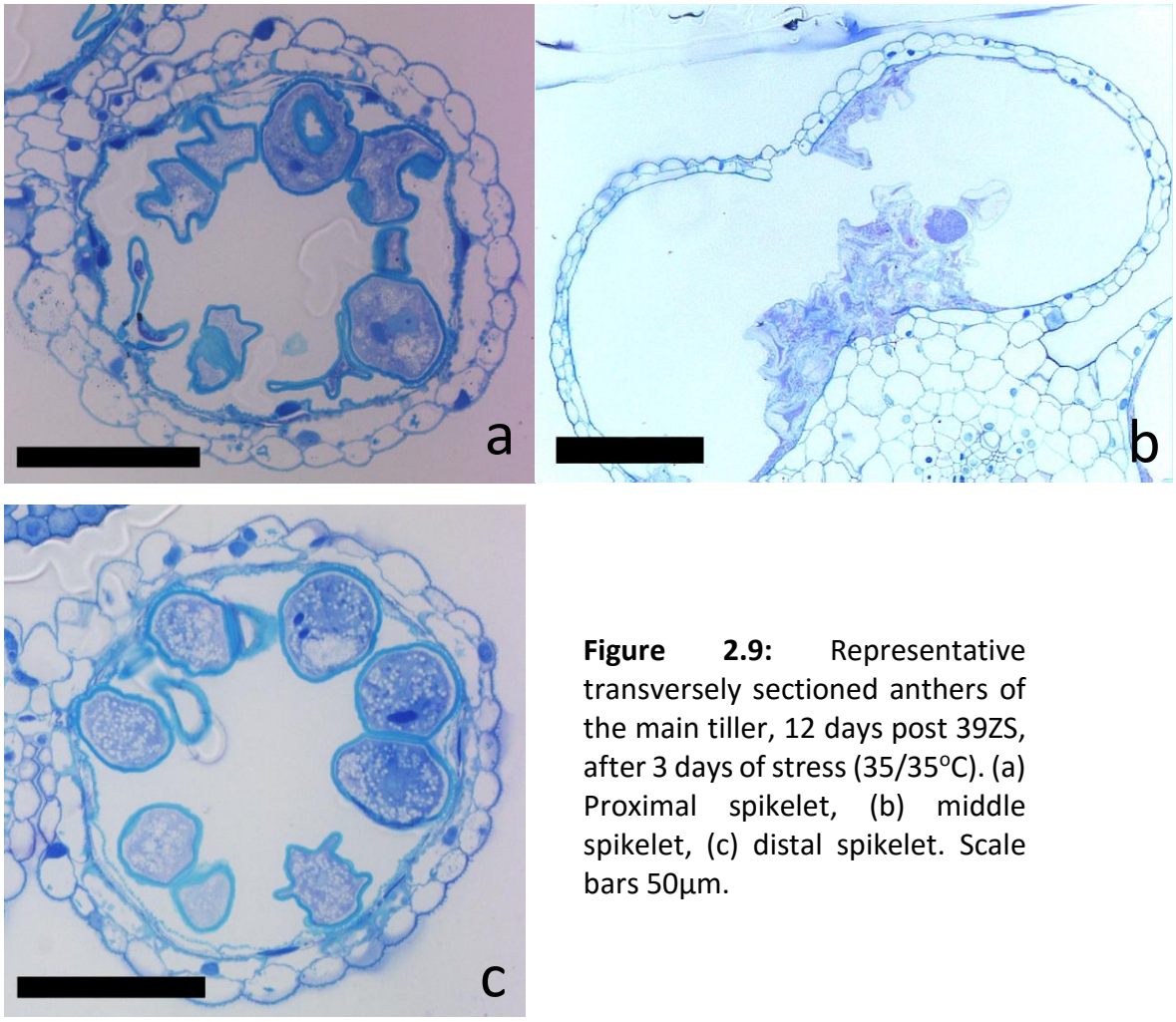


Figure 2.9: Representative transversely sectioned anthers of the main tiller, 12 days post 39ZS, after 3 days of stress (35/35°C). (a) Proximal spikelet, (b) middle spikelet, (c) distal spikelet. Scale bars 50µm.

2.4.2.3 Post floret 'e'

Temperature stress imposed at different times throughout booting, had a significant (p=0.015) effect on the number of florets above the relatively distal position of the spikelet that is floret 'e'. When inflicted at the stages both, approximately at the end of (6-9 days), and directly following booting (9-12, 12-15 days), the eventual number of florets above floret 'e' was significantly less that the control. However, when inflicted at earlier stages (0-3, 3-6 days)

there was no significant difference between the numbers of these florets, when compared to the control (Figure 2.10a). In addition, the earlier stresses possessed more grains in a post 'e' position after the earlier stresses, when compared to the later stresses. However, neither stress grouping had more grains than the control in these positions (Figure 2.10b).

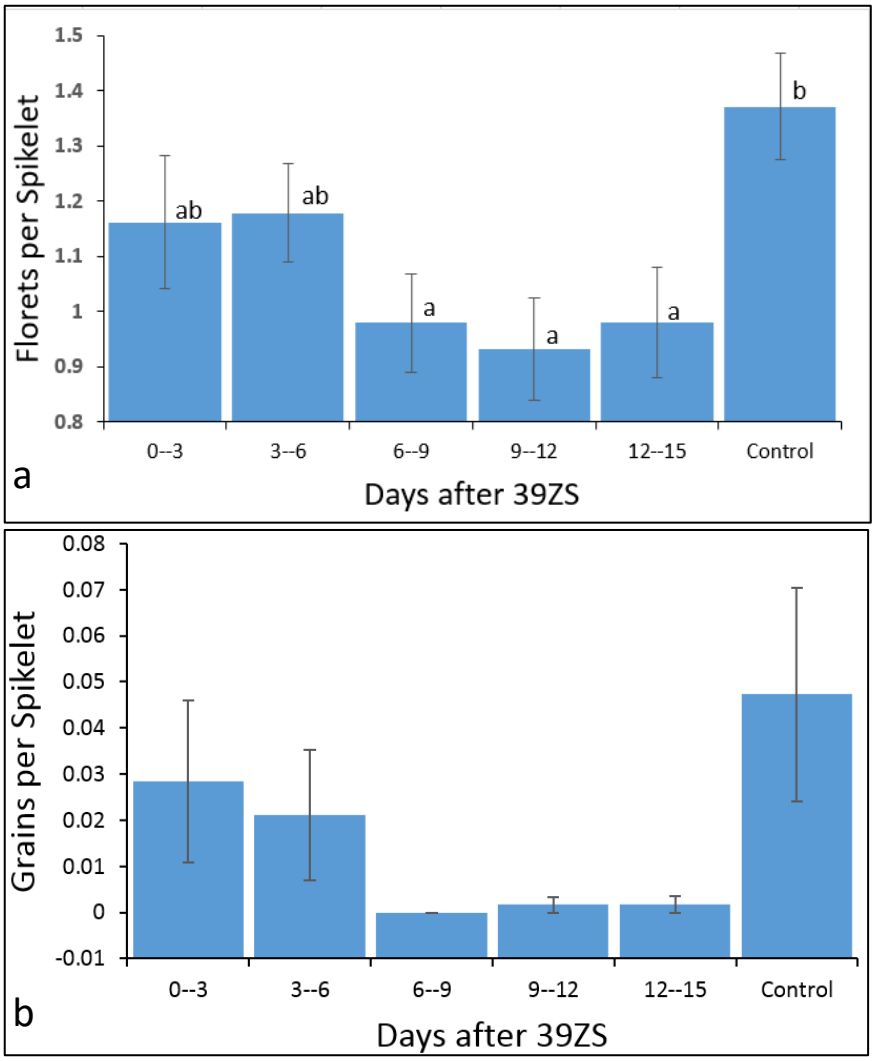


Figure 2.10: The effect that heat stress (35/35°C; 3 days), given at five different developmental stages, had on main tiller (a) floret, and (b) grain number per spikelet, above floret 'e'. Identical letters indicate non-significant difference (p=>0.05). Error bars indicate standard error. n= 21-24, df= 135.

2.4.2 Field experiment

This experiment was devised to assess the possibility of finding a correlation between pollen development and wheat temporal/gross developmental progression. The dry weight of ears (p=<0.001) and average spikelets (p=<0.001) differ significantly between approximately mid-booting (3 days after 39ZS), the end of booting, and anthesis (Figure 2.11b,d). The gap between each of these stages was approximately 4 days. Additionally, on a finer scale (daily measurements), there were significant differences between the weights of ears (p=<0.001) and average spikelets (p=<0.001) at the early stages of booting (Figure 2.11a,c).

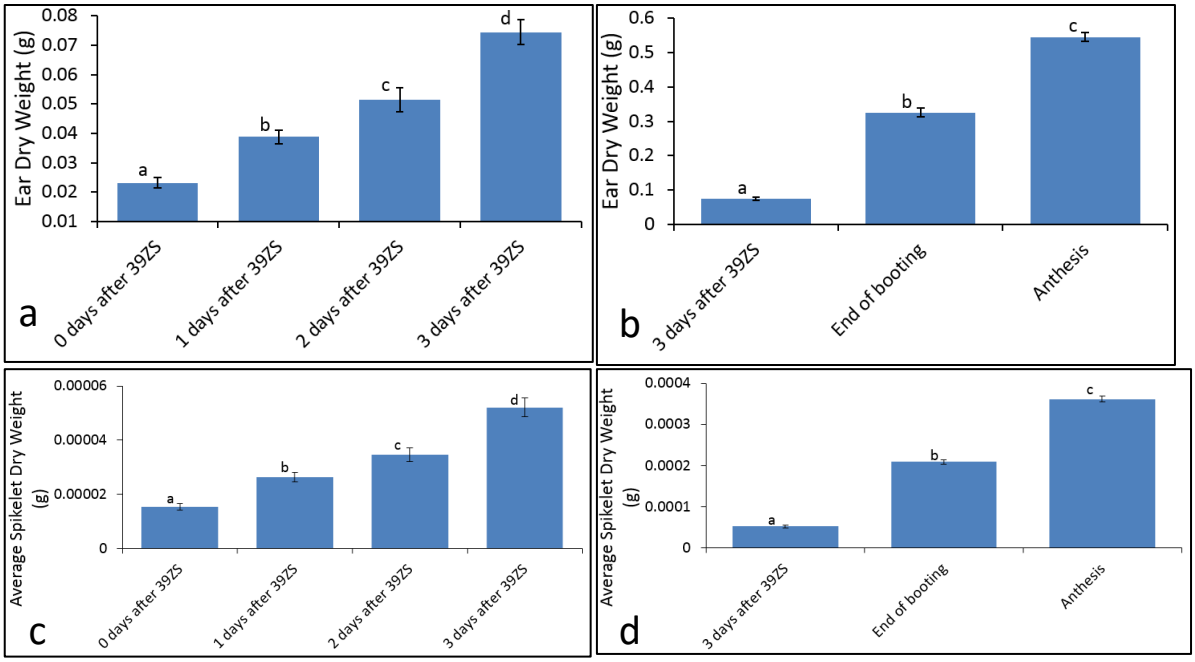


Figure 2.11: Ear (a,b) and average spikelet (c,d) dry weights, at different time points in gross plant development. Identical letters indicate non-significant difference (p=>0.05). Error bars indicate standard error. n= 19-28, df= 99 (a,c), n= 27-35, df= 94 (b,d).

2.5 Discussion

2.5.1 Stage of pollen development most sensitive to heat stress

There was no significant difference between the spikelet numbers of any of the six treatments imposed upon the wheat ears. This indicates that heat stress, at any stage after 39ZS, does not have a negative effect on spikelet number, which may already have already been determined before the start of booting, a conclusion supported by Rawson & Bagga (1979). Additionally, this would also suggest that any change in grain number is not due to a change in spikelet number.

According to this primary research, and contrary to what has been previously reported (e.g. Saini & Aspinall, 1982; Jäger *et al.*, 2008), the early stages of booting, around meiosis, even though highly sensitive to heat stress, in relation to both resulting yield loss, and initial anther/ reproductive cell disturbance, are not the most sensitive stages. Instead, when inflicted with heat stress from 6-12 days (post 39ZS), around the time of booting end, with booting lasting typically between 7-9 days, both the grain number and pollen development are more significantly affected. However, one must not disregard the detrimental influence that the damage to the endothecium, during the early stages of pollen development, will have on eventual dehiscence (Wilson *et al*., 2011), and therefore eventual yield.

With the damage to the anthers, during 9-12 days (post 39ZS), being primarily seen in the form of the premature pollen grain/microspore detachment from their normal peripheral location in the anther lumen, this is, in light of current literature, a clarification/reiteration of the importance of the tapetum. As well as being reduced when the heat stress is imposed nearer the start of booting (0-3 days post 39ZS), the tapetum is relatively reduced in the stressed anthers, in the later stages of pollen development.

The tapetum is largely responsible for exine formation (Yang *et al.*, 2007_a). This may explain the collapse of these microspores, as they lack the essential rigidity provided by the exine. This essential role is in spite, and in fact because of, the tapetum, during this time, being well into the processes of naturally degrading, through programmed cell death (PCD) (Vizcay-Barrena & Wilson, 2006). When considering the timing of tapetal PCD, and the associated release of the '*full complement of compounds*' for wall formation (Wilson & Zhang, 2009), Dolferus *et al.* (2011) characterise it as '*critical*' for determining eventual pollen viability. However, perhaps the collapse of these microspores, due to heat stress, has little to do with

the premature degradation of the remnants of the tapetum, per se, but the negative effects such heat stress has on the assimilation of '*compounds*' released by the tapetum, either before or during the heat stress event.

The highlighting of a differing stage of pollen development being more sensitive to heat stress, as has been previously reported (e.g. Saini *et al.*, 1984), is perhaps this experimentation's most notable departure from/addition to the current body of knowledge. Despite Saini *et al.* (1984) observing some similar effects of heat stress at around meiosis (e.g. middle layer and endothecium reduction and premature tapetal degradation), they did not document the same negative effects heat stress had on the later stages of pollen development (e.g. microspore/ pollen grain collapse and detachment from the inside wall of the lumen), and the associated yield reductions.

Apart from a genuinely greater sensitivity to heat stress, there are a number of possible reasons for the finding of a later pollen developmental stage with greater susceptibility to heat stress. Perhaps foremost amongst these is:

- Differing genotypes - where Saini & Aspinall used a variety (Gabo) bred for growth in Australia, Paragon was bred for a temperate British climate. Therefore, due to presumably different selection criteria by their respective breeders, the physiological responses such heat stress may have invoked, may have been markedly different between the two varieties.
- Differing stress temperatures - Saini & Aspinall stressed at 30^{o}C (day & night). However, the stress temperature used in this experiment was 35^{o}C (day & night).
- Shading, and the interaction between heat stress and shading – with plants grown for this experiment grown at light levels below those recommended, and the knowledge that shading, during pre-anthesis, even in the absence of other stresses, reduces grain number, most likely due to reduced assimilate supply (Fischer & Stockman, 1980; Demotes-Mainard *et al*., 1995), the possible interaction between the heat stress and the potential stress inflicted by reduced light intensity, may be a factor to consider.

The finding that those anthers stressed between 9-12 days (post 39ZS) were in the early stages of dehiscence, whereas their un-stressed counterparts, even though possessing secondary thickening in the endothecium, had yet to dehisce, further clarifies that heat stress not only effects microspore/pollen grain integrity, but also the other component parts of the anther.

This finding supports the conclusions of Zinn *et al.* (2010), in that the heat stress seemed to accelerate anthesis. However, unlike rice (Matsui & Omasa, 2002) and tomato (Sato *et al.,* 2002), the stressed anthers were not characterised by the tight closure of locules, which were responsible for reduced pollen dispersal in both of these species. The ability to maintain the ability to dehisce was a key feature in heat tolerant cultivars in rice (Prasad *et al*., 2006).

Conceivably, the significant reductions in grain number, seen as a result of being stressed from day 12-15 (post-39ZS), was due to the heat stress having a negative effect on both pollen germination and pollen tube growth (see Wahid *et al*., 2007; Zinn *et al*., 2010). The sectioned anthers, collected after this time of heat stress, not only showed apparently healthy pollen grains, in addition to apparently damaged grains, but also anthers well into the process of dehiscence, including completely devoid of pollen. This was very much in keeping with stages '*14 a-b*', as outlined by Sanders *et al.* (1999).

2.5.2 Regions of the primary ear most susceptible to heat stress

The results of this primary research are largely in keeping with past results, in relation to regional sensitivity, with the proximal third of the ear, and distal florets of the spikelet, being the least sensitive to yield depletion, due to early booting (0-3 days) heat stress (e.g. Saini & Aspinall, 1982)). However, the results of this primary research also present the progressive changes, with time, in the grain set dynamics, both at the regional (third), and spikelet level, a finding not presented in previous work. Where at the start of booting it was the proximal third and distal florets of the spikelet that were the least sensitive to heat stress, when stressed later on in development, the opposite is apparent, with the distal third and proximal florets of the spikelet being least sensitive.

When comparing the stressed anthers from different regions of the ear, a pollen related explanation for this, at times significant, regional discrepancy, may be unable to be provided. This is due to anthers from different parts of the ear, when stressed, appearing not to display damage, in line with regional yield losses. However, Saini *et al.* (1984) did show, through the periodic harvesting of anthers after a stress event, the presence of some lag effect, in that the damage becomes microscopically apparent only a number of days after the stress. If this were the case, one might expect those anthers, in the stage presented, at the base of the ear, to take on characteristics of damage, perhaps similar to those in the centre of the ear, in order to come in line with regional grain set losses for the stage at which they were collected. The

possibility of this already becoming apparent may be visible, due to some microspore collapse within the anthers of the proximal spikelet.

If, with further examination, it was confirmed that the effect of stress on anthers, from different regions of a stressed ear, were not in keeping with the grain loss levels, this may indicate that there may be one or more other underlying influences on grain set dynamics, resulting from heat stress. Are some areas of an ear, at certain times, intrinsically more capable of withstanding heat stress than others? Or, due, at times, to a relatively long period of time between the stresses and anthesis/grain set, and in light of the importance of resources such as starch in pollen development and germination (Clément *et al*., 1994; Lalonde *et al*., 1997), are resources able to be relocated from damaged areas, in order to support other areas within the ear maintain their grain set? Support for the latter of these possibilities can potentially be seen, in that, the prevalence of eventual florets, and grains, above floret 'e' of spikelets, are higher within those ears resulting from the earliest heat stresses, when compared to the later stresses; something also reported by Bingham (1966).

Perhaps this greater time between stress and anthesis allows for resources to be moved from proximal spikelets florets to more distal ones, leading to not only an increase in post 'e' floret and grain numbers, when compared to later stresses, but also an increasing in the number of grains found in position 'e', when compared to the control. However, it is worth noting that, in addition to the limited size of the grains found in post 'e' positions, due to the limited size of the florets in which they were found (Millet, 1986), these grains were often also small, due to a perceived lack of maturity. This was perhaps due to their filling being aborted, at a relatively early stage, or the grains being shrunken (see Tashiro & Wardlaw, 1990).

These post 'e' grains were, on a few occasions, so small that there was a question whether they were in fact the result of fertilization, or merely the remnant of an unfertilised gynoecium, or the result of parthenocarpy (see Tashiro & Wardlaw, 1990). Due to the limited occurrence of such small 'grains', it is unlikely that a misidentification distorted results. However, this phenomenon, along with the aborted and shrunken grains, may warrant assessment in further similar studies, not least due to them also occurring in more proximal florets in spikelets (position 'e' and below) as well. Does this suggest that grain quality can be affected, even when the heat stress in implemented before fertilization takes place?

It has been reported that floral asynchronicity provides limited protection to short stress events (Dolferus *et al.*, 2011). Even though neither explicitly agreeing, nor disagreeing with this statement, the results of this experimentation, in relation to changes in comparative regional sensitivity, may prove the catalyst for further research in order to understand what role, if any, asynchronicity in floral (including pollen) development has in relation to the avoidance of some of the negative effect of abiotic stress. This work may take the form of collecting anthers from a greater number of spikelets within an ear and even, if possible, collecting anthers from different florets of the same spikelet, instead of just focusing on the most proximal florets within a spikelet (florets a & b).

2.5.3 In-field applications of growth cabinet derived results

When seeking to apply the principles, derived from the results of the experimentation carried out in the growth cabinets, to field grown wheat, in relation to the effects heat stress may have on both pollen damage and associated yields reductions, a number of questions arise. One such question may be whether the consistency of pollen staging, within a temporal/gross developmental framework, would be present within the greater plant heterogeneity found in a field, as it was in the growth cabinets? In relation to this question, it can be seen that, even when collected with as little as a day between cohorts, there was a significant difference in ear/average spikelet dry weight. This result would therefore indicate that there is a strong possibility, under the assumption that ear/average spikelet weight is relatively strongly positively correlated with anther maturity (tentatively supported by the results of chapter 3), that, at least within a genotype, anther staging is uniform when assessed in relation to its temporal distance from the start of booting (39ZS). However, even though this area would need further investigation in the future, in an effort to gain clarity, knowing that those such as Fernández Gómez & Wilson (2012) have found strong correlations between the external features of barley, during booting, and anther stage, there is the potential for the developing of quick and accurate methods of assessing anther stage without the long, costly and relatively difficult methods used in the primary research of this chapter (sectioning from resin).

2.6 Conclusions

Because of this potentially paradigm shifting finding of greater sensitivity being found in later pollen development, and in light of its relatively little support in past literature, further research, including the direct reputation of this experimentation, may be needed before arriving at a firm conclusions. This is especially prudent as the potential implications of defining a new developmental period of ultra-sensitivity to abiotic stress, may be considerable; not least in any future breeding endeavours to create germplasm more capable of withstanding changing climates.

If nothing else, this primary research has further demonstrated the detrimental effects heat stress has on eventual grain set, when inflicted during pollen development and compounded the strong relationship between pollen damage and grain set reduction.

Whist acknowledging the direct effect heat stress has on pollen, there is still some ambiguity in relation to what other reproductive factors heat stress affects. This further highlights the importance of, not only establishing a greater understanding of the mechanisms behind pollen damage, but in a desire to work towards greater wheat yield stability, a more holistic appreciation for the reasons behind yield loss. Such areas of further research may include changes in female reproductive tissue and flowering patterns due to abiotic stress.

Chapter 3

The Sensitivity of Different Wheat Varieties' Pollen to Early Booting Heat Stress

3.1 Introduction

3.1.1 Pre-existing knowledge pertaining to germplasm tolerance to early booting heat stress

Saini & Aspinall (1982) and Saini *et al*. (1984), and the findings of 'Chapter 2', illustrate the highly detrimental effects heat stress, during early booting/pollen development, has on eventual grain number. Since there is likely to be, within the natural genetic variation possessed by *Triticum*, germplasm with increased resistance to early booting heat stress (Khodadadi *et al.,* 2011); establishing the identity of such material would, in light of future climatic challenges to global food security, be of great importance (Bita & Gerats, 2013). Identification of such germplasm would not only allow the expansion of wheat cultivation into geographical areas with a heightened risk to heat stress during early booting, but would also allow for their use in future breeding programmes. Past instances of germplasm selection, in order to propagate desired characteristics through breeding, include the Chinese cultivar Sumai 3 having been used extensively as a source of type II *Fusarium* head blight resistance in wheat (Basnet *et al.*, 2012), and the introduction of reduced height (Rht) semi-dwarfing genes which led to impressive increases in wheat yields during the Green Revolution (Pearce *et al.,* 2011).

Yield susceptibility to abiotic stress (including heat stress) is largely attributable to the dependence of modern agronomy on a comparatively narrow genetic base, with genes imparting resistance to particular stresses more likely to be found in wild species, rather than those in current cultivation (Shivanna & Sawhney, 1997; Xie & Nevo, 2008). Nevertheless, there is merit in exploring the tolerance of existing cultivated varieties to heat stress during early booting. This is not least because such information will enable the easier future

characterisation of relevant quantitative trait loci (QTLs), as there is conceivably a more comprehensive understanding of cultivated varieties' genetic background, than there is of wild species.

The inclusion of *Rht* genes into the germplasm of wheat stocks was largely responsible for increases in wheat yields in several wheat growing areas of the world, between the 1960's and the 1990's (Alghabari *et al.*, 2014). This has meant that lines containing such genes soon became widespread, with 80% of registered wheat cultivars containing a major gene for reduced height (Alghabari *et al.*, 2014). Therefore, in light of 90% of the world's semi-dwarf wheat genes containing either the Gibberellic acid (GA)-insensitive *Rht-B1b* (a.k.a *Rht1*), or *Rht-D1b* (a.k.a *Rht2*), gene (Worland *et al.*, 1998), and the increased sensitivity of such germplasm to '*unfavourable conditions*' (Gale & Youssefian, 1985), a greater understanding of the effects of heat stress on such lines was essential. Alghabari *et al.* (2014) therefore conducted an in-depth analysis of the effects of both heat and drought stress on wheat containing *Rht* alleles, when imposed during both booting and anthesis. They demonstrate, through combined analysis of varying experimental methods, that sensitivity to GA, as suggested by preliminary work (e.g. Law *et al*., 1981; Law & Worland 1985), does not appear to confer tolerance to either heat or drought stress, in relation to the effect on eventual grain set. However, they do suggest that any differences in field scale 'tolerance' may possibly be due to GA-insensitive lines possessing comparatively inferior root architecture and function, and/or the lack of adaptive significance in the timing of the onset, and duration, of susceptible growth stages. In relation to early booting heat stress, one such period of susceptibility is pollen mother cell (PMC) meiosis (Saini & Aspinall, 1982).

Even though it may have been expected, wheat genotypes from the comparatively warmer south of Europe may not possess greater tolerance to heat stress, through an anticipated combination of natural and artificial selection, when compared to their northern European counterparts (Semenov *et al*., 2014). However, in another study, increased levels of both drought and temperature had a greater negative effect, in relation to the production of '*normal*' pollen grains, on one variety, when compared to another, to such an extent that one (Plainsman V) was characterised as 'tolerant' and the other (Cappelle Desprez) 'sensitive' (Jäger *et al.*, 2008). However, whether this was due to tolerance or avoidance remains unclear.

3.1.1 Tolerance vs. resistance to abiotic stress

When seeking to identify germplasm for either expansion of area harvested and/or use in future breeding programmes, it is important to identify what is tolerance/resilience/resistance and what is avoidance. Tolerance, over and above avoidance, would be desired.

Tolerance is more desirable to avoidance, due to avoidance being, especially in relation to climatic conditions, a rather transitory, intra-seasonal, classification. Due to the increasingly unpredictable nature of both current and future heat stress events (Hansen *et al.*, 2012), to breed for avoidance may lead to high yields one year, but very much reduced yields the next, as a developmental period of susceptibility (like those seen within 'Chapter 2') may have been, by chance, avoided in the first but affected, by heat stress, in the second. Instances of heat stress avoidance include the adoption of wheat genotypes containing genes providing insensitivity to photoperiod (e.g. *Ppd-1*). Even though primarily bred into germplasm in order to allow for the ability to grow wheat in a wider range of latitudes, a secondary effect of enabling flowering sooner after sowing, due to not having to wait for longer day lengths, included the likelihood of avoiding deleterious climatic conditions (e.g. heightened risk of heat stress events) associated with the latter stages of the growth season (Worland & Snape, 2001).

Resistance, on the other hand, is a non-transitory classification. Examples of biotic resistance in wheat include the presence of greater levels of DIMBOA glucoside, implicated in increased levels of resistance to insect damage (Elek *et al*., 2009). To date, little is known about resistance to abiotic stress in wheat. However, significantly different rooting lengths and masses of wheat genotypes (Ford *et al*., 2006) may offer resistance to the abiotic stresses that is drought stress. However, further experimentation is ongoing on this front.

3.1.3 The role of the tapetum, and tapetal mitochondria, in pollen development

With Echlin (1971), Laser & Lersten (1972), Shivanna *et al.* (1997) & Wilson & Zhang (2009) all extolling the, potentially unrivalled, importance of the tapetum in angiosperm pollen development, not least due to its nutritive function, it is unsurprising to find that pre-mature tapetum degradation has such negative consequential effects on the eventual pollen viability of wheat (Saini *et al*., 1984). The tapetum is extremely, metabolically active during meiosis (Dolferus *et al.*, 2011). Dolferus *et al.* (2011) go as far as to suggest that it is one, or more, aspects of the tapetum intrinsic nature that makes the male floral anatomy, of many

angiosperms, more susceptible to heat stress, when compared to the female's. Meiosis, both pollen mother cell (PMC) and embryo sac mother cell (EMC), occurs, in wheat, around the start of booting (Zadoks *et al*., 1974).

As well as stating the importance of the tapetum in microspore nutrition and exine development, there is an important role the tapetum plays in the eventual breakdown of callose wall around microspore tetrads, thus enabling microspore release, another vital process in the proper development of pollen (Shivanna *et al.,* 1997).

Defining tapetal degradation as 'pre-mature', as has previously been done (e.g. Saini *et al*., 1984), as a result of abiotic stresses, would correctly imply that the tapetum is by no means an ever present feature in the process of pollen development. Tapetal degradation commences in and around the stages just prior to pollen mitosis in *Arabidopsis* (Sanders *et al.*, 1999), and at a similar stage in wheat (Saini *et al.*, 1984; Mizelle *et al.*, 1989).

Functional pollen development requires the tapetum to degenerate through programmed cell death (PCD) at an appropriate stage of development (Rogers, 2006; Parish *et al.*, 2010). It is thought that mitochondria have a central role in this process (Pelletier & Budar, 2007; Parish *et al.*, 2010). In addition to Parish *et al.* (2010) reporting that pre-mature tapetal degradation, due to the tapetum's sensitivity to abiotic stress, can lead to severe losses in crop yield, they also extol the potential benefits of pre-mature tapetal degradation in hybrid plant breeding, in relation to the creation of male sterile lines.

In a thermo-sensitive rice line, under heat stress, the tapetum undergoes early degeneration via PCD, with processes such as cytoplasmic shrinkage, membrane blebbing, and vacuolation happening within the tapetum earlier than normal (Ku *et al*., 2003). Therefore, conceivably, an intrinsic resistance (or lack of) against pre-mature tapetal degradation in wheat, due to heat stress, would have a knock on effect in relation to yields.

3.2 Objectives

1) To assess whether analogous heat stress treatments, determined by gross plant developmental stage (Zadoks Scale), has a differing effect on the grain sets of different winter wheat varieties.
2) To assess the relationship between varietal grain set reductions, due to heat stress, and varietal anther/pollen developmental stage before and after this stress.
3) To assess the effects heat stress has on the different spatial regions of differing wheat varieties' ears.
4) To assess the possibility of assessing pollen developmental stage, by means of assessing ear/average spikelet dry weight.

3.3 Methods

3.3.1 Germplasm selection

Most of the wheat grown in the UK is winter wheat (Nabim, 2012_a). Therefore, inter-varietal screening studies were carried out, based upon seven winter wheat varieties. These varieties were found within either nabim group 1 or group 2 (Nabim, 2012_b), recommended in the 2013/2014 growing season by the Home Grown Cereals Authority (Table 3.1). The nabim (National Association of British and Irish Flour Millers') groups are an assessment of milling quality, with groups 1 & 2 being the highest quality wheat to be found in the four groups.

Table 3.1: Winter wheat varieties, their breeder, and their National Association of British and Irish Flour Millers' (nabim) classification.

Winter Wheat Variety	Breeder	Nabim Group
Crusoe	Nickerson International Research GEIE	1
Gallant	New Farm Crops Ltd	1
Solstice	CPB Twyford Ltd	1
KWS Sterling	CPB Twyford Ltd	2
Panorama	Advanta Seeds UK Ltd	2
Cordiale	CPB Twyford Ltd	2
Einstein	Nickerson International Research GEIE	2

In addition to these varieties presumably having, due to the regard in which they are held, a significant effect on the UK bread making economy over the next few years, studying these lines also will provide insight into the effect genetic diversity has on the resistance of wheat pollen to heat stress. This is due to the seven chosen varieties possessing markedly different parental backgrounds (Appendix 5).

3.3.2 First year's experimentation

3.3.2.1 Experiment 1

3.3.2.1.1 Plant growth and stress implementation

Pots (12.5cm) were filled with 'PEL mix' (see section 2.3.1.1).

One hundred and ninety eight pots were sown with seeds of eight different winter wheat varieties (Table 3.2).

Table 3.2: Details on genotypes grown for inter-varietal experiment.

Winter Wheat Variety	Number of Pots Sown	Number of Seeds per Pot	Purpose of Plants
Crusoe	20	3	Experimental
Gallant	20	3	Experimental
Solstice	20	3	Experimental
KWS Sterling	20	3	Experimental
Panorama	20	3	Experimental
Cordiale	20	3	Experimental
Einstein	20	3	Experimental
Oakley	58	3	Guard Plants

All pots were arranged in a Latin square, in an unheated polytunnel at the Plant Environment Laboratory, University of Reading, UK (51.413349, -0.93749225). When these plants reached approximately 23ZS, 8 pots, from each experimental variety, were moved to the controlled environmental facilities of the Harborne Building, University of Reading, UK (51.437551, -0.94186842), where they were also arranged in a Latin square, in a walk-in growth chamber. Within this facility they continued their growth, in growth rooms at 20±1°C (day & night), 16h photoperiod and an average irradiance, to booting canopy level, of 360 µmol/m^2/sec, fluorescent and incandescent illumination. A control growth temperature of 20°C was chosen, based upon Bennett *et al.* (1973).

At approximately 31ZS, the plants were thinned down to one plant per pot, and three tillers per plant. Tiller thinning was repeated approximately every 10 days throughout the rest of the growing season.

Upon the start of main tiller booting (39ZS), 4 plants from each experimental variety were placed in a 'heat stress' growth cabinet at 35±1°C (day & night), with otherwise identical environmental conditions, for 3 days (72 hours). They were then returned to their original location within the 20°C environment, to join the other un-stressed plants.

During the high temperature treatments (35°C) and control temperature (20°C) the atmospheric relative humidity was kept above 60% (V/V). In addition, through regular physical examination, the water content of the medium was kept relatively stable, at approximately field capacity.

3.3.2.1.2 Latter stages of plant growth

Both stressed and unstressed plants had 55mm x 190mm cellophane crossing bags (Focus Packaging & Design LTD) sealed over their ears, after their emergence from the flag leaf sheath, and before anthesis, in order to prevent inter-ear cross pollination. After pollination was complete, the crossing bags were removed and the plants were placed within an outside area at the Harborne Building, and surrounded by bird proof netting. During this time they were again arranged in a Latin square.

3.3.2.1.3 Ear collection and analysis

After grain development was complete the ears were collected, individually stored in labelled paper bags, and dried at room temperature. Grain presence/absence was recorded for each of the main ears' florets. Ear third allocations were in line with parameters set forth in 'Appendix 2' and floret labelling followed the scheme of Lukac *et al.* (2012), with the first floret from the lower glume labelled as 'a', and subsequent florets labelled sequentially.

Grain numbers were compared via two way ANOVA. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, *p*-values were drawn from permutation tests (4999 random permutations). Maximum least significant differences (LSDs) were used in order to most conservatively determine significance. Discrete data (e.g. spikelet numbers) was analysed via a Generalised Linear Mixed Model, using a Poisson distribution. All analysis was done using GenStat 16. Differences with p=<0.05 were deemed 'significant'. Models can be seen within 'Appendix 7'.

In order for the accurate analysis of regional grain set levels, those spikelets not possessing at least five florets, most often those at the base and top of an ear, were standardised. This standardisation took the form of 'topping-up' those spikelets with less than 5 florets, with supplementary, non-seed bearing florets. This prevented a loss in floret presence, due to heat stress, distorting the percentages, and instead gave better appreciation for regional sensitivity.

3.3.2.2 Experiment 2

At approximately 33ZS, 36 pots (6 from each experimental variety, excluding Cordiale), which were left in the polytunnel after those plants used in 'experiment 1' were removed, had all but their main tiller removed. De-tillering was continually undertaken throughout the rest of this experiment.

Nine stems, from each variety, were harvested at the start of booting (first day of 39ZS). Ears were collected, dried (80^{o}C for 48 hours), and weighed. Average spikelet dry weight was calculated via the below formula. Viable spikelets were those that visibly looked like they had the potential to possess grains; this often excluded the bottom few spikelets of each ear.

- Ear dry weight
  (Total number of spikelets x % viable spikelets)

Resulting data was analysed via ANOVA. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, *p*-values were drawn from permutation tests (4999 random permutations), and maximum least significant differences (LSDs) were used in order to most conservatively determine significance. All analysis was done using GenStat 16. Differences with *p*=<0.05 were deemed 'significant'. Models can be seen within 'Appendix 8'.

3.3.3 Second year's experimentation

3.3.3.1 Plant growth and stress implementation

Pots (12.5cm) were filled with 'PEL mix' (see section 2.3.1.1).

One hundred and seventy pots were sown with three different winter wheat varieties (Table 3.3).

Table 3.3: Details on genotypes grown for inter-varietal experiment.

Winter Wheat Variety	Number of Pots Sown	Number of Seeds per Pot	Purpose of Plants
KWS Sterling	56	3	Experimental
Cordiale	56	3	Experimental
Oakley	58	3	Guard Plants

All pots were arranged in a Latin square, in an unheated polytunnel, at the Plant Environment Laboratory, University of Reading, UK (51.413349, -0.93749225). When these plants reached approximately 23ZS, 44 pots, from each experimental variety, were moved to onsite controlled environmental facilities. These facilities were in the form of three growth cabinets at 20±1°C (day & night), 16 h photoperiod and an average irradiance, to booting canopy level, of 700 µmol/m^2/sec, fluorescent and incandescent illumination. A control temperature of 20°C was chosen, based upon Bennett *et al.* (1973). Approximately a third, of each variety, were put in each cabinet, with a somewhat equal representation of the cohorts seen within Table 3.4.

Within 24 hours, after being moved from the polytunnel, to the 20°C growth room, the plants were thinned down to one plant per pot, and three tillers per plant. Tiller thinning was repeated approximately every 10 days throughout the rest of the growing season.

Upon the start of main tiller booting for each variety (39ZS), a range of procedures were carried out, as seen within Table 3.4. Where applicable, the use of two 'heat stress' growth cabinets running at 35±1°C (day & night), with otherwise identical environmental conditions to the control cabinets, were used.

Table 3.4: Methods implemented on each variety at the start of booting (39ZS)

Method	**# of Pots**
Stressed for the first three days (72 hours) of booting then returned to control temperature until grain set	16
Left at control temperature throughout booting, and left until grain set	16
Anthers collected from the top, middle and bottom of main tiller at the start of booting	4
Anthers collected from the top, middle and bottom of main tiller, three days into booting (after 3 days (72 hours) of stress)	4
Anthers collected from the top, middle and bottom of main tiller, three days into booting (after no stress)	4

The inter-cabinet movement dynamics during these stressing events is represented within 'Appendix 6'.

During the high temperature treatments (35°C) and control temperature (20°C) the atmospheric relative humidity was kept as high as possible (normally above 40% (v/v)). In addition, through regular physical examination, the water content of the medium was kept relatively stable, at approximately field capacity.

3.3.3.2 Microscopy analysis

Those plants whose ears were going to sectioning were collected, prepared and examined in the following way.

After removing the ear from surrounding leaf sheaths, spikelets/anthers from the lower, middle and uppermost third of the ears (Appendix 1) were removed and placed directly into Karnovsky's fixative (2% paraformaldehyde (v/v), 2.5% glutaraldehyde (v/v), in 0.05m phosphate buffer), for approximately 24 hours, before being stored in 0.1m phosphate buffer at 4^{o}C. Where only anthers were collected, due to increased spikelet maturity, these were from floret 'a', as defined by Lukac *et al*., (2012).

In time, these samples were removed from the buffer and dehydrated through an ethanol series ((10%, 30%, 50%, 70%, 90%, 100% & 100% (v/v)) 1 hour per solution). The samples were then gradually infiltrated with medium grade LR White resin (London Resin Company) through a sequential gradation of ethanol to resin (3:1, 1:1, 1:3 (v/v)) 1.5 hours per solution). Samples were then placed in 100% resin and left for 2 hours, before the resin was replaced and left for another 2 hours. Samples were placed in gelatine capsules (size 00) (Agar Scientific) and polymerised at 58^{o}C for 24 hours.

From the addition of fixative to the infiltration with resin, during working hours, samples were kept moving in solution, using a rotating platform. During non-working hours the samples were stored at 4^{0}C.

Approximately 1.2µm thick transverse sections, of the anthers of florets at the bases of spikelets, florets 'a' or 'b', as defined by Lukac *et al*. (2012), were cut, using a glass knife, and a Leica EM UC6 microtome, and then stained with 0.5% (w/v) Toluidine Blue O. Images were taken with a Leica DM5000 B light microscope.

3.3.3.3 Post-treatment plant growth

Plants going to grain set, both stressed and unstressed, had 55mm x 190mm cellophane crossing bags (Focus Packaging & Design LTD) sealed over their ears, after their emergence from the flag leaf sheath, and before anthesis, in order to prevent inter-ear cross pollination. After pollination was complete the crossing bags were removed and plants were left to reach maturity.

3.3.3.4 Ear collection and analysis

After grain development was complete, the ears were collected, individually stored in labelled paper bags, and dried at room temperature.

After weighting the ears, grain presence/absence was recorded for each of the first three ears' florets. Ear third allocations were in line with parameters set forth in 'Appendix 2' and floret labelling followed the scheme of Lukac *et al.* (2012), with the first floret from the lower glume labelled as 'a', and subsequent florets labelled sequentially.

Whole ear, along with regional grain set levels and weights, were analysed via ANOVA. Regional grain set percentages were transformed to empirical logit. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, *p*-values were drawn from permutation tests (4999 random permutations). Maximum least significant differences (LSDs) were used in order to most conservatively determine significance. Discrete data (e.g numbers of days) was analysed via a Generalised Linear Mixed Model, using a Poisson distribution. All analysis was done using GenStat 16. Differences with $p=<0.05$ were deemed 'significant'. Since there was no significant ($p=>0.05$) effects of the differing cabinet combinations (Appendix 6) on grain number, each pot/plant was treated as the unit of replication. Models can be seen within 'Appendix 9'.

In order for the accurate analysis of regional grain set levels, those spikelets not possessing at least five florets, most often those at the base and top of an ear, were standardised. This standardisation took the form of 'topping-up' those spikelets with less than 5 florets, with supplementary, non-seed bearing florets. This prevented a loss in floret presence, due to heat stress, distorting the percentages, and instead gave better appreciation for regional sensitivity.

3.4 Results

3.4.1 First year's experimentation

3.4.1.1 Experiment 1

3.4.1.1.1 Grain number

Despite the variety having a highly significant (p=<0.001) effect on the number of spikelets possessed by ears, within this screening experiment the temperature treatment did not have a significant (p=0.134) effect. There was also no significant (p=0.690) interaction between variety and temperature treatment. Therefore, any changes in grain number, within a variety, cannot be attributed to different spikelet numbers.

In this screen experiment, devised to highlight varieties with differing levels of sensitivity to early booting heat stress, it is apparent that both temperature treatment (p=<0.001) and variety (p=0.043) had a significant effect on grain number per spikelet (Figure 3.1a). Additionally, there was a highly significant (p=<0.001) interaction between temperature treatment and variety (Figure 3.1a), with there being markedly different percentage losses, due to heat stress (Figure 3.1b).

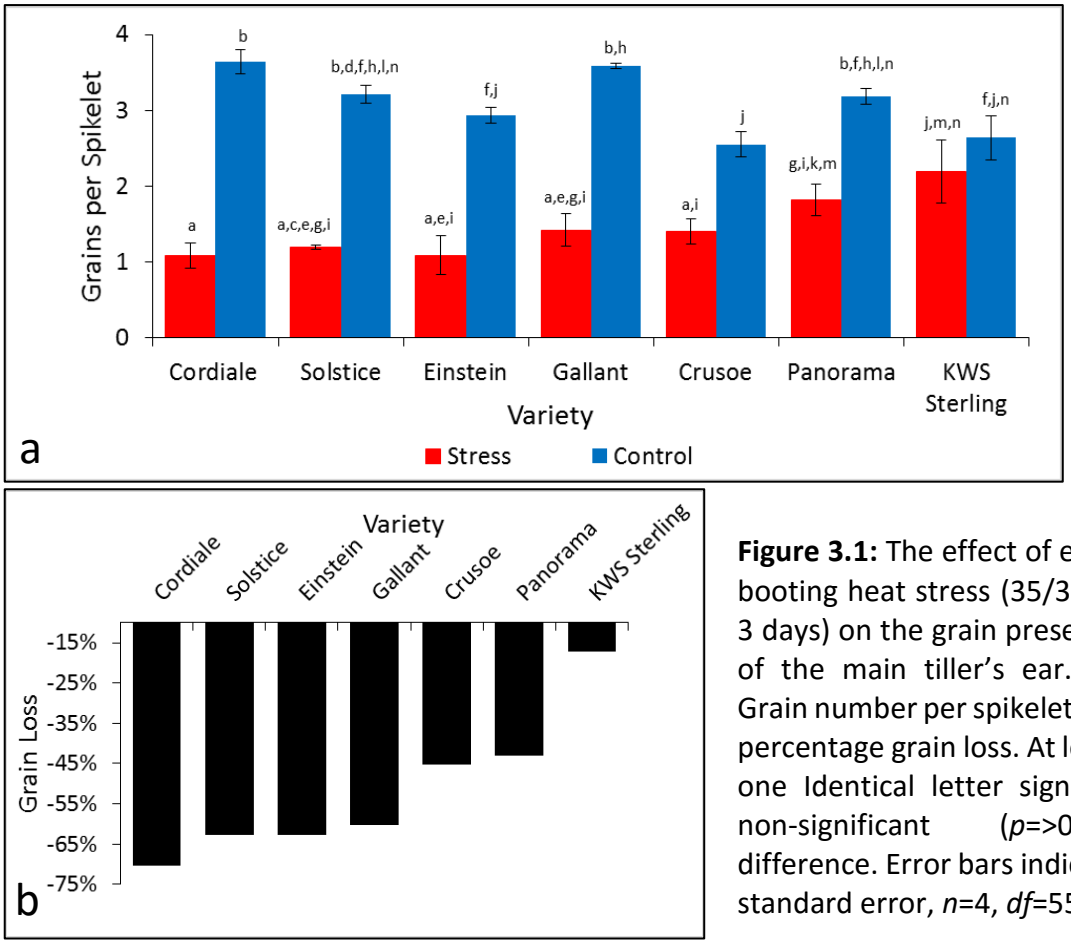


Figure 3.1: The effect of early booting heat stress (35/35°C; 3 days) on the grain presence of the main tiller's ear. (a) Grain number per spikelet, (b) percentage grain loss. At least one Identical letter signifies non-significant (p=>0.05) difference. Error bars indicate standard error, n=4, df=55.

3.4.1.1.2 Regional losses

Across the seven varieties, there was a marked level of inter-varietal similarity in the effect early booting heat stress had on regional grain set (Figure 3.2 & 3.3), with the proximal florets of spikelets and upper third of the ears, being consistently most sensitive.

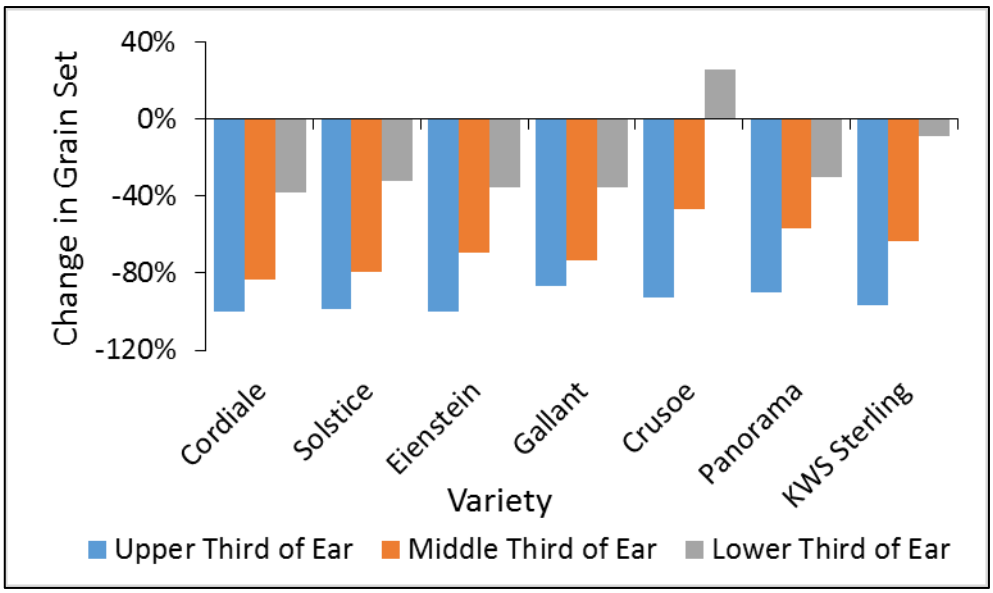


Figure 3.2: The effect that early booting heat stress (35/35°C; 3 days) had on the presence of grains, over the length of main tiller ears, when compared to the control. *n*= 4. Note: only considering the first five florets of each spikelet

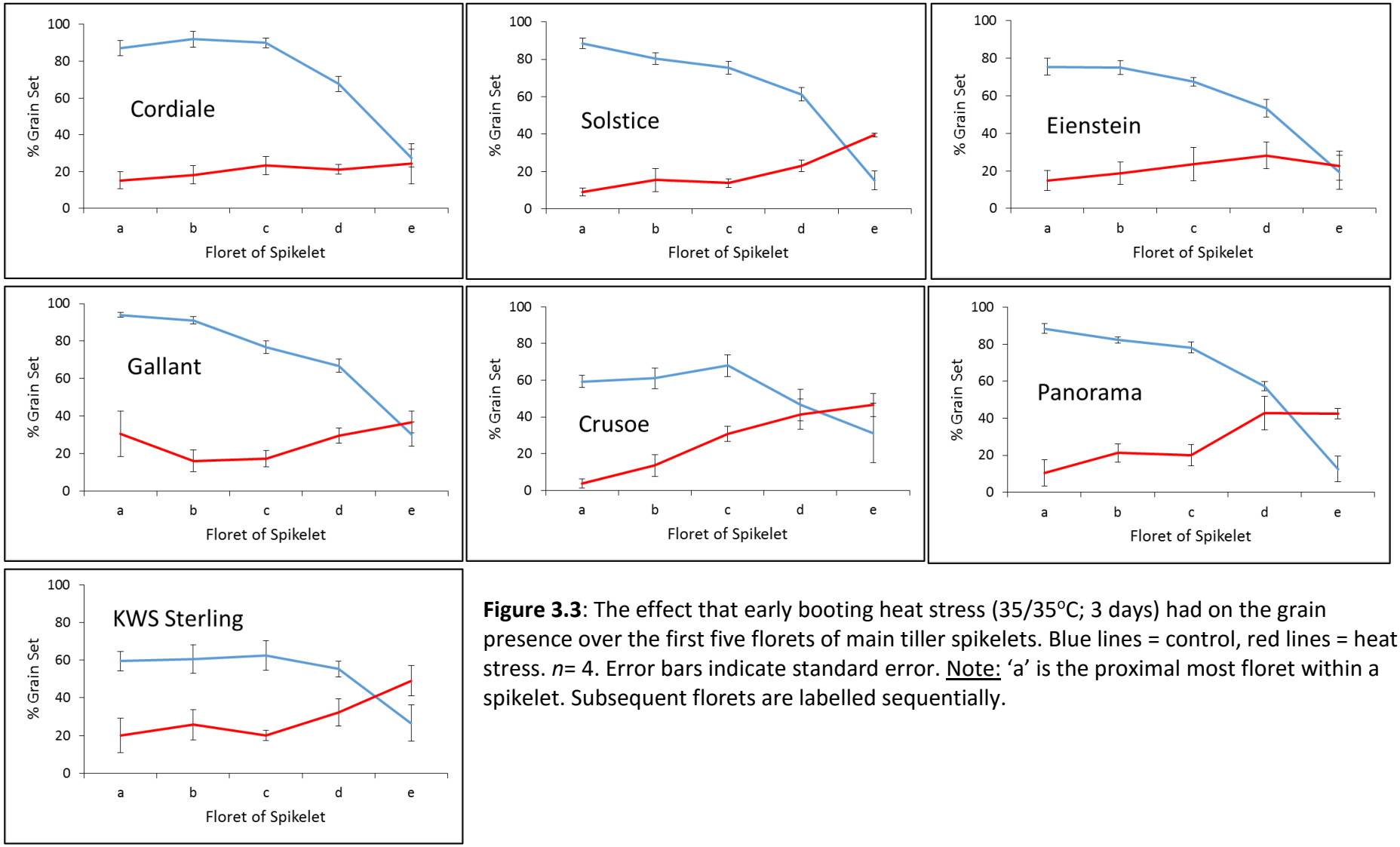


Figure 3.3: The effect that early booting heat stress (35/35°C; 3 days) had on the grain presence over the first five florets of main tiller spikelets. Blue lines = control, red lines = heat stress. *n*= 4. Error bars indicate standard error. Note: 'a' is the proximal most floret within a spikelet. Subsequent florets are labelled sequentially.

3.4.1.2 Experiment 2

Variety had a significant effect on both ear (p=<0.001) and average spikelet (p=0.006) dry weight (Figure 3.4), when measured at the start of booting. These changes in dry weight were strongly positively correlated with sensitivity to early booting heat stress (Figure 3.5 & 3.6).

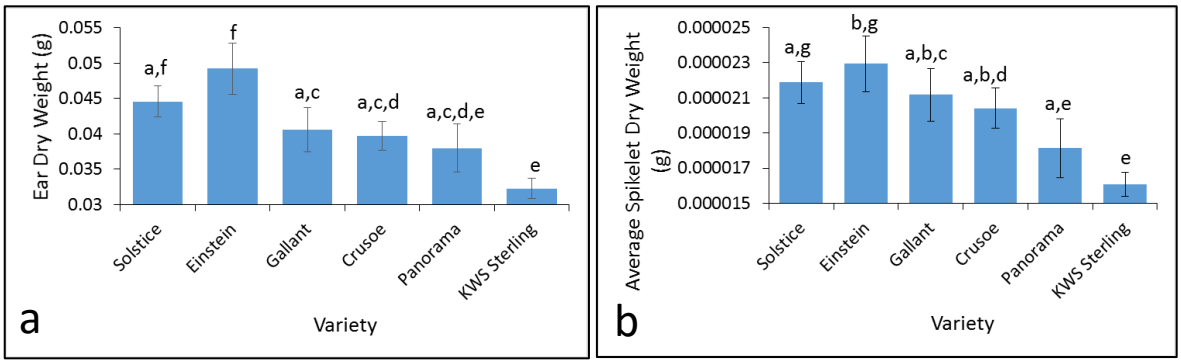


Figure 3.4: The (a) ear and (b) average spikelet dry weights of six winter wheat varieties at the start of booting (39ZS). At least one Identical letter signifies non-significant (p=>0.05) difference. Error bars indicate standard error. n= 8-9, df = 51.

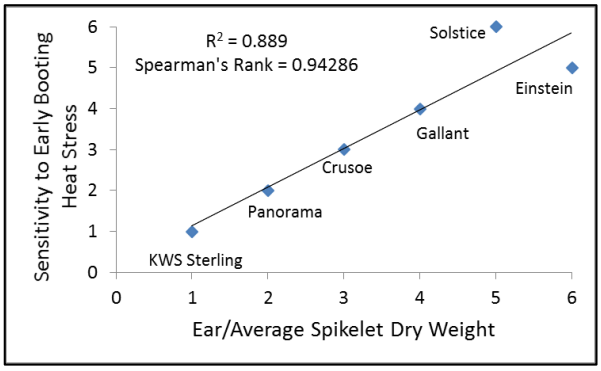


Figure 3.5: Correlations between ear/average spikelet dry weight, at the start of booting (when ranked), and sensitivity to early booting heat stress (when ranked). 1 = lightest ear/average spikelet & least sensitive to early booting heat stress, 7 = heaviest ear/average spikelet & most sensitive to early booting heat stress.

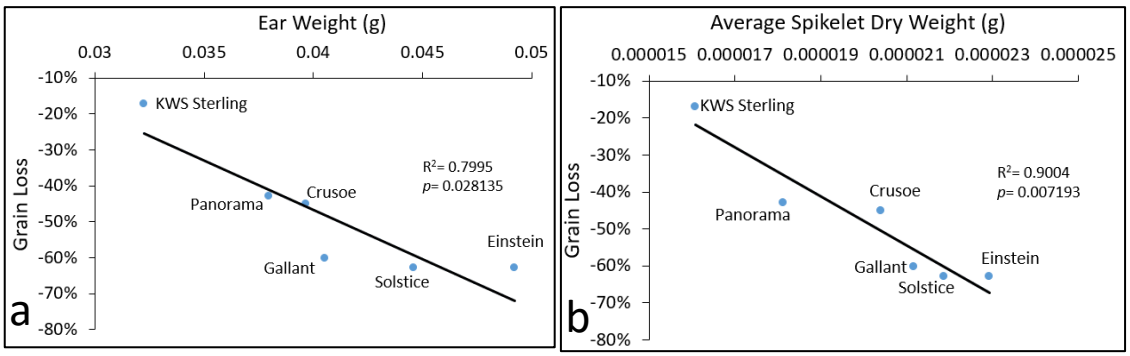


Figure 3.6: Correlations between percentage grain loss, due to early booting heat stress, and (a) the ear, and (b) average spikelet dry weight, at the start of booting (39ZS). n= 6, df = 4.

3.4.2 Second year's experimentation

3.4.2.1 Grain number

Temperature treatment had a non-significant effect on the spikelet number of both the main tiller (p=0.198) and second and third tillers (p=0.980). The variety had a non-significant (p=0.343) effect on the spikelet number of the main tiller, but did have a highly significant (p=<0.001) effect on those of the second and third tiller, with KWS Sterling having more spikelets. Across both main (p=0.470) and secondary tillers (tiller 2 + 3) (p=0.349), there was a non-significant interaction between temperature treatment and variety. For the purposes of this experimentation, paired spikelets (see Boden *et al*., 2015), which were present in low quantities in both varieties, were considered 'spikelets' alongside primary spikelets.

In this experiment, devised to clarify the effect early booting heat stress had on two winter wheat varieties, both early booting heat stress (p=<0.001) and variety had a highly significant (p=<0.001) effect on main tiller grain set, with there also being a highly significant (p=<0.001) interaction between variety and heat stress. There was no significant difference between the numbers of grains possessed by both controls (Figure 3.7a). All of these phenomenon, and levels of significance, were still present when considering only the first five florets of each spikelet, where the largest, more yield determining, grains may be found (Millet, 1986).

Like the main tiller, both early booting heat stress (p=<0.001) and variety (p=<0.001) had a highly significant effect on the grain set of the secondary tillers. There was also a highly significant (p=<0.001) interaction between variety and heat stress. There was no significant difference between the numbers of grains possessed by both controls and the stressed ear of KWS Sterling (Figure 3.7b). All of these phenomenon, and levels of significance, were still present when only considering the first five florets of each spikelet, except that the stressed ears of KWS Sterling were no longer non-significantly different to the two controls.

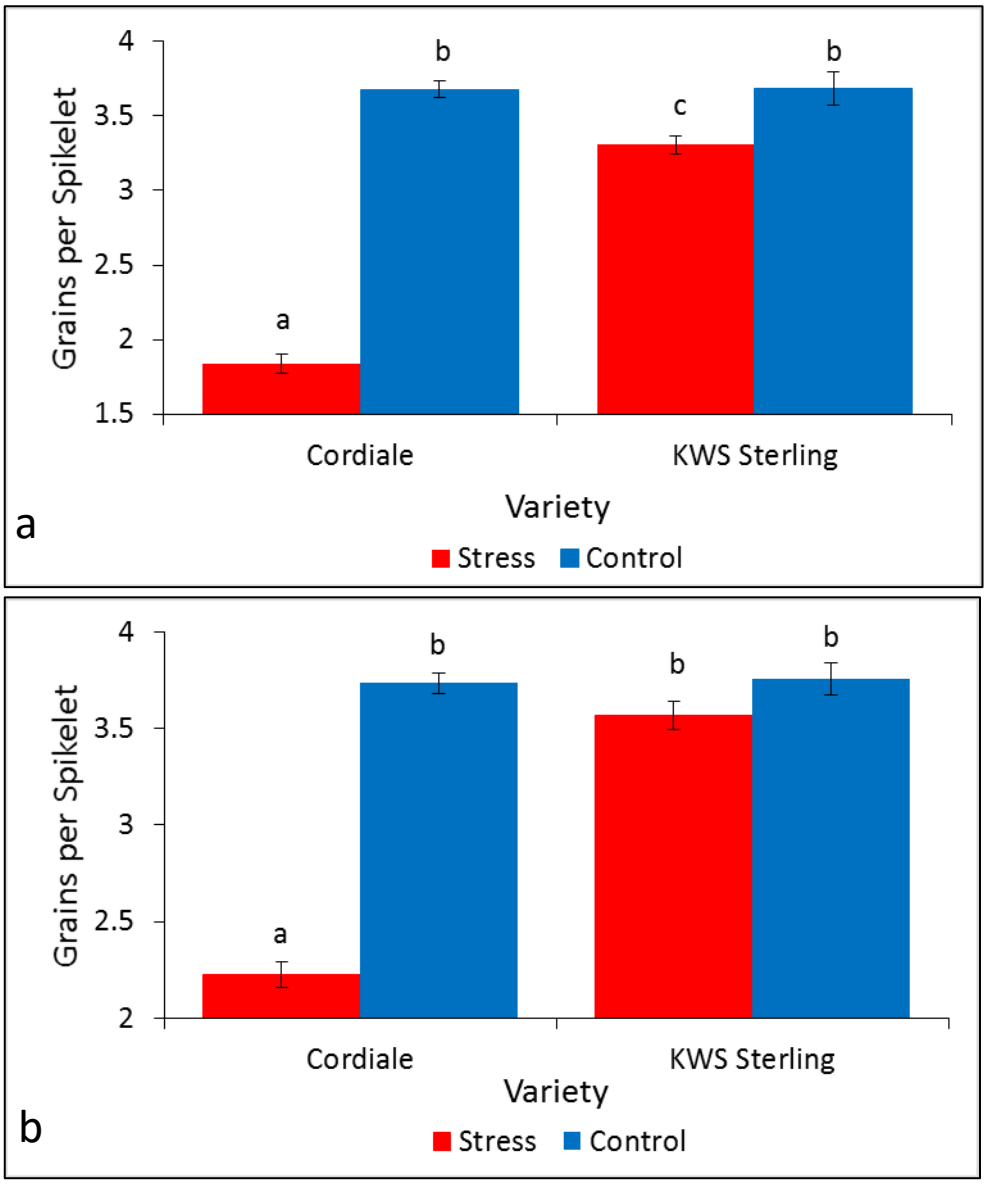


Figure 3.7: The effect of early booting heat stress (35/35°C; 3 days) on the grain number of two winter wheat varieties. (a) Main tiller, (b) tiller 2 & 3. Error bars indicate standard error. Identical letters indicate non-significant difference (p=>0.05), n= 14-15, df = 58.

3.4.2.2 Anther/reproductive cell damage

The inter-varietal differences in main tiller grain numbers (Figure 3.7a) are in keeping with the effects that the heat stress had on anther/pollen development, when compared to their respective controls (Figures 3.8 & 3.9). This is due to the anthers of KWS Sterling tending to be in a less vulnerable stage of development (pre-meiosis), during early booting, when compared to Cordiale's tendency to have a larger proportion of its anthers at the relatively more sensitive stage of meiosis. A key characteristic of this greater sensitively is the effect early booting heat stress has on the tapetum of Cordiale. In all the three regions of Cordiale's

main ear, the tapetum is considerably more degraded, due to heat stress, than their KWS Sterling equivalents.

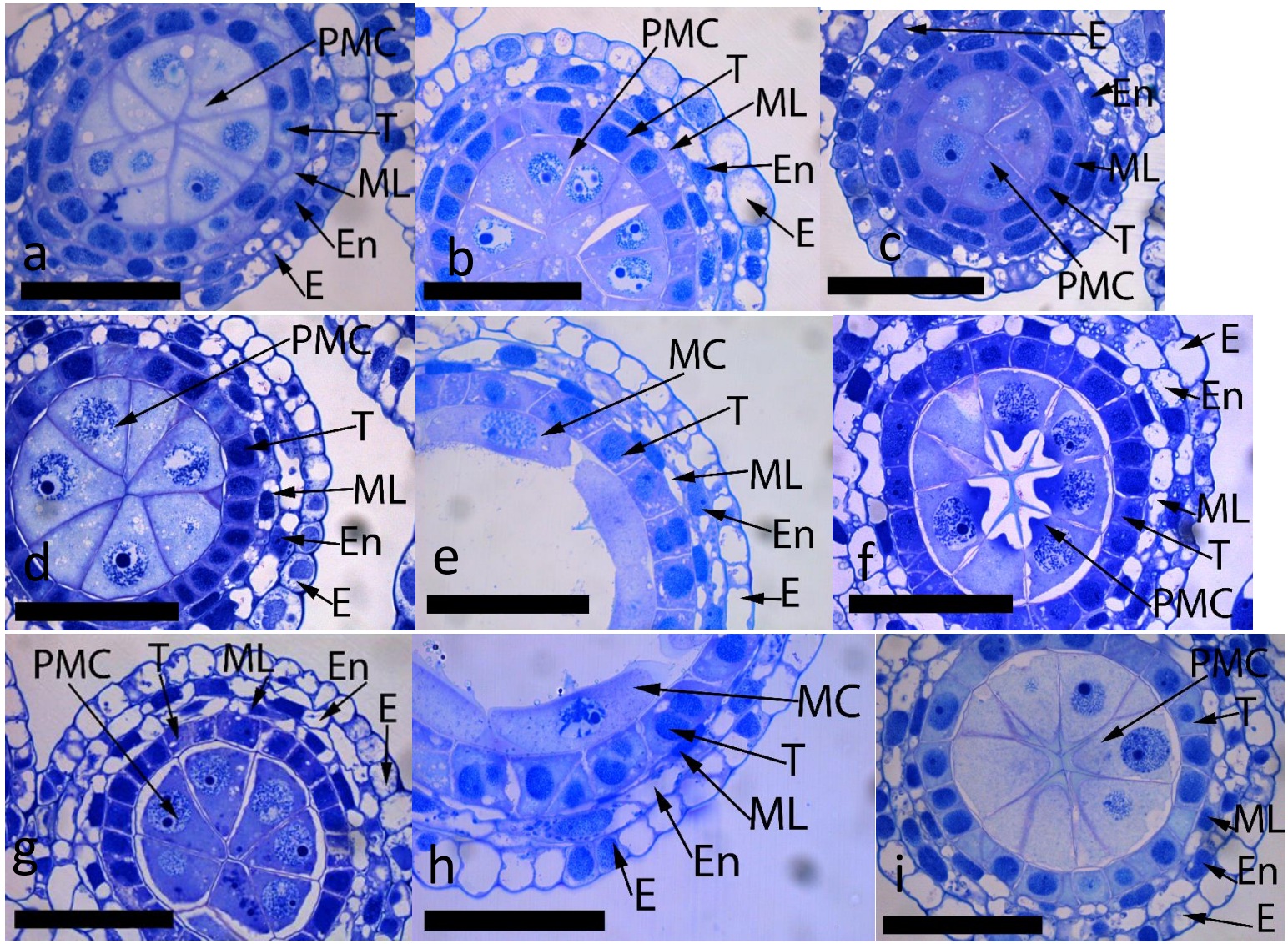


Figure 3.8: Transverse sections of anthers from the main tiller of KWS Sterling. (a-c) 0 days post 39ZS, control (20/20^{o}C), (d-f) 3 day post 39ZS, control (20/20^{o}C), (g-i) 3 days post 39ZS, after 3 days of stress (35/35^{o}C). Anthers from (a,d,g) distal, (b,e,h) middle and (c,f,i) proximal position of ear. E= epidermis, En= endothecium, MC= meiotic cell, ML= middle layer, PMC= pollen mother cell. T= tapetum. X 100 magnification, Scale bars

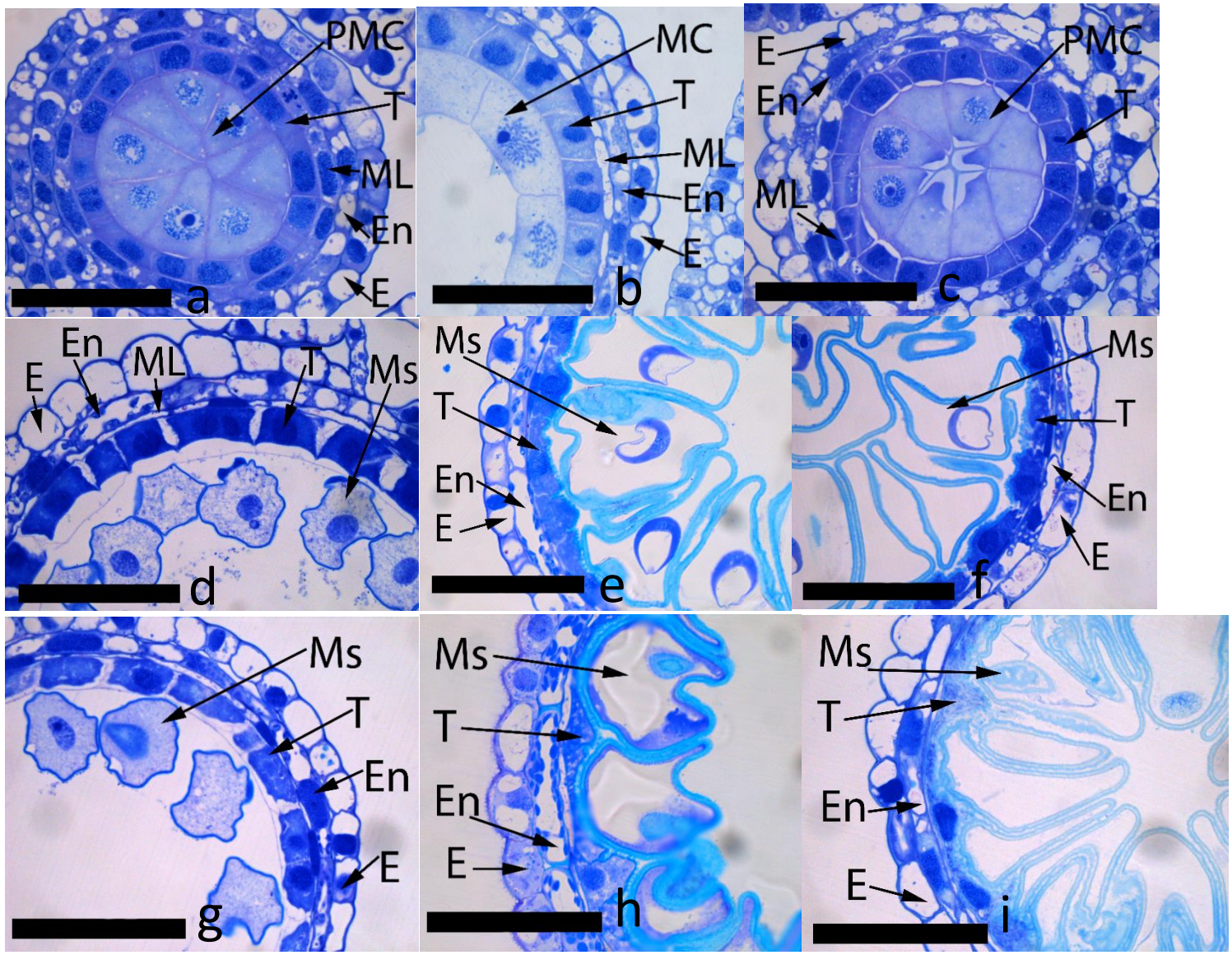


Figure 3.9: Transverse sections of anthers from the main tiller of Cordiale. (a-c) 0 days post 39ZS, control (20/20°C), (d-f) 3 day post 39ZS, control (20/20°C), (g-i) 3 days post 39ZS, after 3 days of stress (35/35°C). Anthers from (a,d,g) distal, (b,e,h) middle and (c,f,i) proximal position of ear. E= epidermis, En= endothecium, MC= meiotic cell, ML= middle layer, Ms= Microspore, PMC= pollen mother cell, T= tapetum. X 100 magnification, Scale bars 50µm.

3.4.2.3 Regional losses due to heat stress

3.4.2.3.1 Thirds of ears

In addition to the temperature treatment imposed at the start of booting, consistently, across both the main (Figure 3.10a) and secondary (Figure 3.10b), tillers having a highly significant (p=<0.001), mostly negative, effect on the levels of regional grain set in the thirds of ears, it is apparent that the variety also consistently has a highly significant (p=<0.001) effect (Figure 3.10a,b). In addition, there is, across both tiller sets, with the exception of the middle third of the main tiller (p=0.773) and the lower third on the secondary tiller (p=0.351), consistently a highly significant (p=<0.001) interaction between the temperature treatment and the variety, with, at times, when applying the LSD, unlike Cordiale, there being no significant difference between the regional grain set levels of the stressed and control plants of KWS Sterling.

With the exception of the secondary tillers of KWS Sterling, where the opposite was present, the upper third of both varieties, and both tiller sets, were most sensitive to heat stress in relation to decreases in grain set, with the lower thirds being the most resistant (Figure 3.10c). When comparing the differences between the two varieties, the greatest discrepancy was found in the upper third of the ear, across both tiller sets (Figure 3.10d).

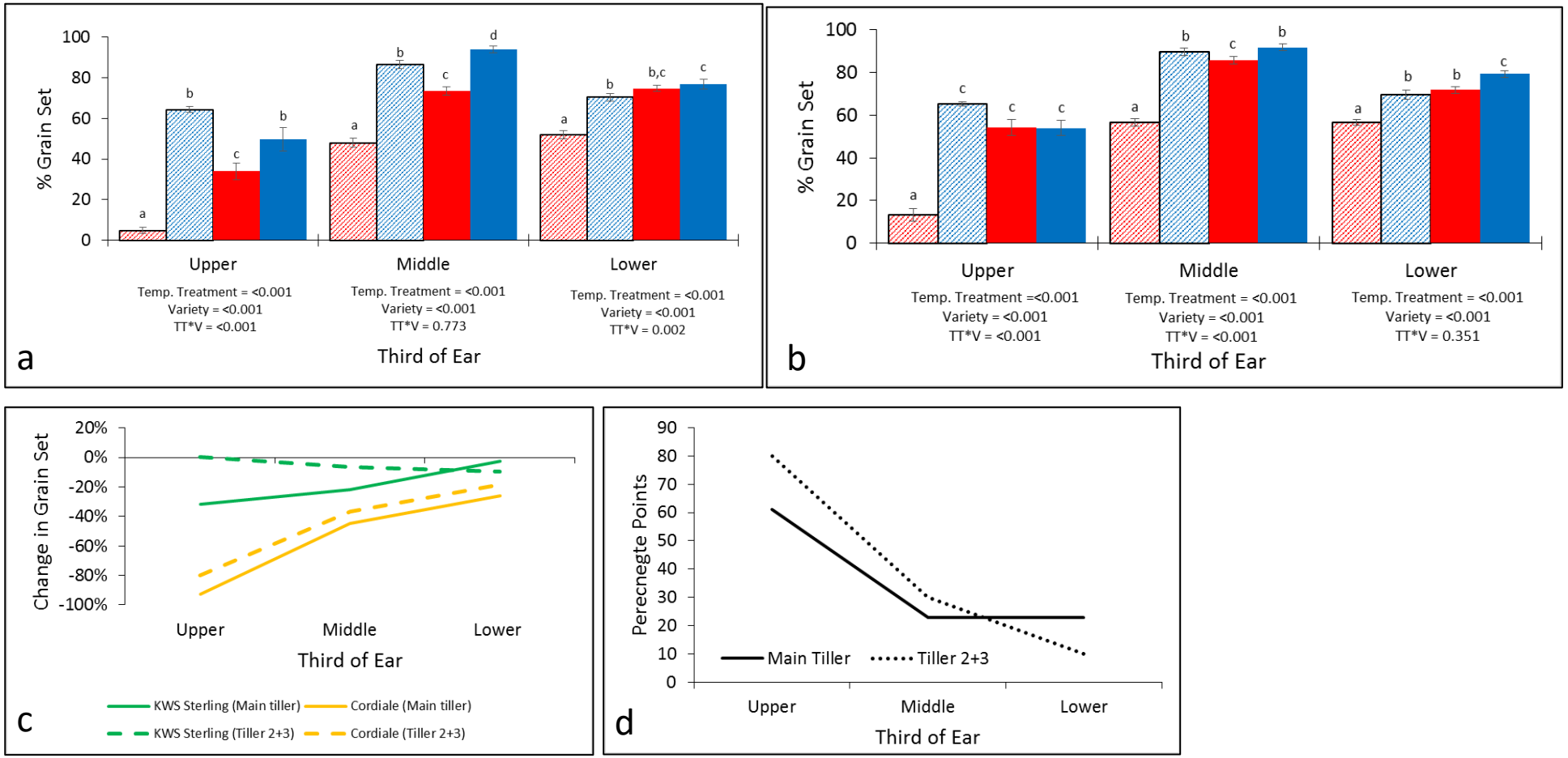


Figure 3.10: The effect of early booting heat stress (35/35°C; 3 days) on inter-regional grain presence. (a) Main tiller, (b) tiller 2+3. Red = stressed, blue = control, dashed = Cordiale, solid = KWS Sterling. Error bars indicate standard error. Identical letter indicates, intra-regional, non-significant difference (p=>0.05). n= 14-15, df = 58.

(c) Percentage change in grain set due to early booting heat stress. (d) Percentage point difference between the regional grain set of KWS Sterling and Cordiale. Note: only considering the first five florets of each spikelet.

3.4.2.3.2 Florets of ears

With the exception of floret ‘d’ of the secondary tillers (*p*=0.116), the temperature treatment imposed at the start of booting, has a highly significant (*p*=<0.001) effect on floret grain set. There was an increase in the grain set in floret ‘e’ of both varieties, and tiller sets, and floret ‘d’ of KWS Sterling’s tillers sets, due to heat stress (Figure 3.11a,b,c) with, at times, these increases being significant (Figure 3.11a,b). With the exception of floret ‘b’ in both the main (*p*=0.916) and secondary (*p*=0.056) tillers, and floret ‘a’ in the secondary tillers (*p*=0.075), the variety had a significant effect on grain set (Figure 3.11a,b).

There is, across both tiller sets, with the exception of floret ‘e’ in both the main (*p*=0.181) and secondary tillers (*p*=0.064), a significant interaction between the temperature treatment and the variety, with, in the majority of florets, KWS Sterling being more resilient to early booting heat stress than Cordiale (Figure 3.11d). This increased resilience of KWS Sterling can also be seen when comparing the losses of each variety, across both tiller sets, with the florets in the middle of the spikelets (e.g. florets ‘c’ & ‘d’) being where the greatest discrepancies were (Figure 3.11d). In both varieties, and tiller sets, the florets at the proximal most regions of spikelets tended to be most sensitive to heat stress (Figure 3.11c).

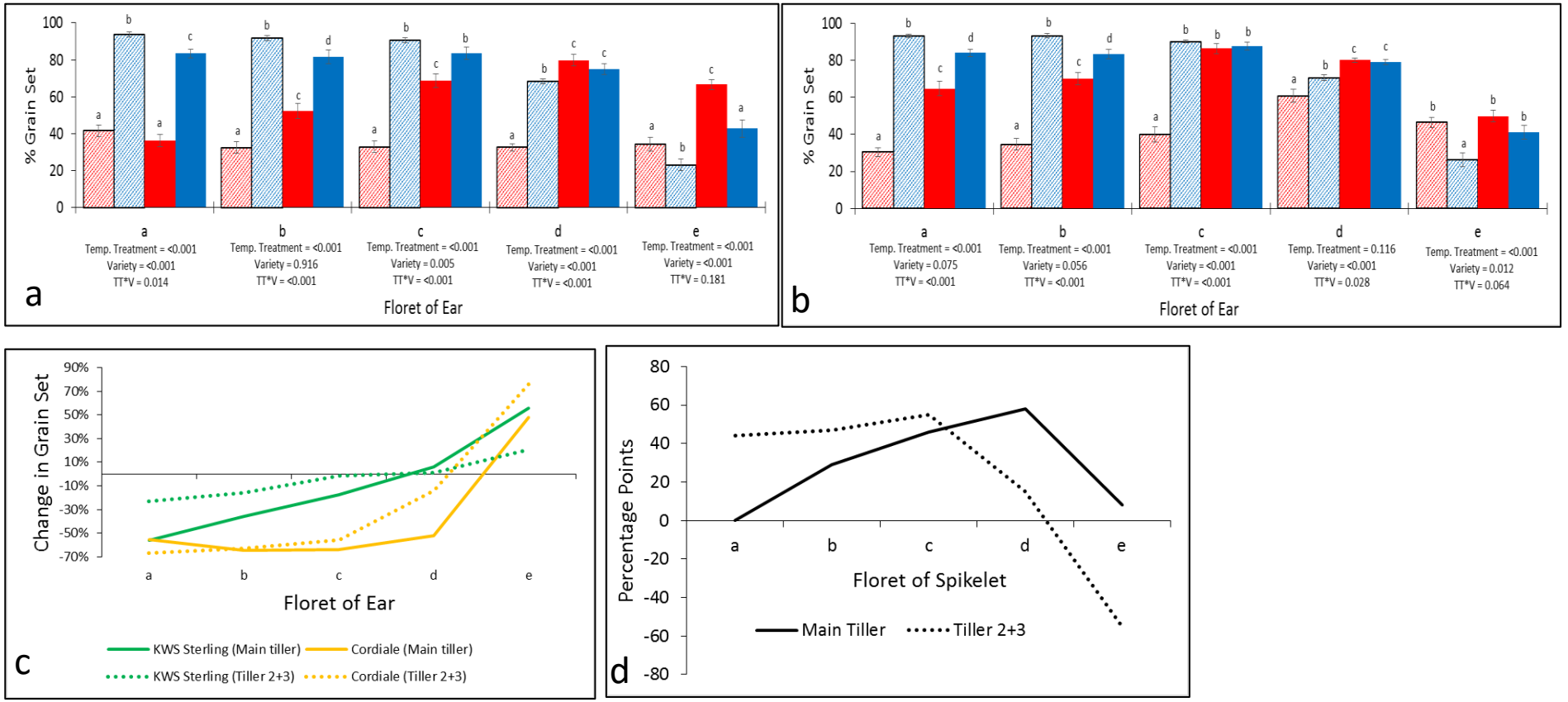


Figure 3.11: The effect of early booting heat stress (35/35°C; 3 days) on intra-spikelet grain presence. Main tiller (a), tiller 2+3 (b). Dashed = stress, un-broken = control. Red = Cordiale, blue = KWS Sterling. Error bars indicate standard error. Identical letter indicates, intra-floret, non-significant difference (p=>0.05). n= 14-15, df = 58.

(c) Percentage change in grain set due to early booting heat stress. (d) Percentage point difference between the regional grain set of KWS Sterling and Cordiale. Note: 'a' is the proximal most floret within a spikelet. Subsequent florets are labelled sequentially.

3.4.2.4 Floret and grain number post floret 'e'

Both the temperature treatment, imposed at the start of booting, and variety had a highly significant (p=<0.001) effect on the number of florets in the upper regions of wheat spikelets (above floret 'e') in both tiller sets. Heat stress consistently caused a significant increase in the number of these florets (Figure 3.12a). In the main tiller there was a significant (p=0.025) interaction between the temperature treatment and the variety. However, in the secondary tillers there was no such significant interaction (p=0.278).

Both varieties, in both their main and secondary tillers, had a greater number of post floret 'e' grains when stressed (Figure 3.12b).

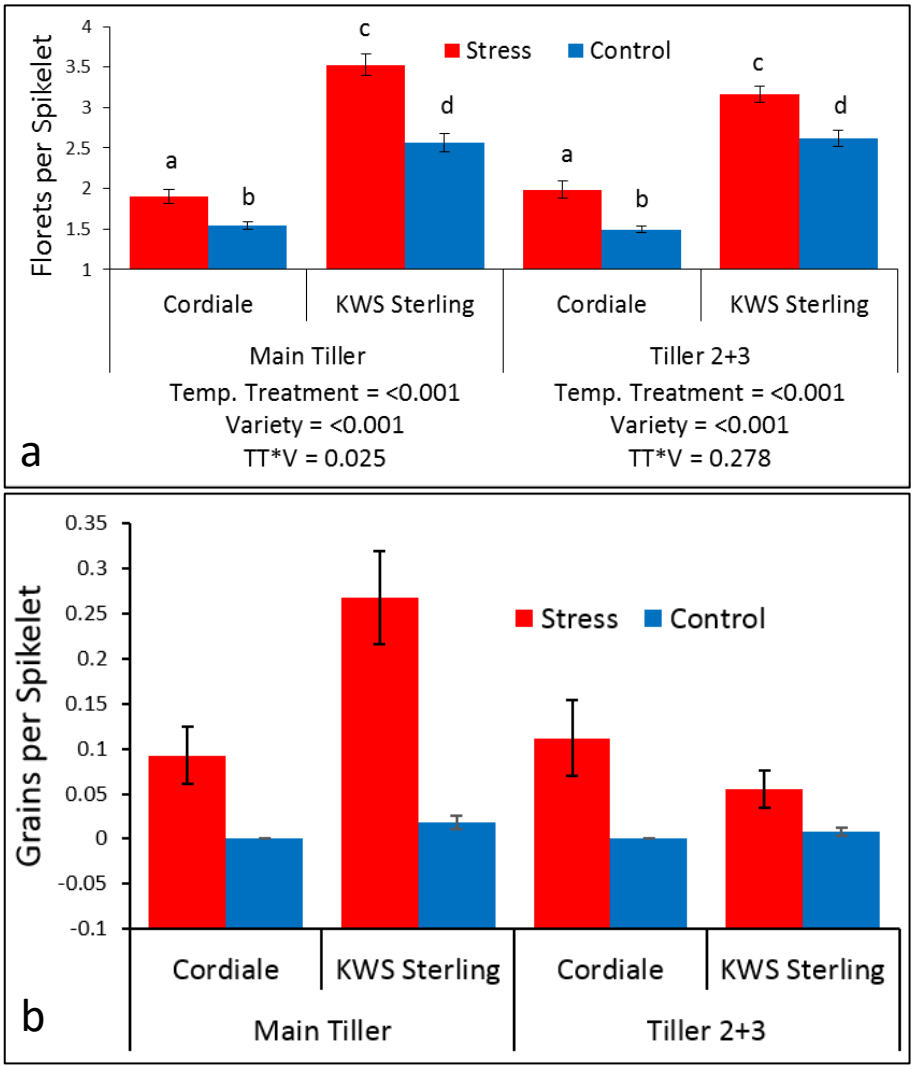


Figure 3.12: The effect of early booting heat stress (35/35°C; 3 days) on post-floret 'e' (a) floret number and (b) grain number. Error bars indicate standard error. Identical letters indicate non-significant, intra-tiller, difference (p=>0.05). n= 14-15, df = 58.

3.4.2.5 Plant phenology

Neither the temperature treatment, at the start of booting, nor the variety, had a significant effect on the length of intervals between the first three tillers reaching the start of booting (39ZS). There was, however, in the intervals between tiller 2 & tiller 3 (p=0.018) and tiller 1 & tiller 3 (p=0.030) a significant interaction between temperature treatment and variety (Figure 3.13a). Where heat stress consistently caused a decrease in the intervals between the start of tiller booting in Cordiale, in KWS the heat stress caused an increase in the time between the commencements of tiller booting (Figure 3.13a).

When considering the length of tiller booting (the number of days between 39ZS and 47ZS), the variety, across each of the first three tillers, had a highly significant effect (p=<0.001), with KWS 'in boot' for longer (Figure 3.13b). Additionally, when applying the LSDs, it appears that where the temperature treatment did not significantly affect the length of tiller booting in KWS Sterling, an increased temperature at the start of booting did lead to a significant decrease in the length of time each of the first three tillers of Cordiale was 'in boot' (Figure 3.13b).

Not only did Cordiale start booting earlier in the calendar year than KWS Sterling, but it also finished booting before KWS Sterling (Figure 3.13c).

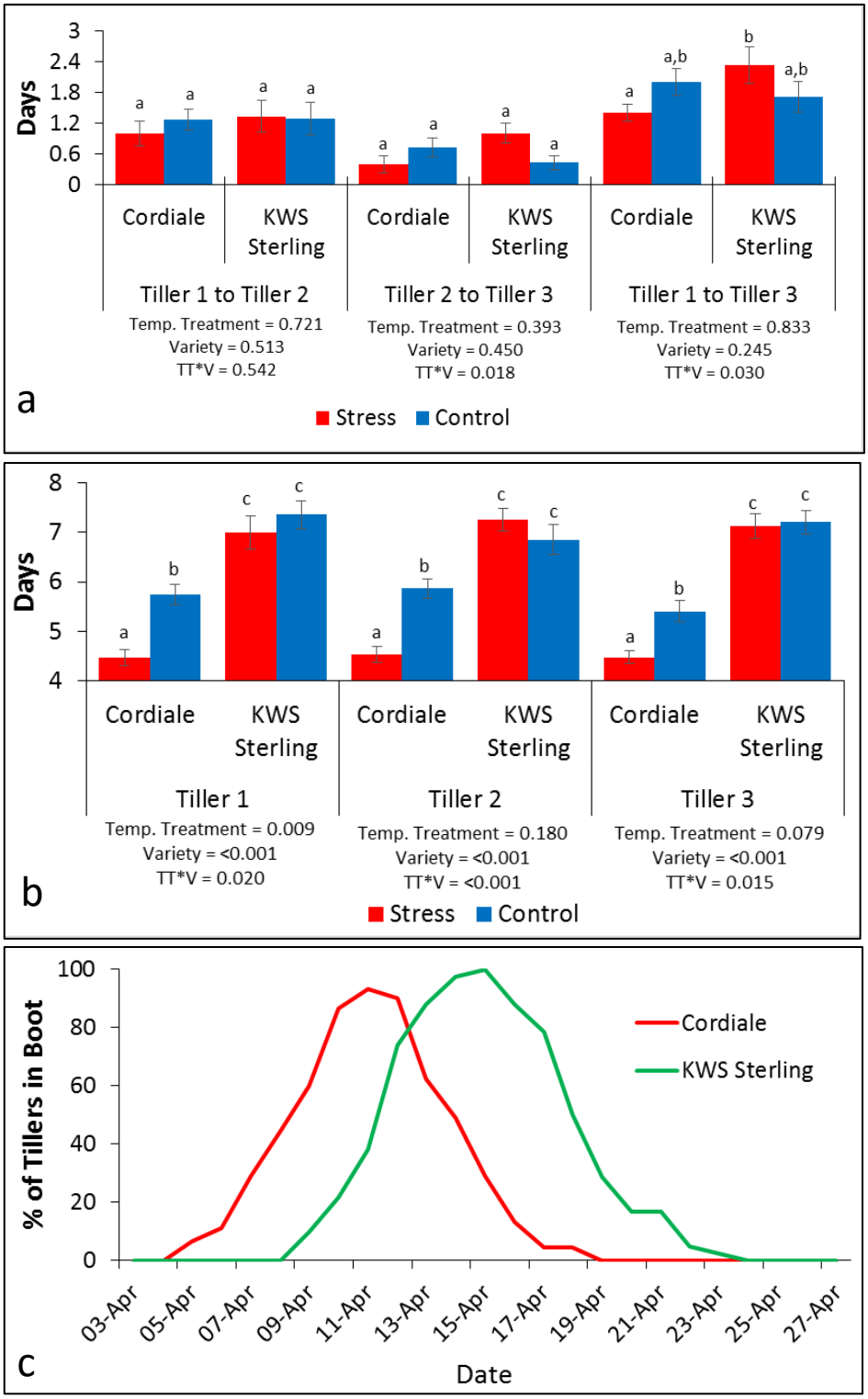


Figure 3.13: The booting dynamics of wheat. (a) Length of time between the start of tiller booting, (b) length of time between 39ZS and 47ZS, and (c) calendar date in which control plants (including all three tillers) were booting. Error bars indicate standard error. Identical letters indicate non-significant difference (p=>0.05). n= 14-15. df = 56.

3.4.2.6 Ear weight

In keeping with the average grain number per spikelet results (Figure 3.7), across both tiller sets, both the the temperature treatment (p=<0.001) and the variety (p=<0.001) had a highly significant effect on the average spikelet weight (Figure 3.14). There was, across both tiller sets, also highly significant (p=<0.001) interactions between temperature treatment and variety with, Cordiale suffering greater percentage weight loss, when compared to its control, than KWS Sterling (Table 3.5a).

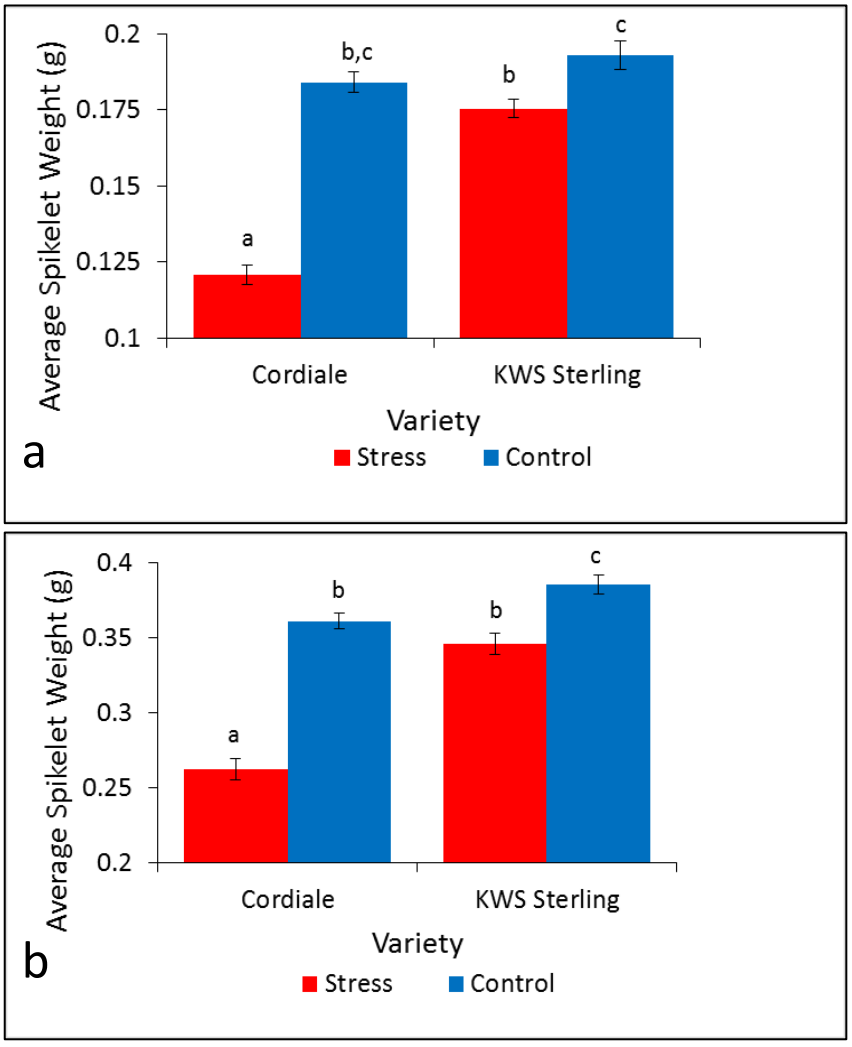


Figure 3.14: The effect of early booting heat stress (35/35°C; 3 days) on the average spikelet weight of two winter wheat varieties. (a) Main tiller, (b) tiller 2 & 3. Error bars indicate standard error. Identical letters indicate non-significant difference (p=>0.05). n= 14-15, df= 58.

Table 3.5: (a) Percentage decline in average spikelet weight, and average grains per spikelet, due to early booting heat stress (35/35°C; 3 days), and (b) percentage point difference between the two.

a		Main Tiller	Tiller 2+3
Average Spikelet Weight	Cordiale	-34%	-27%
	KWS Sterling	-9%	-10%
Average Grains per Spikelet	Cordiale	-50%	-41%
	KWS Sterling	-10%	-5%

b	Main Tiller	Tiller 2+3
Cordiale	-16	-14
KWS Sterling	-1	+5

Figure 3.15 shows there to be, even when taking into account both varieties and both temperature treatments, a highly significant (p=<0.001) correlation between ear weight and grain number. Table 3.5b may suggest that ear weight and grain number may not be as strongly correlated in Cordiale, when compared to KWS Sterling.

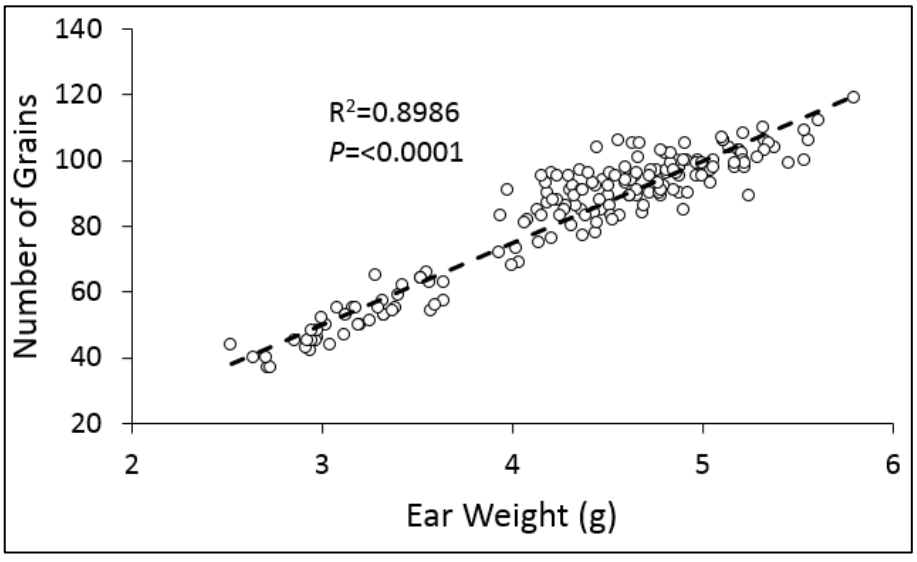


Figure 3.15: Correlation between ear weight and grain number, including both varieties (KWS Sterling & Cordiale), both tiller sets (primary & secondary) and both temperature treatments (control & heat stressed). n=176, df = 174.

3.5 Discussion

3.5.1 Effect of early booting heat stress on the grain number of winter wheat varieties

The inter-varietal screening results of the first year showed that, despite all the varieties being similar in their regional sensitivity to heat stress, there was a significant interaction between early booting heat stress and the variety, in relation to total grain number loss.

Therefore, based upon the preliminary results of this screening, the most (Cordiale) and least sensitive (KWS Sterling) varieties were re-examined, within the subsequent year, at a higher level of experimental replication. These subsequent results demonstrated that the preliminary first year designations of sensitive, to early booting heat stress, could be supported, due to KWS Sterling having significantly more grains than Cordiale across both tiller sets after early booting heat stress. In fact, tillers 2 & 3 of KWS Sterling, did not have a significant reduction in grain set, when stressed on the first three days of the main tiller's booting.

As this secondary tiller result could not be attributed to these tillers being significantly, temporally, behind the main tiller, when compared to those of Cordiale, due to there being no significant effect of variety on the intervals between booting commencement, both this, and the main tiller result, need an explanation. Palynological reasons, if only potentially partial, may offer such an explanation.

Existing literature (e.g. Saini & Aspinall, 1982; Saini *et al*., 1984) describes the sensitivity of wheat anthers to heat stress during meiosis. Therefore, seeing that a larger proportion of Cordiale's florets, unlike KWS Sterling's, went through PMC meiosis during the first three days of booting, this may help explain the significant differences in main tiller grain number loss.

However, to state that KWS Sterling's pollen is more resilient to heat stress, when compared to Cordiale's, would be highly remiss, as the stresses are not being directed towards analogous stages of pollen development, with Cordiale's pollen/anthers being approximately three days ahead of KWS Sterling's, in relation to maturity.

A resulting question, therefore, may be, '*is KWS Sterling's pollen resilient, when inflicted with heat stress at analogous times to other varieties, including Cordiale?*' Nothing within the results of this work would give any indication that this would be the case, except to presume that the secondary tillers of Cordiale and the main tiller of KWS Sterling would potentially be

at the same palynological stage. As Cordiale's secondary tillers had a 40% reduction in grain number, where KWS Sterling's main tiller only had a 10% reduction in grain number, following a heat stress event, this may indicate that KWS Sterling's pollen is more resilient to heat stress, when directed towards the same stage. However, this would certainly need further examination through experimentation.

When considering that Cordiale reaches booting approximately 4 days before KWS Sterling, there is a strong possibility that if these plants were stressed on a particular calendar day, instead of according to their gross development (the commencement of booting), the results of such an experiment may be markedly different. It is conceivable that KWS Sterling reaches PMC meiosis approximately 7 calendar days after Cordiale. In fact, Cordiale's tendency to reach booting before KWS Sterling, which was seen over two years of experimentation, would perhaps make the most of the benefits espoused by those such as Mahan *et al.* (1995) & Worland *et al.* (1998), who state the benefits of early maturation in warmer areas in relation to avoiding heat stress events during the early stages of pollen development.

3.5.2 Effect of heat stress on booting dynamics

Interestingly, despite having no significant difference between the intervals between individual control tillers reaching booting, the length of time the control tillers spend 'in boot' is, across all three tillers, significantly longer in KWS Sterling. This finding, of different booting lengths, may indicate that the relatively immature ear of KWS Sterling, at the start of booting, takes longer to acquire the biomass to push its way through the flag leaf sheath, and hence end booting, when compared to the relatively mature ear of Cordiale. Despite not officiously documenting the interval between the end of booting (47ZS) and the start of anther extrusion (60ZS) in each variety's tillers, there did not seem to be any major difference between the varieties. However, some varieties enter anthesis as early as late booting (Komaki & Tsunewaki, 1981). Therefore, it may be possible that variation in varietal booting length can also be somewhat mirrored by variation in post-booting developmental dynamics.

In contrast to there being no significant difference between the intervals of tiller booting commencement in the control plants, when each variety is compared to their corresponding stressed plants, a marked difference can be seen. Tillers became far more temporally compacted in Cordiale, and more, even though in cases slightly, prolonged in KWS Sterling. This may offer further insights into related grain number reductions. A similar pattern can be seen within individual tiller booting length change due to stress, where Cordiale had a marked

reduction in booting duration, due to early booting heat stress, when compared to KWS Sterling. A greater reduction in booting length may potentially offer a screening tool, with the possibility of changes in phenology offering a better understanding of interactions between an abiotically stressful environment and a plant's wellbeing (Inc. yield levels) (Wahid *et al.*, 2007). High temperatures during the grain filling period of wheat (70-92ZS) impose limitations on kernel weight and grain yield through reducing grain filling duration (Sayed & Gadallah, 1983).

3.5.3 Regions of the primary ear most susceptible to heat stress

When considering the inter-spikelet asynchronicity of pollen development within both varieties' main ears, it seems relatively similar, approximately 2-3 days, thus limiting any heat stress resilience that Cordiale may possess over and above that of KWS Sterling, in this regard. The grain set of Cordiale's main ears may have benefited if the asynchronicity had been greater than 2-3 days, as this would have meant that the anthers in the extremities would have been at a stage (e.g. pre-mitosis) more conductive with having a greater resilience to heat stress.

When observing this asynchronicity in KWS Sterling, and seeing that those anthers within florets 'a' or 'b' at the distal and proximal regions of the ear are at approximately the same stage, it indicates that even though the general stage of pollen development may have a significant effect on determining grain number loss, the stages of the anthers within the ear have, potentially, relatively little bearing on the grain loss dynamics of these ears. However, within Cordiale, it is clear that not only are the anthers at the distal and proximal regions of the ear, at slightly different stages of development, with those at the distal end apparently more mature, but those at the distal end seem, by means of increased tapetum degradation, to be the more damaged. This is in keeping with the regional grain set results. One factor to consider, in relation to the greater sensitivity of the upper third of the ear, is that spikelets found in this region of stressed plants (both varieties) where, unlike the other thirds, often in both size and shape, so malformed/shrunken, at maturity, that it would have been inconceivable that they could have possessed grains. With there being no significant difference between the temperature depression (TD) between the thirds of wheat ears, at anthesis, when under stress (Steinmeyer *et al*., 2013), TD may not be an explanation for this phenomenon. However, whether disproportional inter-third TDs are present at early booting has yet to be established.

In relation to the different grain set dynamics reported here, and the possibility of other underlying factors, other than pollen damage affecting these, further work on this area may be needed. This further work may also provide further clarity into such questions as '*why, across all three tillers, is Cordiale, when compared to KWS Sterling, most sensitive to early booting heat stress in the upper third of ears and middle florets of the spikelets?*' One possible, if only partial, answer to this question may be due to the relative immaturity of KWS Sterling's ears at the start of booting.

If building upon the findings of chapter 2, the observation that a greater level of percentage increase in main tiller post-floret 'e' florets is seen in KWS Sterling, compared to Cordiale, (38 and 23% respectively), may give an indication that the more immature the ear when stressed the greater its ability to relocate its resources into the production of extra florets within spikelets, however, limited these florets' yield may be. When relating this hypothesis to the secondary tillers however, it is apparent that Cordiale has a greater increase (33%) in post-floret 'e' floret number, due to heat stress, that KWS Sterling (21%), whose secondary ears would be presumably less mature. This therefore calls the validity of this hypothesis into question.

In relation of the size of grains in post 'e' positions, it is worth noting that, in addition to the limited size of the grains found in these positions, due to the limited size of the florets in which they were found (Millet, 1986), these grains were often also small, due to a perceived lack of maturity. This was perhaps due to their filling being aborted, at a relatively early stage, or the grains being shrunken (see Tashiro & Wardlaw, 1990).

These post 'e' grains were, on a few occasions, so small that there was a question whether they were in fact the result of fertilization, or merely the remnant of an unfertilised gynoecium, or the result of parthenocarpy (see Tashiro & Wardlaw, 1990). However, due to the limited occurrence of such small 'grains', it is unlikely that a misidentification distorted results. However, this phenomenon, along with the aborted and shrunken grains, may warrant assessment in further similar studies, not least due to them also occurring in more proximal florets in spikelets (position 'e' and below) as well. Does this suggest that grain quality can be affected, even when the heat stress in implemented before fertilization takes place?

3.5.4 Effect of heat stress on mature average spikelet weight

Yield, to the grower, is not merely grain number, even though this is the primary constituent factor (Dolferus *et al*., 2011), but also grain weight. The data presented indicates that, like grain number per spikelet, Cordiale is significantly more sensitive than KWS Sterling to early booting heat stress, in relation to average spikelet weight, when such spikelets are filled with mature grains. Even though the resulting percentage point differences between decline in grain number per spikelet and average spikelet weight are marked in Cordiale, and unlike grain number, the controls of tiller 2+3 are statistically different, results show that there is a strong correlation between grain number and ear weight. This raises the possibility that weighing ears, instead of threshing them and counting the resulting grains, can thus lead to a more rapid means of assessing the effect of stress on yield. Agar *et al.* (2015) also present a strong correction between grain number per wheat ear and grain weight per wheat ear. This correlation was also with both control and heat stressed plants included in the analysis.

In seeing that Cordiale's average, filled, spikelet weight was less dramatically affected by heat stress than its seed number per spikelet, this may suggest that there is a compensation for the loss of seed number by an increase in the size of the relatively limited number of grains remaining.

3.5.5 Potential for non-invasive assessment of pollen staging

Considering the time, skill, materials and money needed to produce those staging images seen within Figures 3.8 & 3.9, any potential of establishing a non-invasive framework for assessing anther staging would be beneficial. In this regard, results show that by assessing the dry weight of the ears and/or average spikelets, there may be, in the future, the possibility of quickly and accurately assessing anther stage, and therefore vulnerability to heat stress. Even though this would need further investigation, the possibility of finding a critical weight for vulnerable stages of pollen development (e.g. PMC meiosis) would aid with the quick and efficient screening of genotypes.

3.6 Conclusions

From the outset, the analysis of varying genetic backgrounds' responses to early booting heat stress was a key objective of this chapter. Therefore to acknowledge that KWS Sterling and Cordiale have similar genetic backgrounds (Appendix 5), with Cordiale being one of the parents of KWS Sterling, is of interest. Therefore, the key question of '*what characteristics, palynological or otherwise, does KWS Sterling's other parental lineage possess?*' may need answering in the future.

As highlighted, from the results of this chapter, the differences between, and identification of, germplasm able to resist a heat stress event, verses germplasm likely to avoid a stress event, is key to identifying truly resistant varieties. Avoidance, even though possibly more easily achieved by means of breeding (Semenov *et al*., 2014), is of limited merit due to the unpredictability of short, transient, stress events (Dolferus *et al.*, 2011). A variety able to avoid one season may be the most susceptible in subsequent seasons. Like the methodologies of this chapter, the collection of anthers, in addition to the assessments of grain set, is the primary way of distinguishing temporal avoidance and true resilience, and will facilitate the avoidance of the current trend towards breeding for escape, rather than tolerance (Semenov *et al*., 2014). However, the ear/average spikelet dry weight analysis conducted, in league with the strong correlations with sensitivity to heat stress, may provide more immediate insights in the future.

In the search for truly resistant wheat germplasm, it would be remiss to exclude related species (e.g. Rye) and land races, and only focus on commercial varieties. Whilst acknowledging their limitations, in relation to yield and grain quality, land races appear to have better abiotic stress tolerance than commercial lines (Dolferus *et al*., 2011). However, this should not lead to the complete disregarding of cultivated lines as, despite not dealing directly with heat stress tolerance, but rather the putatively associated drought tolerance, both Oliver *et al.* (2005) and Ji *et al.* (2010) demonstrate that there are cultivated varieties able to tolerate stress directed towards the initial stages of pollen development, for rice and wheat, respectively. Both Oliver *et al.* (2005) & Ji *et al.* (2010), conclude that their respective varieties of tolerance, to early microspore drought stress, are due to their ability to control and maintain sink strength and carbohydrate supply to anthers. Where Firon *et al.* (2006) also conclude that the ability of tomato anthers to maintain appropriate levels of carbohydrate content, during, or after heat stress, during pollen development, may be a key factor in

imparting tolerance, Matsui *et al.* (2001) associate temperature tolerance in rice to increased locule thickness.

Despite not providing data adequate to infer the palynological qualities of 'sensitive' and 'resilient' to heat stress, the results of this chapter show, based on two years of data, that when inflicted at the same gross developmental stage, one variety is significantly more resilient than another, an indication of avoidance rather than resistance. However, perhaps the most meaningful finding, in relation to eventual resilient germplasm screening, is that gross plant development, as per the Zadoks scale, is not a good indicator of pollen stage. This greatly reduces the possibility of totally non-invasive, inter-varietal screening.

Chapter 4

Examining the Effect of Inter-ear Pollen Movement on Yield after Early Booting Heat Stress

4.1 Introduction

4.1.1 The extent of cross pollination/fertilization in wheat

Modern wheat varieties are predominantly self-fertilizing, with Tsunewaki (1969) (cited in Komaki & Tsunewaki, 1981) estimating that outcrossing can be as low as 0.24%. However, many of the early ancestors of these varieties probably had/have the tendency to cross-fertilise, via wind, more easily (De Vries, 1971), with there even being a possibility of pollination via thrips (Cook, 1913). However, due to its cultivation primarily outside its area of origin, over time there may have been the tendency towards the isolation of self-pollinating/fertilizing forms of wheat (De Vries, 1971). A greater incidence of reproduction via functional cleistogamy, a breeding system characterised by in-flower self-pollination, may also be largely due to closed flowering wheat having a greater physiologically derived resistance to some diseases of the ear (Gilsinger *et al.*, 2005). Cleistogamy, in one form or another, has been documented in 693 angiosperm species, distributed over 228 genera and 50 families, having been thought to have evolved approximately 34-41 times, and being found in higher proportions in families such as the grass family, Poaceae (Culley & Klooster, 2007).

Intra-floret pollination would avoid the effects of variables such as wind direction, wind velocity and inter-floral synchronicity on yield, as changes in one or more of these variables may affect pollination levels in inter-floret pollination. However, the ability to cross-fertilise, even as a 'backup' strategy, may be desirable in light of the effects pollen damage can have on wheat yields, in the scenario where a floret has been rendered male sterile from the effect of stress, but the more resilient female anatomy remains relatively un-affected (Saini & Aspinall, 1982). Additionally, the ability to cross-fertilise will enable the generation of greater genetic diversity.

Therefore, a highly relevant question is, '*would inter-ear pollen movement mitigate, even to a*

small extent, the yield loss associated with heat stress during booting?' This question is especially relevant, since the greatest intra-spikelet/ear yield losses, due to early booting heat stress, occur in the more proximal florets of spikelets and the upper third of the ear (Saini & Aspinall, 1982). The increased vulnerability of these regions is coincident with these regions also being more able to receive pollen from external sources (Lukac *et al.*, 2012; Rajki, 1960; De Vries, 1971).

The current literature regarding the reproductive dynamics of wheat brings to light a number of other wheat floral/palynalogical features of interest in relation to the cross-pollination/fertilisation potential, after periods of early booting heat stress.

4.1.2 Amount of wheat pollen production

With the amount of pollen produced by an anther potentially positively correlated with the probability of cross-fertilisation, it is of interest to note that the wheat variety has a significant effect on the number of pollen grains per anther (Khan, 1968; Beri & Anand 1971). However, this is dependent on the number of anthers possessed by an ear, and the proportion of their pollen they release into the aerial environment.

There is a positive correlation between wheat anther dimensions and number of pollen grains per anther (Beri & Anand, 1971; De Vries, 1971), as well as a considerable relationship between pollen grains per anther and pollen donor capacity to the aerial environment (De Vries, 1974_a). The latter of these findings is unsurprisingly contradicted by Beri & Anand (1971), not least because there is no evident relationship between the number of extruding anthers and amount of pollen released into the aerial environment (De Vries, 1974_a). Between varieties, anther extrusion can vary greatly, with Joppa *et al.* (1968) observing a spring wheat with 72%, and a durum wheat with 22% of their anthers extruding.

4.1.3 Wheat pollen's post-dehiscence viability, and dispersal

The pollen of wheat, like most grasses, has a comparatively greater level of vulnerability to the negative effects of post-dehiscence desiccation, when compared to the pollen of other groups of plants (Barnabás & Kovács, 1997). Wheat pollen viability is quickly reduced by dehydration under atmospheric, and even under conditions of heightened relative humidity (Heslop-Harrison & Heslop-Harrison, 1992), thus supporting the understanding that the pollen of self-fertilizing cereals appear to have a short period of post-dehiscent viability (Dolferus *et al.,* 2011). However, to some extent, this rapid decrease in post-dehiscence viability may be

somewhat offset by wheat pollen having an initial high percentage viability (Hucl, 1996).

Obermayer (1916) (cited in De Vries, 1971), considered it probable that the pollen of later flowering wheat florets have less viability than those at the peak of flowering. This therefore means that, if in the presence of floral synchronicity between stressed and control plants, the proximal florets of spikelets, which suffer most from grain set loss, due to early booting heat stress, may receive pollen from highly viable florets, found in the proximal regions of other, un-stressed, ears' spikelets.

In addition to the high level of post-dehiscence viability loss, the proportion of anthers' pollen content released may contribute to the amounts of pollen reaching the stigmas of other ears (De Vries, 1971). This variation in proportional pollen release, either as an effect of the variety or the environment, may be due to differences in the process of anther extrusion from florets, as outlined by Peterson (1965). In this process, Peterson suggests that flowering begins with the lemma and palea moving apart, and the filaments extending to such an extent that the anthers are pushed outside the floret. With filaments lengthening, anthers begin to split open with a proportion of the pollen falling inside the floret, before the anther is extruded from the floret and releasing the remainder into the aerial environment (Peterson, 1965). How much does the variety/environment influence when in the process of filament elongation the anther begins to dehisce, and therefore determine the proportion of pollen deposited inside a floret?

Wheat has comparatively high pollen weight, due to its high ploidy level (De Vries, 1971). This high weight therefore suggests that the distance pollen, shed from the anther, may be able to travel, under the normal air movement dynamics within a field, is limited. Subsequently, a greater concentration of pollen can be found below ear level than at, or above, ear level (De Vries, 1972).

The grain set of male sterile wheat plants could, dependent on wind direction, become less than 5% of typical, un-sterile plants, even when less than 1m from a pollen source (De Vries, 1974_b). The relatively high weight of wheat pollen has been one reason why, in hybrid wheat production, selection has been for male parents to be taller than the male sterile plants that would be receiving the pollen to their ovules (De Vries, 1972; Beri & Anand, 1971).

Since, as shown within chapter 3, tillers at differing stages of their development have differing levels of yield loss, when exposed to a period of heat stress, if pollen can move the short distance between less male sterile, and more male sterile ears, on the same plant, which

would be a matter of centimetres, the inter-plant movement of pollen movement, and the greater distances associated, may not be needed.

4.1.4 Wheat floral opening and stigma receptiveness

Despite the fact that up to 86% of all wheat pollen is eventually dispersed into the general aerial environment (Anon, 1999b cited in Eastham & Sweet, 2002), inter-plant cross-pollination (cross-fertilization), under field conditions, can involve less than 2% of all florets (Wiese, 1987). Even though this percentage varies, due to both genotype and environment, it tends to remain relatively low (De Vries, 1974_b). Even with optimal wind direction, and a distance of only 25cm from a pollen source, a male sterile wheat plant only has, on average, 36.2% of the eventual grain set of a self-fertile plant (De Vries, 1974_b). This would therefore suggest that somewhere between pollen release into the aerial environment, and the recipient stigma, there is one or more factors limiting fertilization.

Therefore, in acknowledging that pollen dispersal dynamics, and post dehiscence viability, are only one side of a two sided cross-fertilisation 'equation', the stigma receptiveness and the dynamics of the opening of florets must also be considered.

In addition to climatic conditions having an influence on the length, and extent, of floral opening (De Vries, 1974_b), a key factor in pollination, due to it allowing atmospheric pollen to come in contact with the enclosed stigma, genotype has a considerable effect as well. For example, *T. durum* has a greater amount of floral opening than *T. aestivum* (Rajki, 1960). The finding that *T. durum* has a greater tendency towards chasmogamy (open flowering) is unsurprising, due it being less evolutionarily derived that *T. aestivum* (De Vries, 1971; Cook 1913). In contrast to many factors standing in the way of cross-fertilization after stress, one potential physiological feature that may count in its favour is that, when lacking the ability to self-pollinate/fertilise, cytoplasmic male sterile (CMS) plants keep their florets open for longer (De Vries, 1974_b).

In league with the temporal and spatial extent of floret opening, the relative position of the stigma, within a floret, must also be considered. Despite a few exceptions, especially in the later stages of floral maturation (De Vries, 1971), the two stigma lobes of wheat flowers never protrude from beyond the lemma and palea, thus limiting the possibilities for cross-fertilization.

As well as a high stigmatic surface, the length of time a stigma remains receptive to pollen

from an external source is very important, as it maximises the chances of cross-fertilisation (De Vries, 1971), especially since the male and female components, contained on different ears, may not be exactly temporally overlapping (Lukac *et al.*, 2012). Climatic conditions have an effect on the length of flowering processes, including the length of stigmatic receptiveness, with dry and warm weather shortening the process (De Vries, 1971; Cerović *et al.*, 2000).

4.2 Objectives

1) To assess whether an un-stressed ear depends upon pollen from alternative sources to contribute to its grain set and, if so, assess in which spatial region of the ear this occurs.
2) To assess whether the movement of viable pollen can restore the grain set loss associated with early booting heat stress, and, if so, in which spatial regions of the ear is this restoration present.

4.3 Methods

4.3.1 Pot experiment

4.3.1.1 Plant growth and treatment implementation

Pots (12.5cm) were filled with 'PEL mix' (see section 2.3.1.1).

260 pots were sown with 780 untreated seeds (3 per pot) of the spring wheat variety, Paragon, (Plant Breeding International Cambridge Ltd). All pots were placed in an unheated polytunnel at the Plant Environment Laboratory, University of Reading, UK (51.413349, -0.93749225).

When these plants reached approximately 23ZS, 56 pots were moved to onsite controlled environmental facilities. These facilities were in the form of three growth cabinets at 20±1°C (day & night), 16 h photoperiod and an average irradiance, at booting canopy level, of 700 µmol/m^2/sec, fluorescent and incandescent illumination. A control growth temperature of 20°C was chosen, based upon Bennett *et al.* (1973). Approximately a third of each, of the four eventual cohorts, were represented within each cabinet.

Within 24 hours of being moved from the polytunnel to the 20°C growth cabinets, the plants were thinned down to one plant per pot, and three tillers per plant. Tiller thinning was repeated approximately every 10 days throughout the rest of the growing season.

When half the plants reached the start of booting (39ZS), they were placed in one of two 'heat stress' cabinets at 35±1°C (day & night) for three days (72 hours), with otherwise identical climatic conditions, before being returned to their previous position in the 20°C environment. The other half of the plants remained within the control cabinets during this time. The inter-cabinet movement dynamics during these stressing events is represented within 'Appendix 6'.

During the high temperature treatments (35°C) and control temperature (20°C) the atmospheric relative humidity was kept as high as possible (normally above 40% (v/v)). In addition, through regular physical examination, the water content of the medium was kept relatively stable, at approximately field capacity.

At full ear emergence (59ZS), half of those plants that were stressed, and half of those that remained within the control cabinets, during the first three days of booting, had 55mm x 190mm cellophane crossing bags (Focus Packaging & Design LTD) placed, and sealed, over their ears, in order to prevent inter-ear cross pollination/fertilization. During anthesis, pollen

movement depended on the movement of air (1.8Km/hr) provided from the inbuilt ventilation system of the growth cabinets. This air originated from below the plants and departed from the top of the growth cabinets.

Throughout anthesis, informal observational notes, in relation to flowering dynamics, were taken to supplement the analysis of grain set results. Such observations included levels of anther extrusion, anther maturity when extruded, stigmatal maturity and apparent stigmatal health.

4.3.1.2 Ear collection and analysis

After anthesis, the experimental plants were left to reach maturity within the control cabinets. After grain development was complete, the ears were collected, individually stored in labelled paper bags, and dried at room temperature.

After weighting the ears, grain presence/absence was recorded for each of the first three ears' florets. Ear third allocations were in line with 'Appendix 2' and floret labelling followed the scheme of Lukac *et al.* (2012), with the first floret from the lower glume labelled as 'a', and subsequent florets labelled sequentially.

Whole ear, along with regional grain set levels and weights, were analysed via ANOVA. For means of statistical analysis, regional grain set percentages were transformed to empirical logit. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, *p*-values were drawn from permutation tests (4999 random permutations). Maximum least significant differences (LSDs) were used in order to most conservatively determine significance. Discrete data (e.g. spikelet number) was analysed via a Generalised Linear Mixed Model, using a Poisson distribution. All analysis was done using GenStat 16. Differences with p=<0.05 were deemed 'significant'. Models can be seen within 'Appendix 11'.

In order for the accurate analysis of regional grain set levels and weights, those spikelets not possessing at least five florets, most often those at the base and top of an ear, were standardised. This standardisation took the form of 'topping-up' those spikelets with less than 5 florets, with supplementary, non-seed bearing florets. This prevented a loss in floret presence, due to heat stress, distorting the percentages, and instead gave better appreciation for regional sensitivity.

4.3.2 Field experiment

4.3.2.1 Experiment 1

4.3.2.1.1 Plant growth and treatment implementation

Within a pre-existing LIBERATION study (http://www.fp7liberation.eu) being conducted at Sonning Farm, University of Reading, UK (51.472935, -0.90414518), 40 ears from principal stems, 10 ears per block, of both the winter wheats Santiago and Scout (Appendix 10), had 55mm x 190mm cellophane crossing bags (Focus Packaging & Design LTD) placed, and sealed, over them, directly before anthesis. This was done in order to prevent inter-ear cross pollination/fertilization.

After anthesis was complete, the crossing bags were removed, and ears labelled.

4.3.2.1.2 Ear collection and analysis

After grain development was complete, the labelled ears, along with 40 control ears, 10 ears per block, from each variety, were collected and stored in labelled paper bags. These samples were dried in an 80^{o}C oven for 48 hours, split into thirds, in line with 'Appendix 2', and threshed. Grain weight and number were recorded.

Whole ear, along with regional grain set levels and weights, were analysed via ANOVA. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, *p*-values were drawn from permutation tests (4999 random permutations). Maximum least significant differences (LSDs) were used in order to most conservatively determine significance. Discrete data (e.g. spikelet numbers) was analysed via a Generalised Linear Mixed Model, using a Poisson distribution. All analysis was done using GenStat 16. Differences with p=<0.05 were deemed 'significant'. Since there was no significant effect of the cabinet (Appendix 6), each pot/plant was treated as the unit of replication. Models can be seen within 'Appendix 12'.

4.3.2.2 Experiment 2

When at full ear emergence (59ZS), pollination bags were placed upon the ears of a group of pot grown winter wheat plants (var. Savannah) located at the Plant Environment Laboratory, University of Reading, UK (51.413349, -0.93749225). These plants were grown outside in a bird proof cage and in identical growth media to that outline within 'Experiment 1' of the potted experiment of this chapter.

At the same time the pollination bags were added, five thermocouples were added within five

separate bags, thus logging the temperature in the area between the inside surface of the bag and the outside surface of the ears. To complement these, six other thermocouples were added to a similar position outside the ears of six stems, but with no pollination bag covering them.

After a 9 day period (3-12 June), over which time temperatures were logged every ten minutes (the average temperature recorded over the last ten minutes), the thermocouples, along with the pollination bags, were removed from the ears.

The average temperature, over a three hour time periods within a day, was, per thermocouple, averaged over the 9 days. Then an average across the thermocouples, within a treatment, for the daily time period, was taken.

Temperature data was analysed via ANOVA. In light of, at times, the non-normal distribution, and/or un-equality of the variance of residuals, that could not be rectified with transformation, *p*-values were drawn from permutation tests (4999 random permutations). Maximum least significant differences (LSDs) were used in order to most conservatively determine significance. Differences with $p=<0.05$ were deemed 'significant'. Models can be seen within 'Appendix 13'.

All analysis was done using GenStat 16.

4.4 Results

4.4.1 Pot experiment

4.4.1.1 Grain number and regional grain set

Temperature treatment had a non-significant effect on the spikelet number of both the main tiller (p=0.194) and second and third tillers (p=0.289). The presence/absence of pollination bags also had a non-significant effect on the spikelet number of the main tiller (p=0.982) and second and third tillers (p=0.501). Across both main (p=0.0.533) and secondary tillers (tiller 2 + 3) (p=0.729), there was a non-significant interaction between temperature treatment and presence/absence of the pollination bag. Therefore, any changes in grain number/weight per spikelet cannot be attributed to differing spikelet numbers.

In this experiment, devised to assess the effect of preventing the possibility of inter-ear pollen movement restoring grain set after early booting heat stress, despite there being, across both tiller sets, a highly significant (p=<0.001) negative effect of temperature treatment on grain set, both in the main (p=0.933) and secondry tillers (p=0.616), the pollination bag did not have a significant effect on grain set. Likewise, there was no significant interaction between the temperature treatment and the pollination bag for either the main (p=0.333), or secondary tillers (p=0.614) (Figure 4.1).

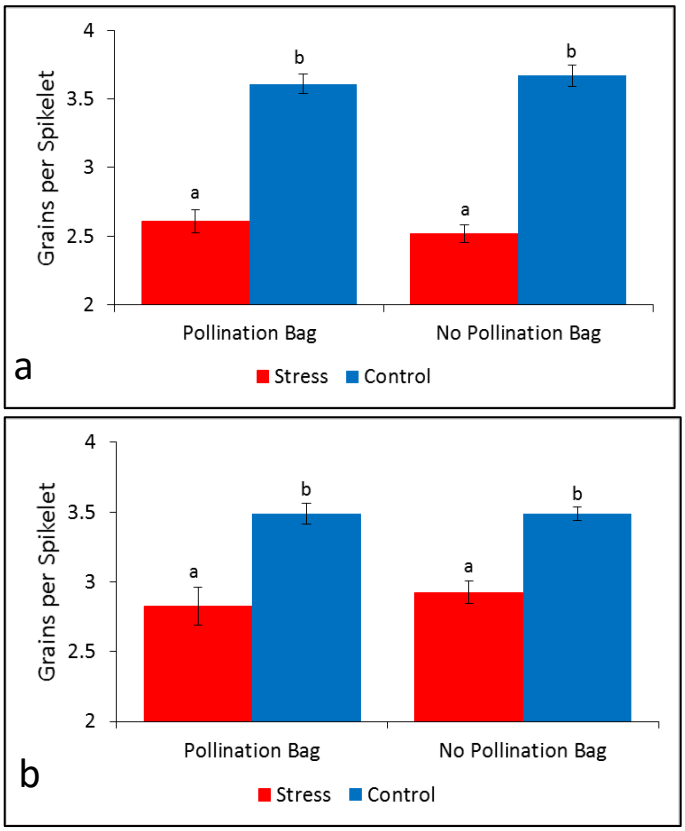


Figure 4.1: The effect of early booting heat stress (35/35°C; 3 days), and the addition of pollination bags, on grain number per spikelet. (a) Main tiller, (b) tiller 2 & 3. Error bars indicate standard error. Identical letters indicate non-significant (p=>0.05) difference. n= 10-14, df= 47.

At the regional level (Figure 4.2 & 4.3), the presence of a pollination bag consistently did not have a significant effect on grain set. Differing temperature treatments, on the first three days of booting, with the exception of floret 'd' of both the main (p=0.107) and secondary tillers (p=0.444) and the lower third of the secondary tiller (p=0.091), had a significant effect on grain set. Additionally, with the exception of the upper third (p=0.013), and floret 'b' (p=0.020) of the main tiller, there was no significant interaction between the pollination bag and temperature treatment. However, instead of such limited significant interactions indicating a regional yield restoration, due to a lack of a pollination bag, and hence enabling the ability of stressed ears to receive pollen from an external source, it appears that the presence of the pollination bag actually had a positive effect on grain set in these two regions.

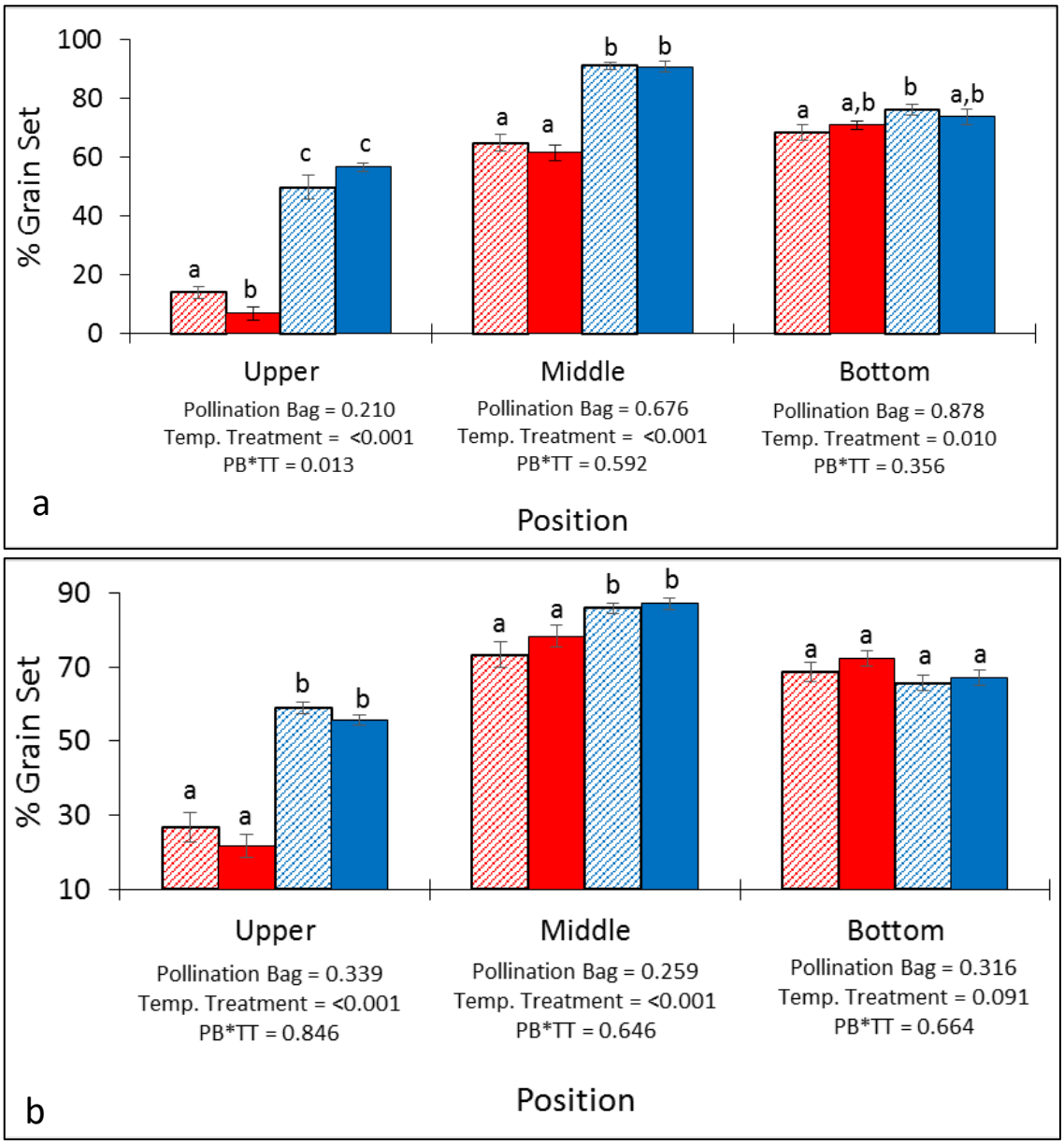


Figure 4.2: Effect of early booting heat stress (35/35°C; 3 days), and the addition of pollination bags, on grain set in different regions of the ear. (a) Main tiller, (b) tiller 2 & 3. Blue = control plants, red = stressed plants, solid colour = no pollination bags, dashed = pollination bags. Error bars indicate standard error. Identical letters indicate, intra-regional, non-significant (p=>0.05) difference. n = 10-14, df = 47. Note: only considering the first five florets of each spikelet.

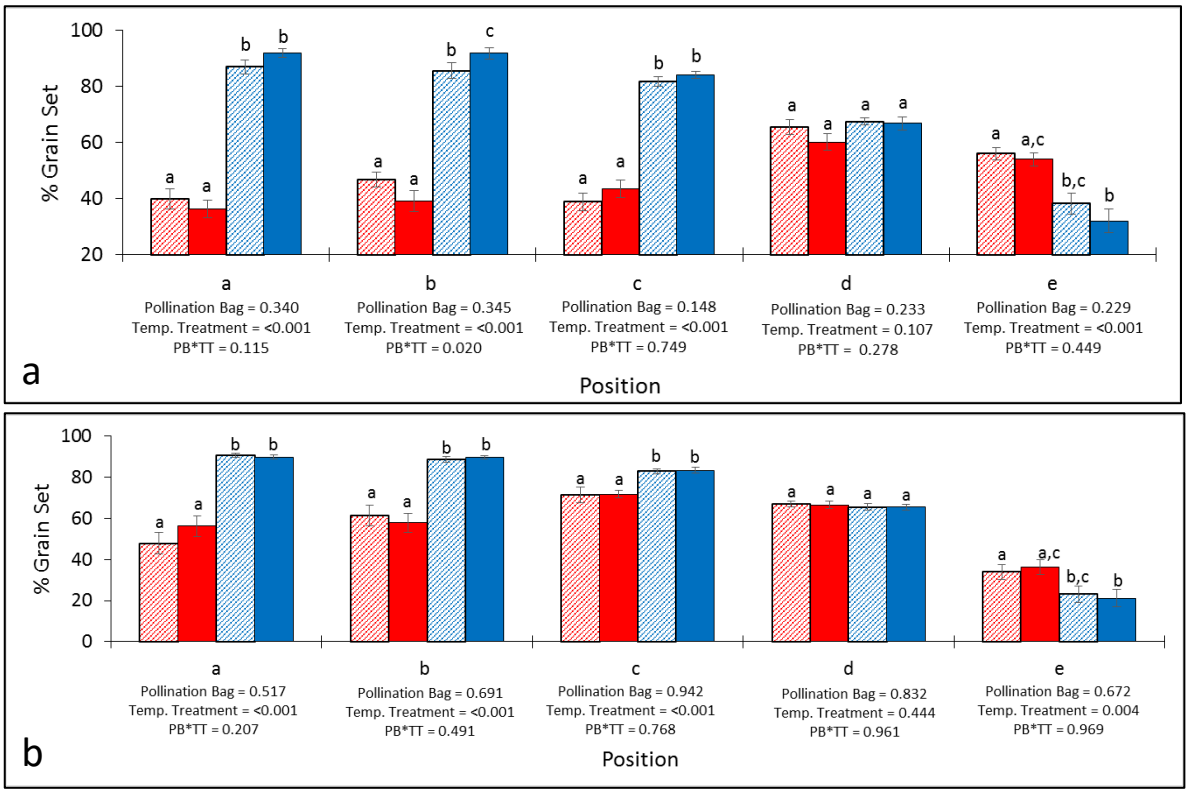


Figure 4.3: Effect of early booting heat stress (35/35°C; 3 days), and the addition of pollination bags, on intra-spikelet grain set. (a) Main tiller, (b) tiller 2 & 3. Blue = control plants, red = stressed plants, solid colour = no pollination bags, dashed = pollination bags. Error bars indicate standard error. Identical letters indicate, intra-floret, non-significant (p=>0.05) difference. n = 10-14, *df* = 47. Note: 'a' is the proximal most floret within a spikelet. Subsequent florets are labelled sequentially.

Figure 4.4 shows that, across all the ears, the distal third of ears and proximal florets of spikelets are the most negatively affected by early booting heat stress.

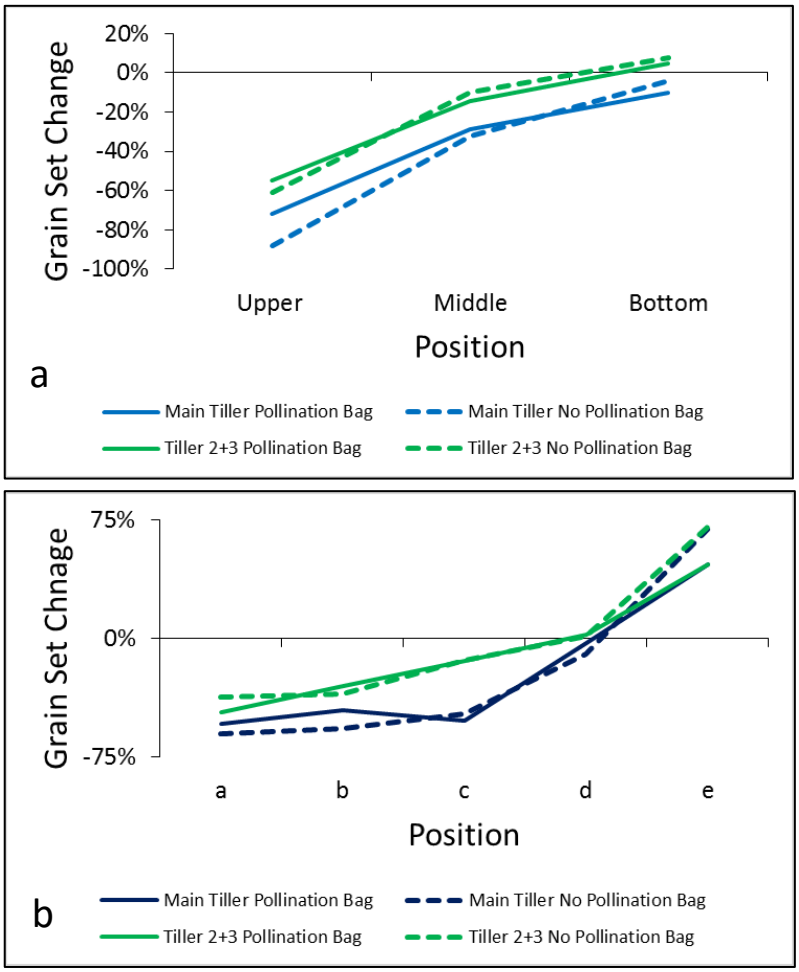


Figure 4.4: Percentage grain set change, due to early booting heat stress (35/35°C; 3 days), in the (a) thirds of the ear, and (b) florets of the spikelet.

4.4.1.2 Anther dehiscence

In light of the lack of yield restoration associated with the absence of pollination bags during anthesis, Figure 4.5 shows images from un-stressed anthers at two different time points before they were extruded from their respective florets. These show that, at approximately day 10 (post 39ZS), anthers in the central part of the ear, which were approximately 3mm long, started to dehisce. Even though not dehisced as yet, a great deal of secondary thickening can be seen within the endothecium of Figure 4.5a. Figure 4.5b shows an anther region devoid of pollen after dehiscence. However, often the distal and middle regions of an anther, can be devoid of pollen, whilst the proximal regions still contains a limited amount, with dehiscence often not happening along the length of the anther all at once, but instead progressively.

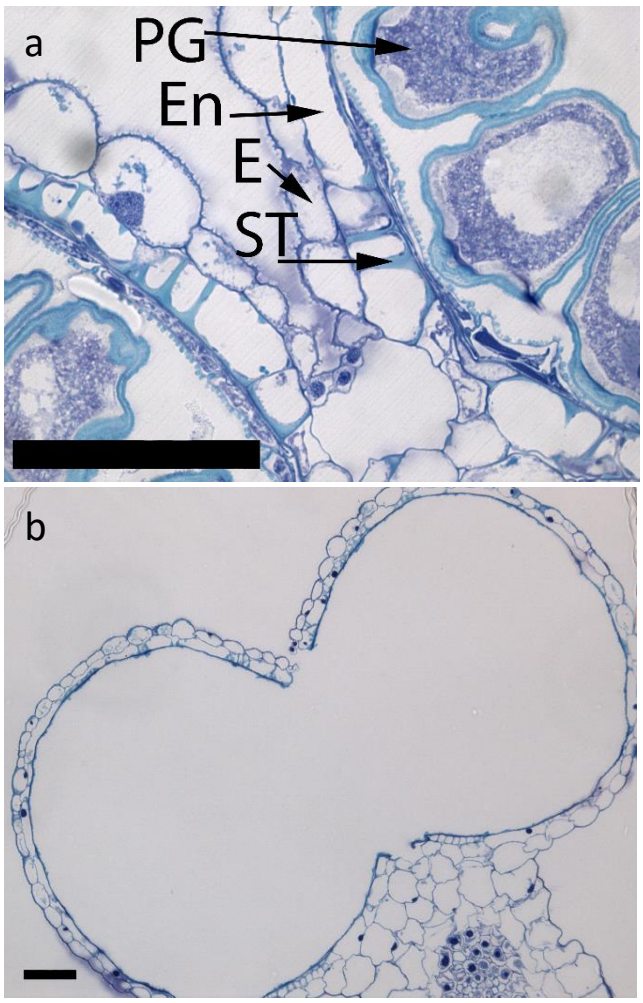


Figure 4.5: Transverse sections from the anthers of un-stressed (20/20°C) main tiller's central florets. These anthers are yet to be extruded from their florets. (a) 9 days post-39ZS (x100), (b) 11 days post-39ZS (x20). E=epidermis, En= endothecium, PG= pollen grain, ST= secondary thickening. Scale bars 50µm.

4.4.1.3 Spikelet weight

Unlike grain number, where there was no significant effect, in relation to ear weight (Figure 4.6), the addition of a pollination bag had a significant effect on both the main (p=0.020) and secondary (p=0.005) tillers. However, like grain number, the temperature treatment had a significantly negative effect on both the main (p=<0.001) and secondary (p=0.002) tillers. Where there was a significant interaction between the pollination bag and the temperature treatment for the main tiller (p=0.023), there was not one for the secondary tillers (p=0.542).

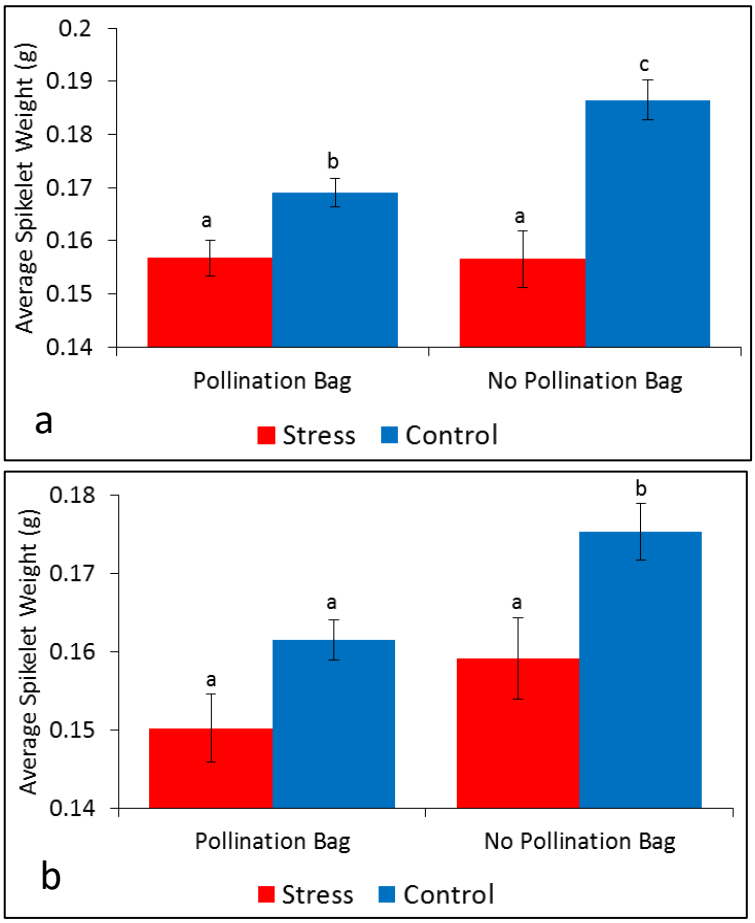


Figure 4.6: The effect of early booting heat stress (35/35°C; 3 days), and the addition of pollination bags, on average spikelet weight. (a) Main tiller, (b) tiller 2 & 3. Error bars indicate standard error. Identical letters indicate non-significant (p=>0.05) difference. n = 10-14, df= 47.

4.4.2 Field experiment

4.3.2.1 Experiment 1

Despite the variety having a highly significant effect (p=<0.001) on spikelet number, with scout having more spikelets, the addition of pollination bags did not have a significant effect (p=0.771) on spikelet number. Additionally, there was no significant interaction (p=0.908) between pollination bag and variety. Therefore, any changes in grain number/weight per spikelet, within a variety, cannot be attributed to a differing spikelet number.

In this experiment, devised to assess the effect of the pollination bag, it is worth noting that in relation to both the number of grains possessed by spikelets (p=0.023) and weight of average spikelets (p=0.025), the additon of a pollination bag during anthesis had a significantly negative effect (Figure 4.7a,b). However, the additon of a pollination bag did not significantly (p=0.323) decrease the average grain weight (Figure 4.7c). Unlike average spiklet weight

(p=0.816), the variety had a significant effect on both the number of grains per spiklet (p=0.027) and average grain weight (p=0.009). Across none of the three features represented within Figure 4.7, grains per spiklet (p=0.516), average spikelet weight (p=0.613), nor average grain weight (p=0.829), was there ever a significant interaction between the pollination bag and the variety.

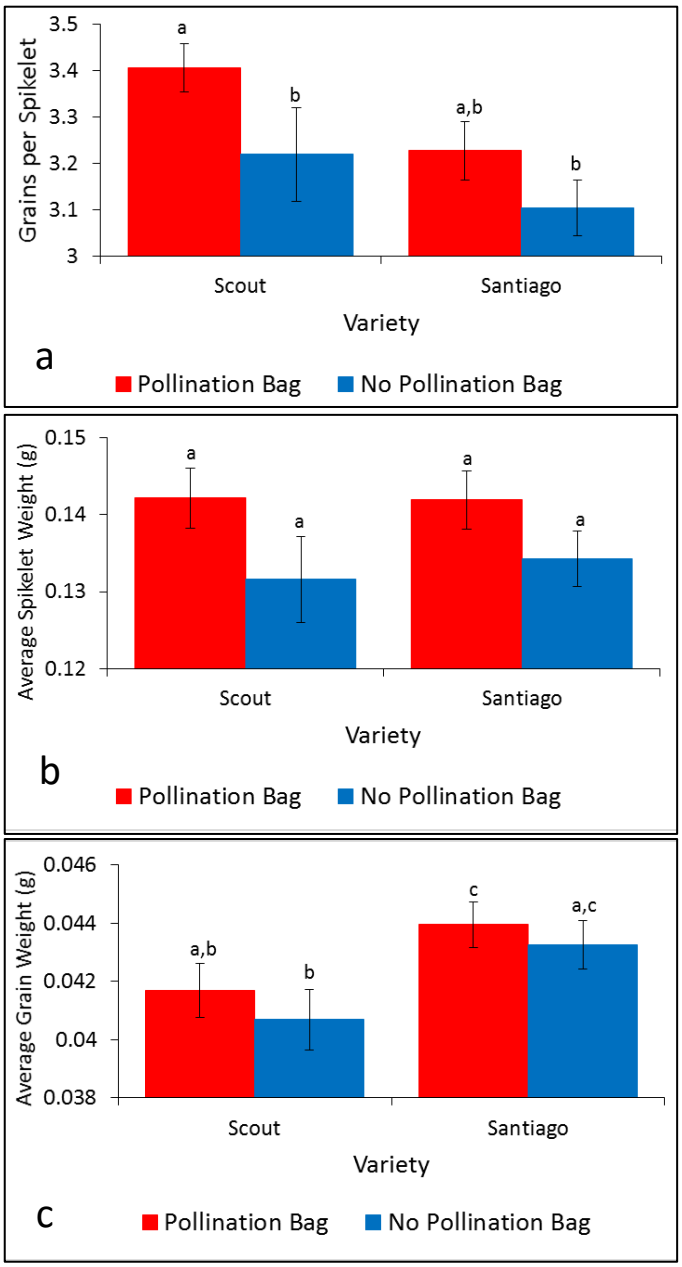


Figure 4.7: The effect of the addition of pollination bags on the (a) grain set per spikelet, (b) average spikelet weight, and (c) average grain weight. Error bars indicate standard error. Identical letters indicate non-significant (p=>0.05) difference. n = 30-36, df = 131.

When separating the ears into three distinct regions, a lack of statistical consistency was apparent, in that neither the pollination bag, nor the variety, consistently showed either a

significant, nor non-significant effect. However, what was consistent, across the nine analyses within Figure 4.8, is that there was no significant interaction between the pollination bag and the variety. Additionally, when applying LSDs, even though normally not significantly different, the presence of a pollination bag consistently had a positive effect on grain number (Figure 4.8a), average spikelet weight (Figure 4.8b) and average grain weight (Figure 4.8c).

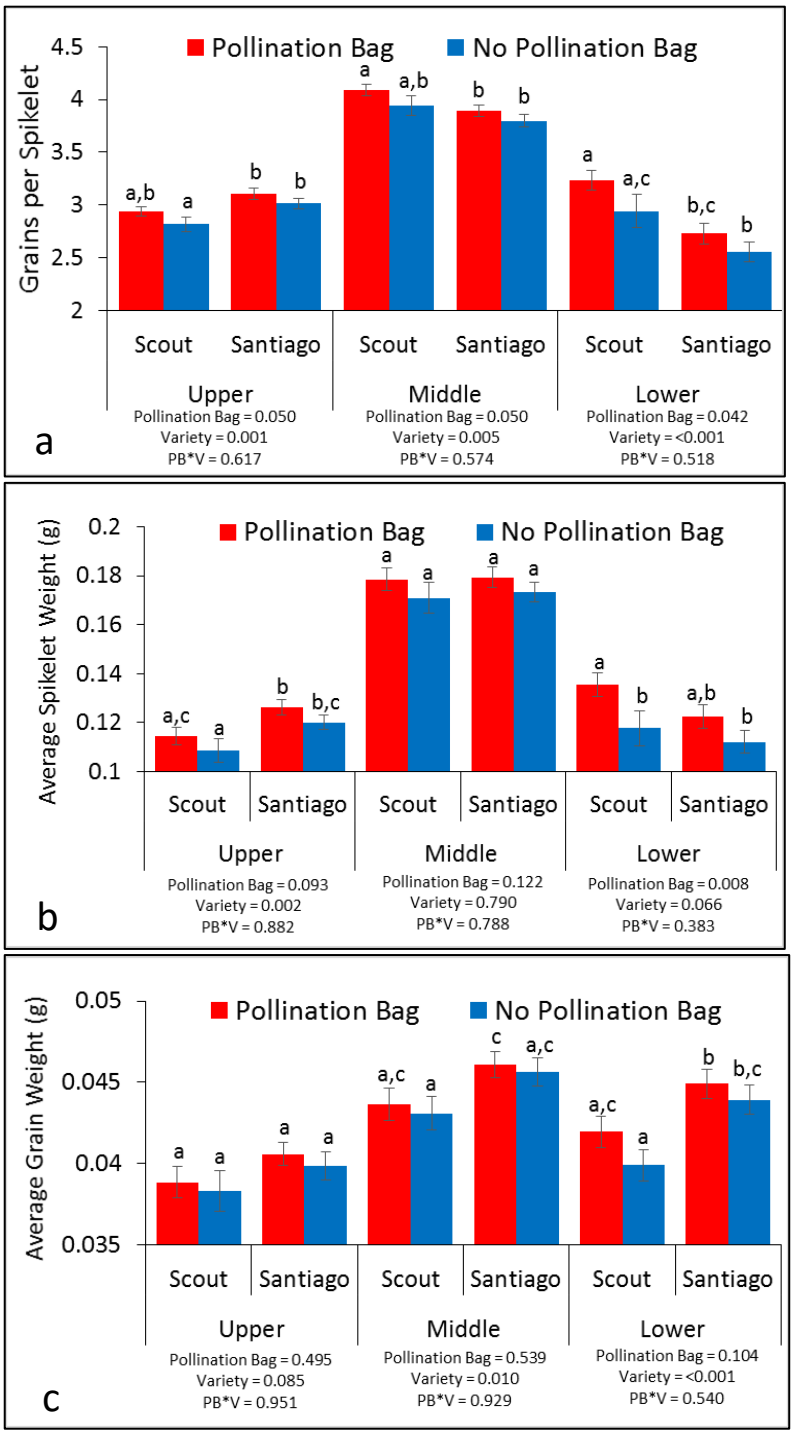


Figure 4.8: The effect of the addition of pollination bags on the (a) regional grain set per spikelet, (b) regional ear weight per spikelet, and (c) regional average grain weight. Error bars indicate standard error. Identical letters indicate, intra-regional, non-significant (p=>0.05) difference. n = 30-36, df = 131.

4.4.2.1 Experiment 2

This experiment was designed to assess the effect that a pollination bag had on the temperature surrounding a wheat ear. As would be expected, not only did the time of day have a highly significant (p=<0.001) effect on the temperature surrounding wheat ears, but the presence of the pollination bag also had a highly significant effect (p=<0.001). There was also a highly significant (p=<0.001) interaction between the two. Whereas, at night (21.00-06.00) the temperature was reduced inside the pollination bags, during daylight hours (06.00-21.00) the temperature increased. This led to the significant interaction between time of day and pollination bag (Figure 4.9).

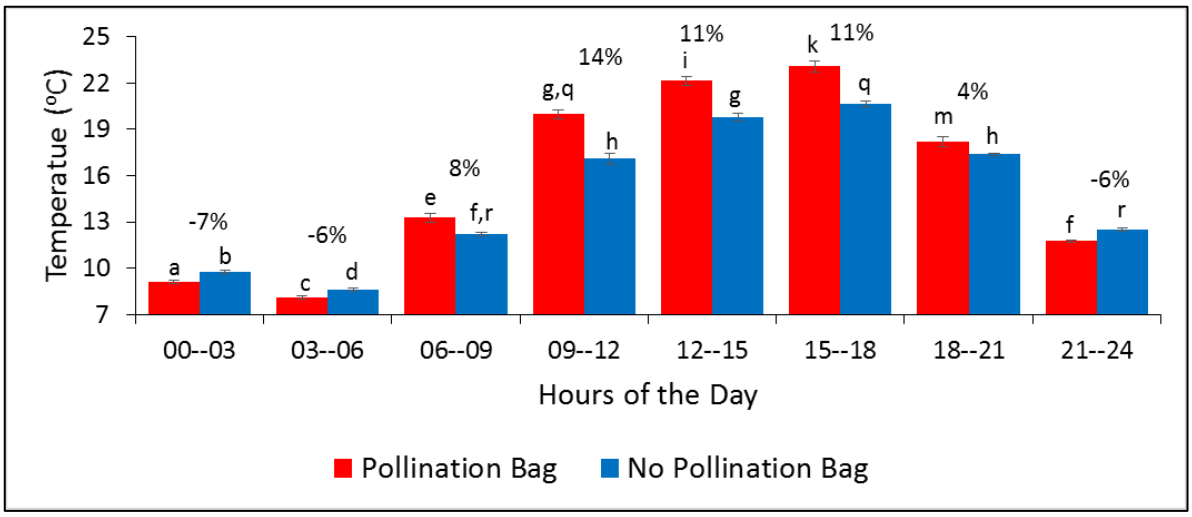


Figure 4.9: The effect of time of day and pollination bag on the atmospheric temperature around a wheat ear. Error bars indicate standard error. Identical letters indicate non-significant (p=>0.05) difference. Percentages show the effect of the pollination bag on the temperature surrounding the ear. n = 5-6, df = 87.

4.5 Discussion

From the pot experiment results it is clear that, despite efforts to encourage the movement of pollen from unstressed ears into stressed ears, there was no significant restoration of yield at any position within the first three ears of this variety (Paragon), within these experimental conditions. This lack of yield restoration was even lacking in areas (proximal florets of spikelets and distal regions of ears) where it might have been, based on previous findings (e.g. Lukac *et al.*, 2012), most expected. In addition, it is apparent that control plants do not need inter-ear cross fertilization to sustain a high grain number, with very few empty florets, where one might otherwise expect to find grains.

4.5.1 Genotype X Environment

There are numerous possible reasons for the finding that the opportunity of cross-pollination does not restore yield in heat stressed plants. However, a large number of these are based around the fact that both genotype, and the surrounding environmental conditions, influence many of the constituent parts of the wheat flowering process, which determine the likelihood of cross-fertilization (De Vries, 1971; Langer *et al.*, 2014). These constituent parts include:

4.5.1.1 Pre-extrusion anther dehiscence

With the use of microscopic assessments, from both transverse sectioned and freshly harvested anthers, it is apparent that the phenomenon of anthers releasing a large amount, even though not all, of their pollen, before they are extruded from florets, is present. This therefore reduces the amount of pollen released into the aerial environment. This is by no means a new finding, with those such as De Vries (1971) documenting results from the early twentieth century, in which some level of pre-extrusion anther dehiscence/pollen release, including total dehiscence/release, were present. This phenomenon is also supported by more recent research (e.g. Whitford *et al.*, 2013).

Considering the results of this pot experiment, whilst not overlooking the effect of genotype, which can even determine the number of anthers which are extruded (De Vries, 1971), the environmental conditions within the growth cabinets in which anthesis took place, may also have played a role in pre-emergence dehiscence. De Vries (1971) supports this conclusion, in that they state that anther filament elongation, which is as rapid as tripling from its original length in about 3 minutes (Peterson, 1965), and positively correlated with the quantity of pollen shed into the aerial environment (Beri & Anand, 1971), may be hindered by unfavourable environmental conditions. If filament elongation were affected, this may mean

that any elongation that does take place may not take the anthers outside the lemma and palea, and hence would not allow pollen to be shed into the aerial environment. Despite not observing a lack of anther extrusion, it may be possible that the particular conditions within these growth cabinets meant that when the anthers were extruded the majority of the pollen had already been shed inside the florets. This hypothesis is based upon the assumption that the processes of anther dehiscence and filament elongation are, at least partially, independent of one another, despite filament elongation and anther dehiscence often being synchronous in wheat (Percival, 1921). However, in contrast to this hypothesis, De Vries (1972), shows that the most pollen appeared to be released, into the aerial environment, at temperatures and relative humidity, somewhat analogous to those found surrounding the experimental plants during anthesis (20^{o}C & relatively high rH).

4.5.1.2 Floral opening

As well as conceivably affecting whether the anthers get an opportunity to protrude from their florets in time to distribute some of their pollen into the external environment, the timing and extent of floret opening will also affect how much external pollen has the opportunity to reach enclosed stigmas.

The lodicule has been thought to be important in the process of floret opening, a process characterised by the separation of the lemma and palea (Peterson, 1965, De Vries, 1971). However, the effect of heat stress on the essential lodicule turgor, derived from water acquired from ovary, is not clear (De Vries, 1971). However, having established that heat stress, during booting, detrimentally affects the ovary, in relation to eventual viability (Saini & Aspinall, 1982), it is conceivable that this movement of water between the two structures may be affected, therefore leading to the very limited floret opening observed within the stressed plants of this experiment. Despite the findings of those such as Bennington & McGraw (1995), Uphof (1938), Obermayer (1916), & Kandaurov & Belkovskaja (1966) (the latter two cited in De Vries, 1971), showing that both heat and drought stress lead to the proportion of cleistogamous flowers being increased, this does not explain the observed lack of floret opening, also within the control plants of this experiment. In seeking further resolution around the area of floret opening, it may be important to distinguish between the amount of florets opening that would allow one or more anthers to be extruded from a floret, and potentially release pollen into the aerial environment, and a sufficient amount of openness, presumably more, to greatly increase the likelihood of external pollen reaching stigmas.

Despite Rajki (1962) (cited in De Vries, 1971), finding a much larger decrease (15.6%) in open flowering of one variety, as a result of dry weather conditions, when compared to another (5.3%), upon the microscopic examination of lodicules from both Paragon, and wheat varieties, it seems, that this much reduced corolla, would have little more than a vestigial presence within florets. Despite its position, somewhat near the axis of the lemma and palea, it is hard to conceive that, even when fully swollen, it would play more than a secondary role in mediating floret opening.

As previously stated, one of the features that would potentially stand in favour of yield restoration via means of viable pollen reaching the stigmas of florets whose own anthers are damaged, would be cytoplasmic male sterility (CMS) plants keeping their florets open for longer (De Vries, 1974_b). However, whether abiotically stressed florets would do the same, due to the reason outlined above, remains to be experimentally quantified.

4.5.2 Proportionality of female damage, and detrimental effects on synchronicity due to early booting heat stress

Despite not assessing the proportionality of stress damage between the females found in differing regions of the ear, it may be the case that the ovules of the proximal florets of spikelets (florets a & b), and the distal third of the ear, were disproportionately affected by early booting heat stress, therefore meaning that their greater likelihood to allow cross-pollination (De Vries, 1971; Lukac *et al*., 2012) may have been somewhat redundant. Knowing that stigma receptiveness is reduced in length due to heat stress (De Vries 1971; Cerović *et al.,* 2000), it may be possible that a disproportional negative effect on stigma receptiveness, in these florets, may be the form this damage took.

As well as the effects that early booting heat stress has on both the male and female components of wheat, the synchronicity between the two, needed for inter-ear cross fertilization, may also be detrimentally affected. Even though this was not assessed during this experimentation, it was noted that, through limited sampling, after both stress and control treatments, stigmas tended to be receptive before the anthers were extruded from the florets. Hedhly *et al.* (2009) suggest that elevated temperatures have a '*complementary effect*' on the temporal progress of both male and female components, with both increasing in rate of development. The primary observations of this experimentation do nothing to contradict this.

4.5.3 Experimental issues

In light of pre-existing knowledge pertaining to cross-pollination/fertilization in wheat, as outlined within the introduction, it is apparent that both genotype and environmental conditions need to be suitable for even a relatively small level of crossing to occur. Therefore, if adding the factor of early booting heat stress, with its associated detrimental effects, into the pre-existing factors regulating inter-ear fertilization, the likelihood of crossing conceivably further diminishes again. However, when reflecting upon the component parts of the primary experimentation within the pot experiment, a number of these were not very conductive with the restoration of any yield. These include:

1) Levels of donor plant availability: high amounts of disease (Inc. *Blumeria* sp., *Fusarium* sp. *Puccinia* sp.) was found on polytunnel grown wheat plants (var. Paragon) that were intended to be added into the growth cabinets. These plants were intended to be added just before anthesis, at a 1:1 ratio to the experimental plants. This would have been done in order to supply additional pollen sources for the uncovered ears of the experimental plants. This failure to add these plants was due to a concern that they may, through pathogen movement, affect the health of the experimental plants involved in this, and other, experiments. The intention had been to remove these donor plants from the cabinets after anthesis.
2) Method of pollen movement: with the planned availability of donor plants, the original intention was to, on a regular basis throughout the day, hold a donor ear, with anthers protruding, close to an uncovered ear and manually try and blow pollen from the donor into the experimental ear. However, even if this would have been implemented, based on the results of Urzay *et al*. (2009), it would appear that such efforts would have been in vain. Gusts of air movement, as planned, would have been ineffective in liberating pollen from the anemophilous anthers of wheat, when compared to sustained air movement.

   Despite there being sustained air movement within the growth cabinets, the direction (coming from the bottom, and moving in an upwards direction) and the velocity, at ear level (1.8Km/hr), were almost certainly not conducive with the ear to ear pollen movement of such comparatively heavy pollen, having instead been designed to reduce disease establishment within the growth cabinets.

3) Extent of stress: when viewed in relation to current, and even future climatic projections (Hansen *et al.*, 2012), 72 hours of uninterrupted heat stress at 35^{o}C, despite being conducive with established principles in other areas of this thesis, were not, in hindsight, conducive with establishing the possibility of yield restoration. Despite Saini & Aspinall (1982) having previously established that the female anatomy of wheat, even though damaged by early booting heat stress, had a higher damage threshold, when compared to the males, it is likely that the temporal length and magnitude of the temperature may have surpassed both the male and female's thresholds. One consequence of this excessive stress is that the upper third of the ear, which had been previously identified as a possible location for yield restoration in stressed ears, often possessed warped spikelets. Such spikelets were in both size and shape, so malformed/shrunken that it would have been inconceivable that they could have ever possessed grains, even in the presence of vast amounts of external pollen.
4) Genotype: Paragon not only produces relatively small anthers, it also, as illustrated by the sectioned anthers, has a tendency to shed a very large proportion of its pollen before being extruded from its respective floret. This, therefore, makes it a poor choice of male for assessing the possibility of yield restoration.

Based upon observations over the course of this experimentation, even if the issues relating to donor plant number (issue 1) and released pollen movement dynamics (issue 2) had not been present, it would still be unlikely that any significant yield restoration would have been possible. This is stated not to diminish the importance of the effective movement of appropriate levels of viable pollen, but to emphasise the importance of the correct genotype, that will first shed vast amounts of pollen into the aerial environment, and also the level of stress, that even though affecting the male reproductive anatomy, is not to such an extent that it will overly effect the female reproductive anatomy. Additionally, since, unlike heat stress, water deficiency does not affect female fertility (Saini & Aspinall, 1981), and the suggestion that heat stress and water stress may cause male sterility through similar mechanisms (Saini *et al*., 1984), in future, substituting heat for water stress, even though potentially harder to control/quantify, may be a better way of quantifying the possibility of cross pollination restoring yield. However, whether water deficiency truly has no effect on female fertility, or whether it was simply within the temporal and/or environmental thresholds presented by Saini & Aspinall (1981), remains unclear.

4.5.4 The effect of pollination bags on grain number & weight

Even though not affecting the core results of the pot experiment, in that it did not have a significant effect on grain number, one factor which may need to be considered in the future is the method of pollen exclusion. In trying to prevent pollen reaching an ear, pollination bags (Focus Packaging & Design LTD) were placed over ears. However, in addition to preventing pollen reaching the ear, these bags also created somewhat of a micro-climate, with temperatures within the bag rising by up to 3^{o}C in an outdoor environment, and preliminary results within growth cabinets showing similar increases. This may have been a contributing factor in significantly different grain numbers in the field, with the increased temperature, within the bags, perhaps being conducive with the creation/establishment of more grains. Unlike where the average grain weight in the field trial was not significantly affected by the addition of a pollination bag, conceivably (deriving from average spikelet weight), in the cabinet grown plants, the average grain weight may have been significantly, detrimentally, effected by the addition of a pollination bag.

Experimental challenges, in relation to preventing inter-ear cross pollination, whilst not affecting grain number, merely due to the detrimental/positive, undesired, effect the pollen exclusion method may have, may need to be overcome. However, upon seeing that there is no significant interaction between the pollination bag and the variety within the field experimentation, this may indicate that, given the correct experimental design (with temperature treatments having representatives both with and without pollination bags), any micro-climatic/structural effect of the pollination bag may be easier to account for.

Whilst the aerial environment around the ear is of interest, further work on the internal temperature of the ear, especially before anther extrusion, would prove of potentially greater importance. In addition, based upon the knowledge that reduced levels of light interception can reduce yields in wheat (Fischer & Stockman, 1980; Demotes-Mainard *et al*., 1995), a more thorough understanding of what, if any, effect this, and other, pollination bags may have on grain set/weight may be another line of enquiry.

4.6 Conclusions

In considering the four factors that may have played a considerable role in ensuring no significant yield restoration after heat stress, It would be conceivable, that under a very specific set of environmental conditions in which floret opening and anther dehiscence were both present, after a relatively short and mild heat stress, inflicted upon a 'super male' genotype which would shed a lot of pollen, and have the needed plant/tiller/floret synchronicity, yield restoration may be possible. However, this is conditional on all the component parts within this 'equation' being present. In relation to any future experimentation, which would be based on these recommendations, instead of undertaking any future work within growth cabinets, future experimentation could be primarily based within glasshouse facilities. This would allow the greater amount of 'super male' plants to be grown (dealing with factors 1 & 4), allow for the introduction of electronic fans to produce continual, medium velocity, correctly directed, air movement during anthesis (dealing with factor 2), and hopefully avoid the yield distorting influence of the pollination bag observed within the field.

With this need for a wide range of factors to be simultaneously present for any yield restoration to even have the possibility of taking place, for a grower to adopt a growing practice dependent upon the movement of viable pollen to restore yield, after episodes of pollen damaging heat stress, may be highly remiss. Instead, the identification of wheat varieties that can withstand, or at least avoid, early booting heat stress may be a more efficient avenue of exploration in achieving yield stability in this primarily self-pollinating plant.

Chapter 5

The effect of heat stress on different anther/pollen related genes

5.1 Introduction

5.1.1. Gene functioning

Since the young Swiss doctor Friedrich Miescher, working at the University of Tübingen in the winter of 1868/9, discovered the existence of Deoxyribonucleic acid (DNA) (Dahm, 2008), the understanding of how DNA indirectly controls the development of cellular organisms has greatly increased. However, over the last decades, the core understanding of the fundamental processes behind gene functioning remained largely unaltered. Firstly, genes (molecular units of heritability consisting of stretches of DNA) are transcribed into mRNA, which is in turn translated into the amino acids that make up proteins (Karakach *et al*., 2010). Proteins are involved in the majority of organismal functions (Malik *et al.*, 2013).

With the genomes of the majority of eukaryotic organisms containing a vast number of genes; cell type, maturity, and environmental factors, determine if, where, when, and to what extent these genes are expressed (Karakach *et al*., 2010; Armstrong, 2014). There are, at times, explicit relationships between gene expression (transcription) and protein translation (Karakach *et al*., 2010). Therefore, knowledge of the mRNA levels, for a particular gene, may provide an indirect route in assessing the functionality of an organism, organ, or tissue, and/or the importance of a gene in the related cellular processes.

With the advent of microarray platforms over the last decade and, more recently, high throughput next generation sequencing, the availability, and speed, of attaining these gene expression data sets is ever increasing, whilst the cost of attaining them is continuing to reduce (Stein, 2010). Alongside these developments, the advances in comparative genomics (e.g. BLAST comparisons) and genome databases are making the identification of orthologues to genes, identified in model crop species, more robust (Fernández Gómez *et al.*, 2015).

5.1.2. The effect of temperature on gene expression

Temperature, like other abiotic factors, can, even when not at a level that would be deemed 'stressful', influence gene expression. For example, Himalayan rabbits possess a gene whose expression is temperature regulated. The gene in question is inactivated at 35^{o}C, and is at its most active at 15-25°C (Lobo, 2008). As this gene is influential in the development of pigmentation in the fur, skin and eyes, the impact of this sensitivity is that the warm, central parts of the rabbit's body, where the gene is inactive, causes the fur colour to be white, whilst at the rabbit's extremities, where the temperature is lower, the gene's activity produces pigments that give the fur a dark colour (Lobo, 2008).

In wheat, the effect that temperature has on gene expression plays a principal role in the classification of *T. aestivum* cultivars into either spring wheat' or 'winter wheat'. In winter wheat, flowering is induced by a period of low temperature, a process known as vernalisation. Seeds of winter wheat are therefore, in temperate regions, sown in late autumn, as the relatively cold winters provide vernalisation. In contrast the transition from vegetative to reproductive development, in spring wheat, cannot be induced by vernalisation (Yan, 2009). Advances in the understanding of vernalisation in wheat has greatly benefited from insights provided by molecular biology, for example the successful cloning of three major vernalisation genes, *VRN-1* (Yan *et al.*, 2003), *VRN-2* (Yan *et al.*, 2004) & *VRN-3* (Yan *et al.*, 2006).

Elevated temperatures can have both a positive and neutral effect on plant wellbeing, including productivity in crop plants. However, for example, after an initial increase in both protein synthesis and shoot growth in sorghum, due to progressively increased temperatures, levels of both drop off very quickly, when a temperature threshold is breached (Howarth & Ougham, 1993).

An example of the negative effect elevated temperatures can have on wheat is in relation to its influence on starch accumulation and, consequently yield. Wheat grains are comprised of 65-75% starch, and at times starch exceeds 80% of endosperm weight (Hurkman *et al*., 2003). Reduced levels of the transcription of genes, associated with starch biosynthesis in wheat grains, have been observed due to heat stress, which can, in turn, lead to reductions of up to 58% in starch content, when stressed during grain development/filling (Hurkman *et al*., 2003).

In light of the detrimental effect heat stress can have on plant wellbeing, and where relevant, productivity, of the 16,000 genes exhibiting plastic expression in response to cold or heat

stress in *Arabidopsis thaliana*, only between one and four percent were associated with significant evidence of adaptive value (Swindell *et al.*, 2007). Adaptive value, to aid in the mitigation of detrimental phenotypic responses, would be desired.

The expression of heat shock proteins (HSPs) in cereal species, produced in response to exposure to stressful conditions, in order to mitigate negative effects, has been documented since the 1980's. Thirteen HSPs were detected in five cereal species, including both common and durum wheat (Necchi *et al.*, 1987). Additionally, it has also been reported that differing levels of acquired thermal tolerance, between two wheat species, were associated with significant differences in the synthesis of numerous HSPs (Krishnan *et al.*, 1989).

Despite numerous studies highlighting physiological assays that reveal inter-varietal variation, in relation to general plant thermotolerance, including increased levels of RUBISCO in some rice varieties (Gesch *et al*., 2003), and prolonged grain filling in wheat (Sayed & Godallah, 1983), relatively little, follow-up, quantitative genetic analysis has been reported. This is largely due to general plant thermotolerance not being a characteristic with a single facet/gene of influence, but instead being a multi-faceted characteristic, controlled by a wide range of genes (Maestri *et al.*, 2002) and therefore, in the past, far more difficult to assess. However, with recent advances in molecular technology, the assessment of such traits is becoming more achievable, both practically and financially.

5.1.3. The effect of temperature on the expression of genes associated with anther/pollen development.

Larkindale *et al*. (2005) reported that the complexity of the reproductive process is due to multiple gene effects, with approximately 13,977 genes expressed in the male gametophyte of *Arabidopsis*, with 9.7% of these genes being specific to the male gametophyte (Honys & Twell, 2004). Much like a change in gene expression in other localities, changes in expression of those genes exclusively, or disproportionately, expressed in the anther, can have a negative effect on the functionality of anthers. For example the over-expression of the *MYB24* gene, which is primarily expressed within flowers, resulted in defects including abnormal pollen grain development and non-dehiscent anthers (Yang *et al*., 2007_c).

Much in keeping with Dolferus *et al.* (2011) calling pollen formation '*the Achilles tendon of reproductive development*', numerous authors, have documented the negative effects of the changes in anther/pollen related gene expression on reproductive efficiency (Giorno *et al.*,

2013), but few mention the effect of temperature stress. Those that have looked at anther orientated gene expression change, due to elevated temperatures, have reported deleterious effects including both general and specific transcriptional alterations in response to temperature changes (Giorno *et al.*, 2013). These changes have included the premature up-regulation of numerous genes, including the meiosis specific *Asy1* gene in barley, which in turn corresponded to the premature, and damaging, progression of anther/pollen development, including premature tapetal degradation (Oshino *et al*., 2007). Premature tapetal degradation has long been associated with irreparable microspore damage (Saini *et al.*, 1984). Endo *et al.* (2009) present correlations between anther developmental health and related gene expression, under elevated temperatures, in rice.

Due to these examples of correlations between physiological/anatomical changes, and changes in associated gene expression levels/patterns, there is the potential for molecular biology to be a useful tool in assessing the effects heat stress has on the pollen production of wheat. Therefore, the presence of an existing anther/pollen-related gene regulatory framework (Figure 5.2), derived from numerous independent studies, and comprising a variety of characterised genes, gives a good platform for assessing the effect heat stress has on pollen development. Despite regulatory networks such as this being primarily derived from model species (e.g. *Arabidopsis* and rice), and not wheat, there is good evidence that these networks are relatively well conserved across evolutionary distance (Fernández Gómez *et al.*, 2015), and therefore applicable/transferable across taxa (Figure 5.1). This is especially the case when the model species is rice which, like wheat, is a monocot found in the family Poaceae. A comparison between the pollen transcriptomes of rice and *Arabidopsis* revealed that 56.6% of the rice pollen preferential genes had homologs in *Arabidopsis* genome (Wei *et al.*, 2010). Chen *et al.* (2005) offer further support for the pollen formation of rice following a similar developmental pathway to that observed in *Arabidopsis*.

An example of the identification of pollen related gene orthologues in a non-rice crop species is that of Fernández Gómez & Wilson (2014), who characterised *MS1* in barley (Poaceae), using genomic information from Arabidopsis (Brassicaceae), rice (Poaceae) and the bridging genome of *Brachypodium* (Poaceae).

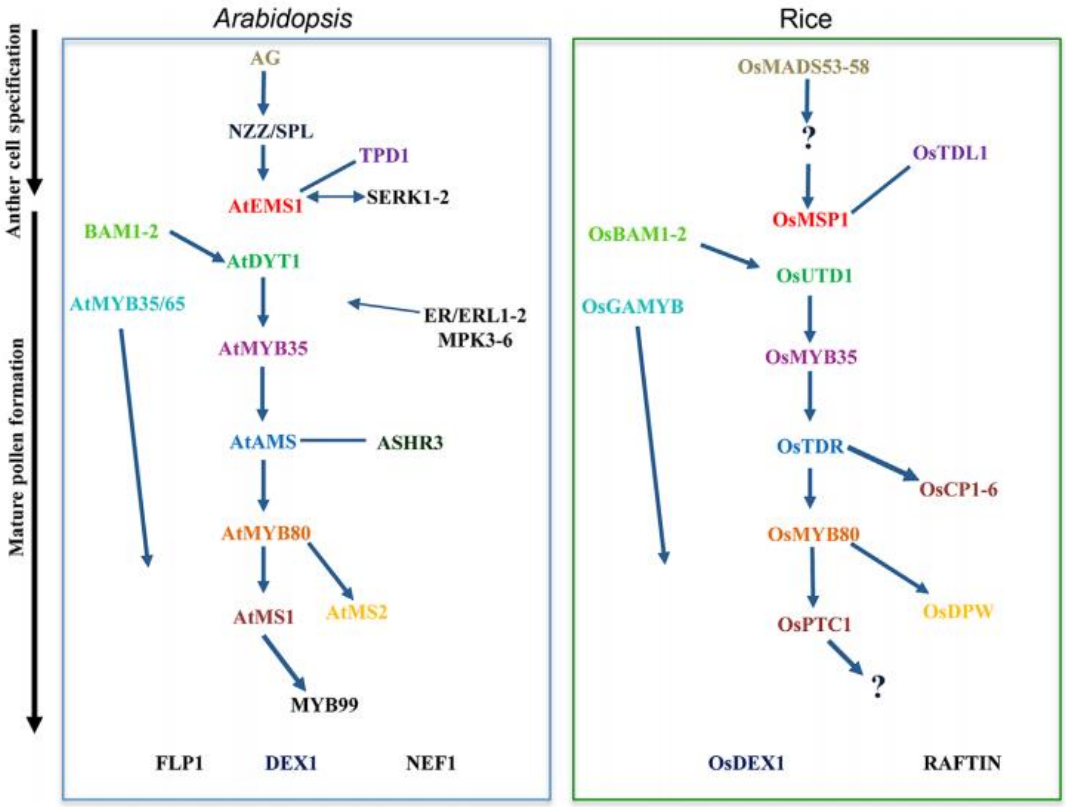
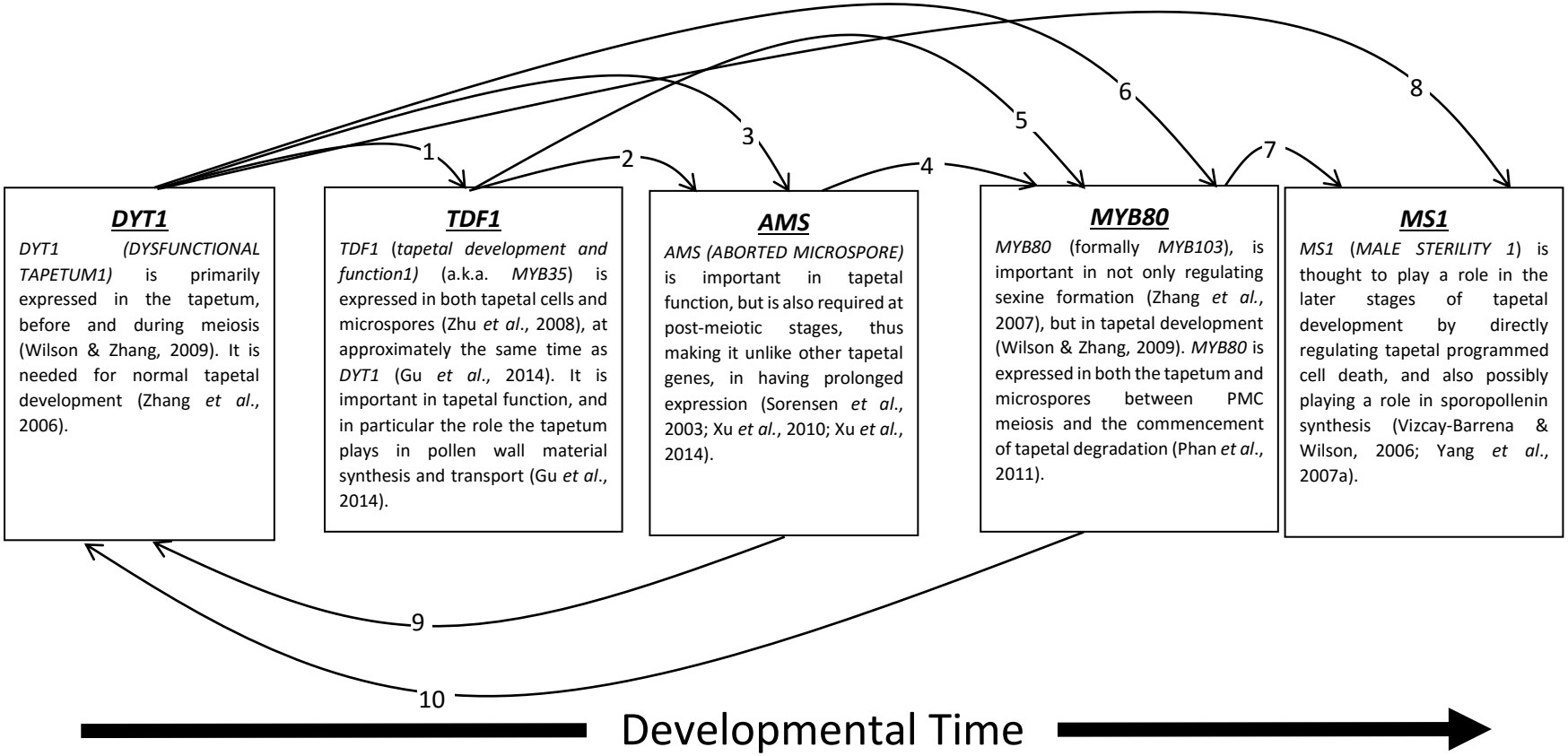


Figure 5.1: Genes involved in anther/pollen development presented within regulatory networks. Unconnected regions and '?' indicate ambiguity. Colours correspond to equivalent orthologues (Fernández Gómez *et al*., 2015).



1= Feng *et al.* (2012), Zhu *et al.* (2011), Gu *et al.* (2014), Zhu *et al.* (2008)
2= Feng *et al.* (2012), Zhu *et al.* (2011), Zhu *et al.* (2008), Gu *et al.* (2014)
3= Zhang *et al.* (2006)
4= Zhu *et al.* (2011), Zhu *et al.* (2008)
5= Zhu *et al.* (2008), Gu *et al.* (2014)
6= Zhu *et al.* (2008), Zhang *et al.* (2006)
7= Zhu *et al.* (2011), Yang *et al.* (2007a)
8= Zhang *et al.* (2006), Feng *et al.* (2012)
9= Feng *et al.* (2012)
10= Feng *et al.* (2012)

Figure 5.2: Regulatory network for five genes associated with tapetal development and the early stages of anther/pollen formation in *Arabidopsis*. Arrows signify direct regulation and/or a relative position.

An additional benefit from investigating the particular gene regulatory network presented in Figure 5.2, in the presence of heat stress, is that these genes are all associated with tapetal development. As the tapetum is not only vital for proper pollen development (Wilson & Zhang, 2009), but is also negatively affected by elevated temperatures (Parish *et al.*, 2010), the knowledge derived from the expression patterns, under heat stress, will potentially offer the possibility of constructing future genotype screening frameworks to assess the comparative effect analogous stresses have on different genotypes.

In addition to the five tapetum expressed genes, described in Figure 5.2, the comparatively late expressed gene, *MYB26*, will also be examined. *MYB26* (*MS35*) expression is limited to inflorescences (Steiner-Lange *et al*., 2003), and is critical for the development of secondary thickening in an anther's endothecium, and subsequent anther dehiscence (Yang *et al*., 2007_b). Given that functional male sterility can often occur due to the failure of viable pollen release from anthers, as well as a lack of viable pollen (Wilson *et al.*, 2011), indehiscence must be seen as a possible major contributing factor to projected future cereal yield reductions, and therefore worth further investigation. This is especially the case since both Sato *et al.* (2002) & Porch & Jahn (2001) report that heat stress leads to indehiscent anthers in tomatoes and string beans respectively. There may be more stage-enriched transcripts associated with defence/stress, signalling transcription and RNA processes in monocots (Inc. rice and wheat) than eudicots (Inc. tomatoes and string beans) (Wei *et al.*, 2010).

5.2. Objectives

1) To assess, across a developmental time course, the expression of six genes associated with anther/pollen development.
2) To assess the effect heat stress has on the expression patterns of six genes associated with anther/pollen development, and how these changes relate to anther/pollen phenotypes under heat stress.

5.3. Methods

5.3.1. Growth and preparation of plant material

Pots (12.5cm) were filled with 'PEL mix' (see section 2.3.1.1).

260 Pots were sown, with 780 untreated seeds (3 per pot) of the spring wheat variety, Paragon, (Plant Breeding International Cambridge Ltd). All pots were initially placed in an unheated polytunnel at the Plant Environment Laboratory, University of Reading, UK (51.413349, -0.93749225).

When these plants reached approximately 23ZS, 96 pots were moved to onsite controlled environmental facilities. These facilities were in the form of two growth cabinets at 20±1°C (day & night), 16 h photoperiod and an average irradiance, to booting canopy level, of 700 µmol/m^2/sec, fluorescent and incandescent illumination. A control growth temperature of 20°C was chosen, based upon Bennett *et al.* (1973). Half of the plants, within each of the 22 cohorts (Table 5.1), were represented within each cabinet.

Within 24 hours of being moved from the polytunnel, to the 20°C growth room, the plants were thinned down to one plant per pot, and three tillers per plant. Tiller thinning was repeated approximately every 10 days until the harvesting of samples.

These 96 plants were assigned to 22 cohorts (Table 5.1). Those plants that were stressed were placed in one of two 'heat stress' cabinet 35±1°C (day & night) for 24 hours, before being sampled. Half of the plants, in each stressed cohort, were placed in each of the two 'heat stress' cabinets.

Apart from the temperatures, the conditions (e.g. light levels, day length), between the stress and control cabinets, were otherwise identical.

Table 5.1: The 22 cohorts within this experimentation, and the timing of their processing. 'X' equals 4 plants.

	Days post 39ZS (of the main tiller)												
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Control (20/20°C)	x	x	x	x	x	x	x	x	x	x	x	x	
Stress (35/35°C)		x	x	x	x	x	x	x	x	x	x	x	x

During the high temperature treatments (35°C) and control temperature (20°C) the atmospheric relative humidity was kept as high as possible (normally above 40% (v/v)). In

addition, through regular physical examination, the water content of the medium was kept relatively stable, at approximately field capacity.

Below are details of the process each plant went through, at its allocation time, and after its allocated temperature treatment.

As determined by 'Appendix 14', six spikelets from the centre of the main tiller ear were harvested. One was embedded in resin, for possible sectioning, and 5 were snap frozen, for subsequent gene expression analysis.

5.3.2. Microscopy analysis

After, where relevant, removing the ear from surrounding leaf sheaths, spikelets/anthers (Appendix 14), were removed and placed directly into 4% paraformaldehyde (v/v), in 0.05m phosphate buffer for approximately 18 hours, before being washed twice (2x30min) with a 0.05m phosphate buffer. These samples were dehydrated through an ethanol series (10%, 30%, 50%, 70%). The samples were in each of the aforementioned ethanol concentrations for 1 hour, before being stored in 70% ethanol at 4^{o}C. Where only anthers were collected, due to increased spikelet maturity, these were from floret 'a', as defined by Lukac *et al.*, (2012).

In time, these samples were removed from the 70% ethanol and dehydrated further ((70%, 90%, 100% & 100% (v/v)) 1 hour per solution). The samples were then gradually infiltrated with medium grade LR White resin (London Resin Company) through a sequential gradation of ethanol to resin (3:1, 1:1, 1:3 (v/v)) 1.5 hours per solution). Samples were then placed in 100% resin and left for 2 hours, before the resin was replaced and left for another 2 hours. Samples were placed in gelatine capsules (size 00) (Agar Scientific) and polymerised at 58^{o}C for 24 hours.

From the addition of fixative to the infiltration with resin, during working hours, samples were kept moving in solution, using a rotating platform. During non-working hours the samples were stored at 4^{o}C.

Approximately 1.2µm thick transverse sections, of the anthers of florets at the bases of spikelets, florets 'a' or 'b', as defined by Lukac *et al*. (2012), were cut, using a glass knife and a Leica EM UC6 microtome, and then stained with 0.5% (w/v) Toluidine Blue O. Images were taken with a Leica DM5000 B light microscope.

5.3.3 Preparation and implementation of gene expression analysis

5.3.3.1. Sample harvest

Spikelets were collected for gene expression analysis (Appendix 14), flash frozen, using a dry ice/ethanol bath, and stored at -80^{o}C until required.

5.3.3.2. RNA extraction

RNA from approximately 100mg of floral tissue, from three biological replicates per cohort, was extracted using RNeasy® Mini Kits (QIAGEN). This included an on-column digestion of DNase, with a QIAGEN DNase digestion kit, according to the manufacturer's protocol, except that two treatments, of 45min each, were implemented. RNA quality and quantity was assessed by means of a NanoDrop fluorospectrometer. Samples were stored at -80^{o}C until required.

5.3.3.3. cDNA synthesis

cDNA (0.75µg/µl) was synthesised from total RNA by adding the following components to nuclease free tubes.

- 1µl – Oligo dT (12-18) (0.5 µg/µl) (Life Technologies)
- 1µl – dNTP mix (10mM) (Life Technologies)
- 1.5µg RNA (amount of solution dependent upon concentration)
- Nuclease free water to a total volume of 13µl

The mixture was incubated at 65^{o}C for 5 mins, then placed on ice for a minute. Then the following components were added to each tube.

- 1µl – DTT (0.1M) (Life Technologies)
- 1µl – RNAse Out™ (Life Technologies)
- 1µl – Superscript® III (Life Technologies)
- 4µl – 5x First Strand Buffer (Life Technologies)

The mixture was incubated at 50^{o}C for 1h, then 70^{o}C for 15min. 20µl of nuclease free water was added, and samples were stored at -80^{o}C until required.

5.3.3.4. Primer design

Primers were designed, using Primer3 software (http://biotools.umassmed.edu). Primers were designed with melting temperatures (Tm) of between 58-63^{o}C, and in such a way as that they would result in specific amplicons of <500bp from putative wheat orthologous genes (Dr. Jose Fernández Gómez, personal unpublished data). For the target genes, one of the primers in each set spanned an intron in order to prevent the amplification of any potential genomic contamination that may have remained after the DNase digestion. 'Appendix 15' shows the

position of primers upon putative gene sequences. The target gene sequences were derived from genome B, provided by the Wilson Lab, University of Nottingham (un-published data). The Actin housekeeping gene sequence was obtained from NCBI GenBank (accession KC775780).

5.3.3.5. Reverse transcription polymerase chain reaction (RT-PCR)

The testing of primer, in order to find those that produced a single strong amplicon of the predicted size, was conducted via reverse transcription polymerase chain reaction (RT-PCR). This comprised the following components and protocol, with a varying number of denaturation – elongation cycles.

- 5µl – Red Taq DNA Polymerase 2X Master Mix (SIGMA-ALDRICH)
- 0.3µl – Forward primer (10pmol/µl)
- 0.3µl – Reverse primer (10pmol/µl)
- 0.6µl – cDNA (0.75µg per µl)
- 4.1µl – Nuclease free water

Step	Temperature (°C)	Length of Time
Initialisation	94	3 min
Denaturation	94	30 sec
Annealing	58-64	30 sec
Elongation	72	30 sec
Final elongation	72	6 min
Hold	10	Indefinitely

The products of the above protocol were run on relatively high percentage gels (1.4%) to view amplicon length and strength, especially in relation to the HK gene (*ACTIN*).

5.3.3.6. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Before the quantification of expression; and using dilutions of cDNA (1, 0.2, 0.04. 0.008), primer efficiency was calculated and assessed. For all primers, including for the housekeeping gene, an efficiency of between 1.9 and 2.1 was required in order to use a primer set for the quantification of expression. Additionally, the assessment of melting curves produced by each primer set was examined, one clear peak was required. Both primer efficiency and melting curve analysis were assessed, using a LightCycler® 480 qRT-PCR machine and analysed using LightCycler® 480 (Release 1.5.0) software.

Upon the determination of suitable primers, both stressed and un-stressed floral samples, from across a developmental time course, were examined for the expression levels of *DYT1, TDF1, AMS, MYB80, MS1, MYB26*, using the following reaction components and protocol (55 cycles). In addition to floral samples, leaf samples from unstressed plants were analysed.

- 4.5µl – Maxima SYBR green master mix (2x) (Thermo Scientific)
- 0.2µl – Forward primer (10pmol/µl)
- 0.2µl – Reverse primer (10pmol/µl)
- 0.8µl – cDNA (0.75µg per µl)
- 3.2µl – Nuclease free water

Step	Temperature (°C)	Length of Time
Denaturation	95	30 sec
Annealing	58 - 61	30 sec
Elongation	72	1min

Expression levels were determined using a LightCycler® 480 qRT-PCR machine. For each cohort three biological replicates, each comprising of three technical replicates, were used. Expression crossing points (Cp) were determined using LightCycler® 480 (Release 1.5.0) software and the relative expression for each of the target gene technical replicates was calculated, using the below formula, adapted from $2^{-\Delta\Delta C_T}$ methods (Livak & Schmittgen, 2001).

Relative expression = a ^ (b – c)*100

a = efficiency of target gene primer
b = mean of the Cp's for the housekeeping gene technical replicates
c = Cp of one target gene technical replicate

The mean of the target gene technical replicates was then calculated.

5.4. Results

5.4.1. RT-PCR

Figure 5.3 shows the RT-PCR amplification products, for primers, for six selected genes associated with anther/pollen (Appendix 15). This figure shows strong, single bands, of the appropriate/expected size for the respective primer combinations. These bands not only show the expression of these genes within wheat floral tissue, but also show that the primers are efficient at only amplifying one product. The use of negative controls (nuclease free water) shows the lack of primer dimers.

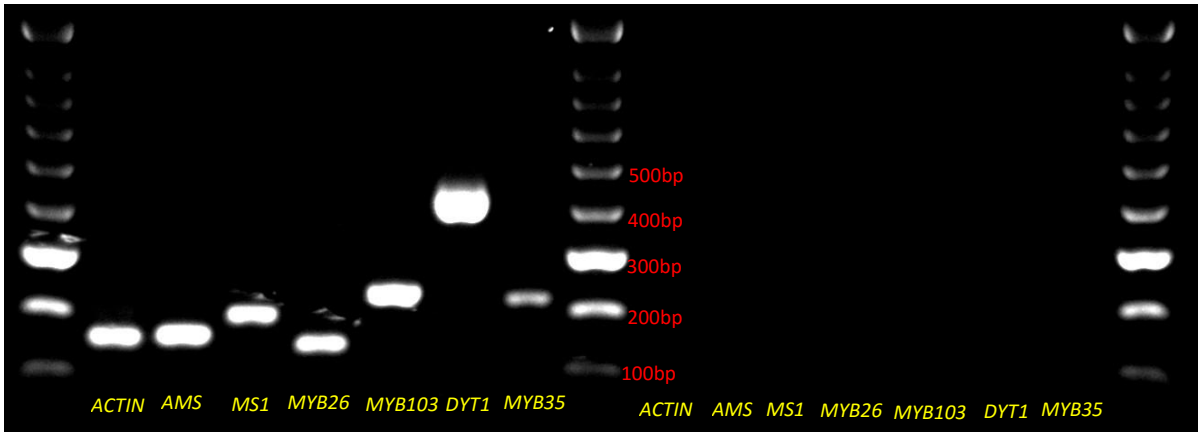


Figure 5.3: Agarose gel (1.4% (w/v)) showing the amplicon length produced using seven primer sets, and cDNA prepared from wheat floral tissue. Left = cDNA, right = negative control (nuclease free water). HyperLadder™ 50bp (Bioline). For primers see 'Appendix 15'

5.4.2. qRT-PCR

Expression levels of six anther/pollen related genes from wheat floral material, under both stressful and non-stressful climatic conditions, across a time course, can be seen within Figures 5.4 & 5.5. The figures are annotated with basic anther/pollen stages, established by microscopic analysis of sectioned material.

Even though leaf tissue was collected from an unstressed plant, it is apparent that none of these genes were expressed, at any significant level, within this tissue (Figures 5.4 & 5.5).

The wild type expression patterns of the wheat genes (Figure 5.4 & 5.5) was largely in keeping with the previously described regulatory network for the putative orthologues (Figure 5.2), with maximal expression seen at the expected corresponding developmental stages. However, heat stress, when inflicted at 11 different developmental times, had a considerable impact on the expression of these genes, when compared to their respective controls.

5.4.2.1. *DYT1*

DYT1 was shown to be pre-meiotically expressed (Figure 5.4). There were three principal trends, relating to this genes expression, as a consequence of heat stress. Firstly, when inflicted with heat stress at the beginning of booting (pre-meiosis), the levels of expression were greatly reduced (-60%) from comparably high levels. Secondly, when exposed to heat stress, at the mid to later stages of booting (4-6 days after 39ZS), *DYT1* expression was elevated. However, these were not to the levels seen in the controls at the start of booting. Thirdly, a phenomenon seen with the control pattern of *DYT1* is a second, if smaller, peak in expression around anther dehiscence (6-9 days after 39ZS). This phenomenon, of a secondary peak, is not seen in any other of the genes' expression patterns.

5.4.2.2. *TDF1* (*MYB35*)

TDF1 is primarily expressed in and around meiosis (Figure 5.4). Heat stress caused a reduction in the expression of *TDF1* on the first three days of booting, when it was primarily expressed.

5.4.3.3. *AMS*

Two principal trends can be seen within the expression patterns of *AMS,* which was primarily expressed in and round meiosis (Figure 5.4). Firstly, heat stress, at the very start of booting (pre-meiosis), causes an increase in expression. Secondly, when stressed at the peak of gene expression around meiosis (3 days after 39ZS), expression levels were reduced by more than a quarter.

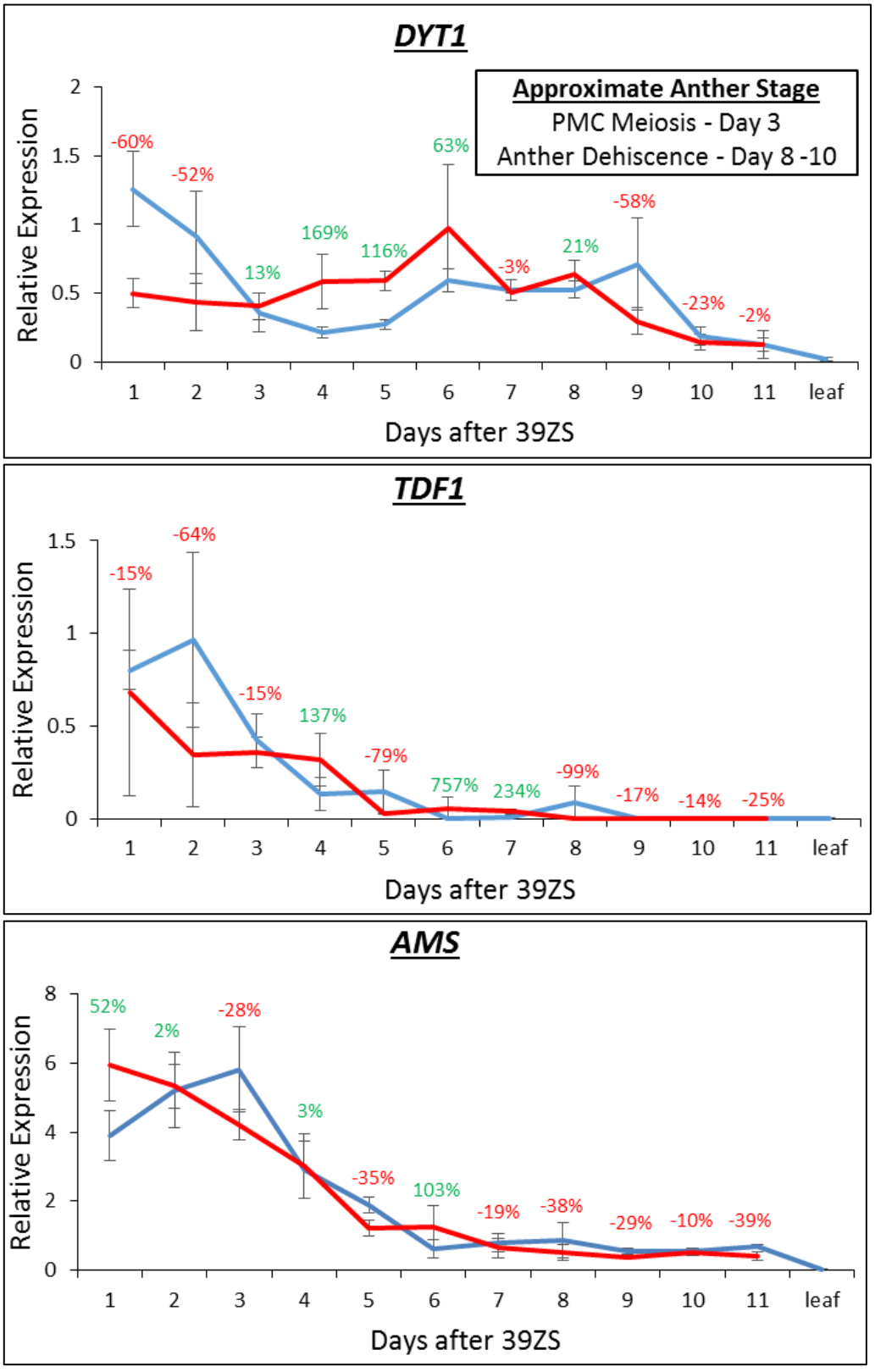


Figure 5.4: Relative expression levels of three genes across an 11 day time course, as a result of medium (blue, 20/20°C) and high (red, 35/35°C) temperature treatments during anther/pollen development. Annotation of anther stage, based on microscopy analysis, is displayed. Error bars show standard error. *n*= 3.

5.4.4.4. *MYB80*

The expression profile of *MYB80* is unique, when compared to the other five genes examined, in that it has a very defined peak around meiosis (three days after 39ZS), before rapidly reducing (Figure 5.5). Heat stress caused a large (-74%) reduction in the expression levels of this gene during its peak expression stage.

5.4.5.5. *MS1*

The two principal trends are seen within the expression patterns of *MS1,* which was primarily expressed in and around meiosis (Figure 5.5). Firstly, heat stress at the very start of booting (pre-meiosis) causes a large increase in expression. Secondly, when stressed around meiosis, the peak of gene expression levels are reduced by around a third.

5.4.6.6. *MYB26*

Unlike the expression patterns of the other five genes examined, *MYB26* was predominantly expressed in the latter stages of booting, which typically lasted around 7 days, and early stages of ear emergence from the flag leaf sheath (Figure 5.5). Anther dehiscence happened around this time as well. Heat stress tended to lead to small reductions in the expression of this gene, when inflicted around the time of anther dehiscence.

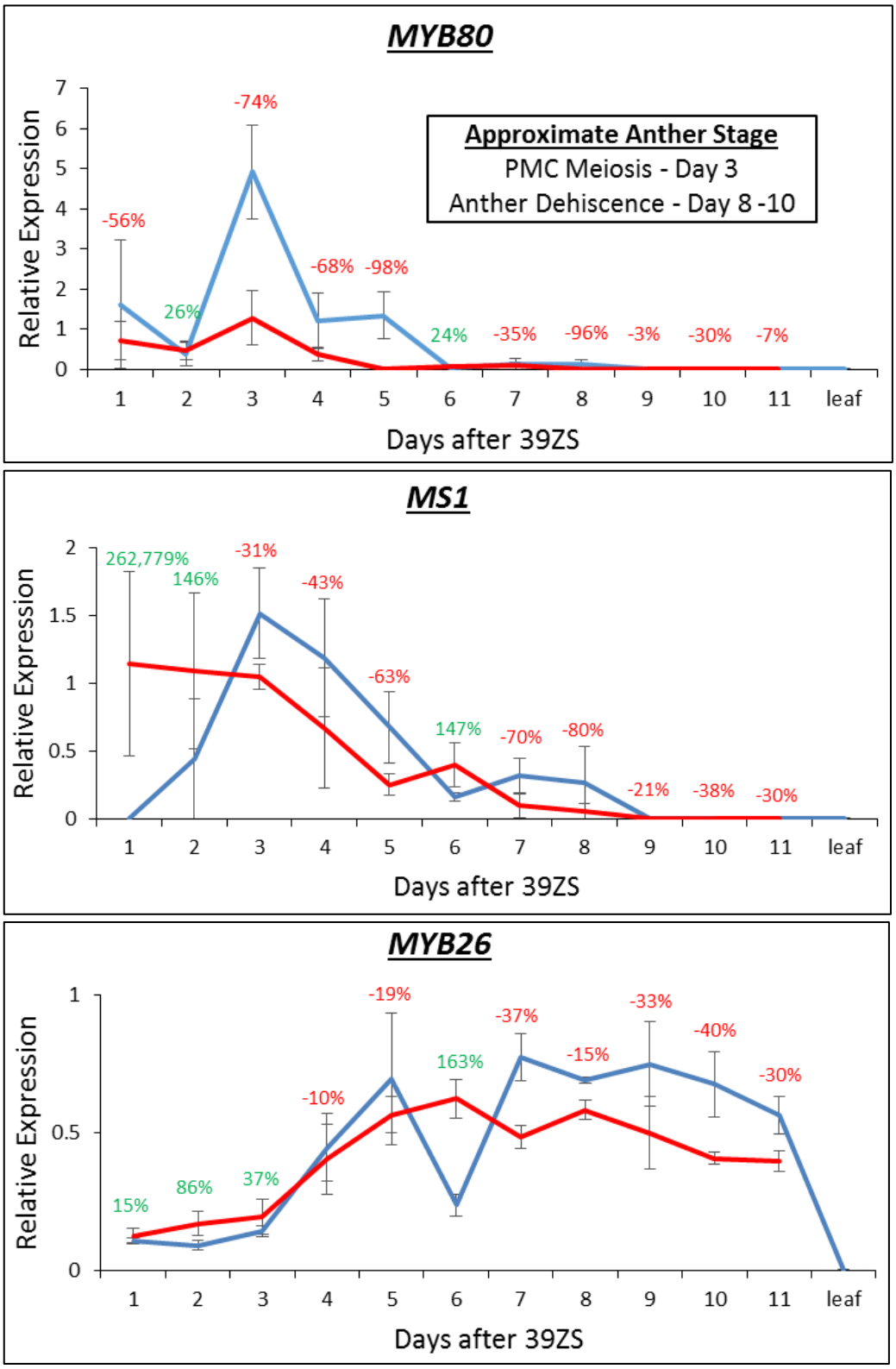


Figure 5.5: Relative expression levels of three genes across an 11 day time course, as a result of medium (blue, 20/20°C) and high (red, 35/35°C) temperature treatments during anther/pollen development. Annotation of anther stage, based on microscopy analysis, is displayed. Error bars show standard error. *n*= 3.

5.4.6.7. Intra-cohort expression

Despite there being quite a profound difference between the levels of expression , across five genes, between three biological replicates within a cohort (Figure 5.6), the relative difference between these expressions, within a plant/sample, was relatively constant, expecially between 'Biological Rep. 1' and 'Biological Rep. 2' (Figure 5.7).

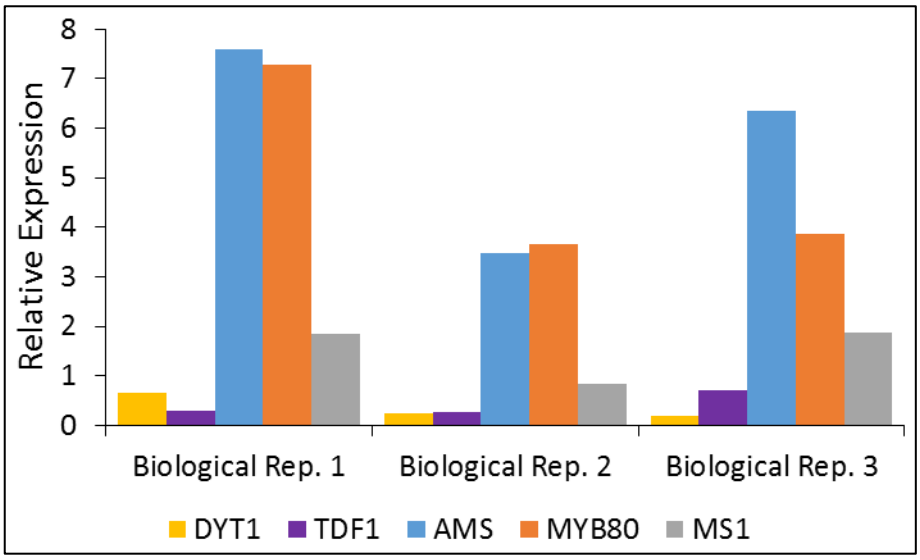


Figure 5.6: Relative expression levels of five tapetal genes, in three biological replicates, within a cohort. These biological replicates were harvested from control conditions (20/20°C), 3 days after 39ZS.

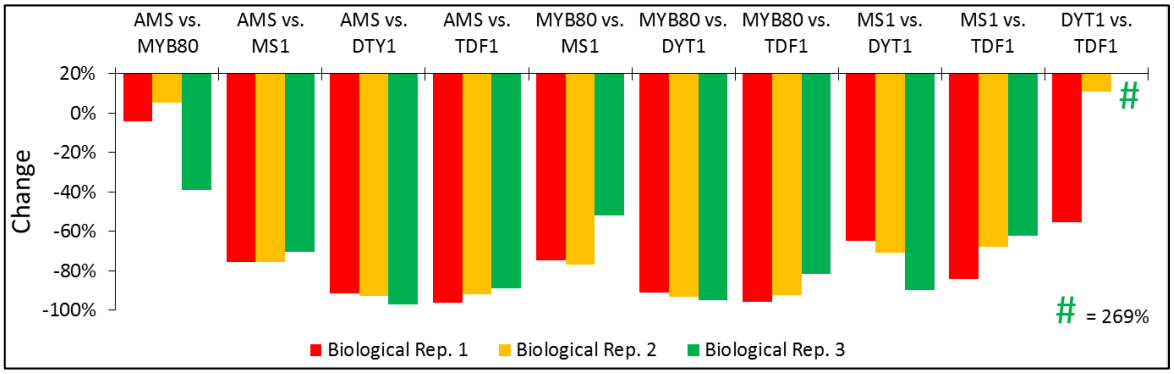


Figure 5.7: Differences between the relative expressions levels of five tapetal genes, in three biological replicates, within a cohort. These biological replicates were harvested from control conditions (20/20°C), 3 days after 39ZS.

5.5. Discussion

5.5.1 qRT-PCR

Despite leaf tissue not being representative of all non-reproductive tissues (e.g. root tissue & stem tissue), from the results of this primary study it is apparent that the six target genes were expressed within reproductive tissues, and not leaf tissues (Figures 5.4 & 5.5). This agrees with the expression patterns of the putative orthologs, in *Arabidopsis*, presented in Figure 5.2, and is also further supported by additional unpublished data from the Wilson Laboratory, The University of Nottingham.

In light of previously published information pertaining to the genes accessed, under both stressed and unstressed conditions, the results of this primary research will be discussed. Despite this being structured, gene by gene, there will be, where relevant, and in light of the regulatory pathway presented within Figure 5.2, the discussion of effects in gene regulation.

5.5.1.1. *DYT1*

Upon seeing, in the control material, that the highest levels of *DYT1* expression are at the very start, or potentially before the start of booting, and thus a few days before meiosis, this would support previous reports (e.g. Zhang *et al*., 2006; Wilson & Zhang, 2009) that suggest that *DYT1* acts at a relatively early stage in tapetal development, before the completion of meiosis, and before the expression of *AMS* & *MS1.*

In light of the material harvested comprising floral material at varying stages of maturity; this variation being primarily found within spikelets, rather than between spikelets; perhaps the second peak observed within the controls was due to less mature florets approaching meiosis. Perhaps the primary reason for this double peak not being seen in any other genes, within the regulatory network of Figure 5.2, is that perhaps, considering the time delay between the peak expression of *DYT1* and other genes (e.g. *AMS & MS1*), at the start of booting (the primary peak), any secondary expression of genes such as *AMS* and *MS1* may be after the last collection (e.g. 12-13 days after 39ZS).

Since both *AMS* and *MS1* are proposed as direct regulatory targets of *DYT1* (Zhang *et al*., 2006; Gu *et al*., 2014), the effect heat stress has on their respective levels is also worthy of note. In seeing that the peak expression of these two genes, in control material, is a number of days after the peak expression of *DYT1*, with them being expressed around meiosis, it is unfortunate that the experimental design does not allow analysis in the days after a heat

stress event. However, what is apparent is that, for *AMS*, and especially *MS1*, heat stress at the very start of booting (1-2 days after 39ZS) caused an immediate increase in expression, perhaps due to the disturbance in the regulatory functioning of *DYT1* and/or *TDF1*, or more generally in the anther. The premature up-regulation of a meiosis specific genes is not unique to *AMS* and *MS1*, as Oshino *et al.* (2007) document the same in *ASY1*.

5.5.1.2. *TDF1* (*MYB35*)

The degradation of the callose wall surrounding developing microspores, allowing their release, is a vital process (Chasan, 1992). Callose breakdown fails to occur in *TDF1* mutants (Zhu *et al*., 2008). Therefore, conceivably the reduced levels of *TDF1* observed at the start of booting, due to heat stress, would have compromised this degradation and thus effected eventual pollen variability, as observed by Saini *et al.* (1984). However, this cannot be confirmed, due to the poor preservation of material intended for sectioning.

It is apparent that, despite the peak expression of *TDF1* being 2 days post 39ZS, meiosis only finished on day 3. This would suggest that there is a delay between the expression of *TDF1* and maximum callose wall degradation. This may suggest a more complex regulatory network involved in callose wall degradation, with other genes acting downstream of *TDF1*.

In addition to the direct effects of a disturbance in the expression of *TDF1*, the fact that *TDF1* is considered a key transitory stage in the regulation of *AMS*, *TEK*, *MS1* & *MYB80* (Gu *et al.,* 2014), the knock on effects, in relation to secondary effects, could be profound.

5.5.1.3. *AMS*

Even though the changes, due to heat stress, in both expression patterns and levels are not as profoundly different in *AMS*, as seen in other genes, knowing the crucial role *AMS* has in the development of tapetal cells (Sorensen *et al.*, 2003), in addition to the post-transcriptional regulation of microspore development and filament elongation (Sorensen *et al.*, 2003; Xu *et al.*, 2014), any changes may have significant effects on pollen development. Such effects on pollen development would inevitably affect eventual yields of this predominantly self-pollinating crop (De Vries, 1971). Because of this diverse range of effects that this gene has, in both location and temporal activity, Sorensen *et al*. (2003) suggest that *AMS* is implicated in the regulation of more than one target gene. Xu *et al.* (2014) confirm, in addition to the large number of direct targets, the secondary, regulatory effects of *AMS*, in that they report that, of 98 candidate genes with specific expression in the anther, 70 are reduced in *AMS* mutants.

5.5.1.4. *MYB80* (*MYB103*)

MYB80 expression was dramatically reduced (12.4% of normal) in *AMS* mutants (Zhu *et al.*, 2008). Therefore, unsurprisingly, the reduced levels of *AMS*, around meiosis due to heat stress, corresponded with a reduction in levels of *MYB80* expression.

When looking at the expression pattern of *MYB80* in the control material, in light of the importance of *MYB80* in the delaying of tapetal programmed cell death (PCD), through promoting the activating transcription of the *UNDEAD* gene (Phan *et al*., 2011), it is unsurprising that levels, after hitting their pinnacle at around meiosis, drop off quite quickly. This is because the initiation signal for tapetal PCD may commence as early as tetrad stage (Kawanabe *et al.*, 2006). In light of the important role *MYB80* plays in preventing tapetal PCD, when seeing the consistently depressed levels of expression in the stressed material, it is unsurprising that the sectioned material appeared to show increased levels of premature tapetal degradation.

5.5.1.5. *MS1*

In light of *MS1* being postulated to be a key gene in modifying the transcription of tapetal specific genes implicated in the latter stages of tapetal PCD (Vizcay-Barrena & Wilson, 2006), seeing its premature expression, due to heat stress, gives cause for concern, as this would lead to, as observed, the premature degradation of the tapetum. However, in addition to the premature expression of *MS1*, perhaps as significant is the premature down regulation of the gene, due to heat stress. This is because *MS1* may also play a role in co-ordinating the incorporation of the tapetal cell debris, left after tapetal PCD (e.g. pollenkitt and tryphine), into the walls of maturing pollen grains (Vizcay-Barrena & Wilson, 2006).

5.5.1.6. *MYB26*

In light of numerous accounts of *MYB26* being inextricably involved in the synthesis of secondary thickening in the endothecium (e.g. Dawson *et al*., 1999; Steiner-Lange *et al*., 2003; Yang *et al*., 2007_b), it is surprising to find that despite heat stress causing the premature dehiscence of anthers collected during the course of this experimentation, and that of 'Chapter 2' of this work, it also causes a downregulation, if comparatively small, in *MYB26* expression. In this regard, an over expression in *MYB26* would have been expected. However, as well as Yang *et al*. (2007_b) postulating that *MYB26* carries out this function via *NST1* and *NST2*, in addition to IRX1, IRX3, IRX8, IRX12, Li *et al.* (2007) documents that the over expression

of HDG3 resulted in the downregulation of *MYB26*. Therefore, expressional data for these genes may prove insightful in relation to this apparent anomaly.

When considering the *MYB26* expression pattern of the control material, it is clear that expression is present, admittedly at relativity low levels, from the very start of booting, and increases even before the apparent, through anther sectioning, start of secondary thickening in the endothecium. Yang *et al.* (2007_b) offer an explanation for this in that they suggest that *MYB26* acts at the early stages of endothecium development, primarily in relation to cell expansion, before the deposition of the secondary thickening. In fact, it may be apparent that these two roles may be temporally separated, with 6 days after the start of booting signifying a point in which neither of the gene's roles is being fulfilled, therefore explaining the reduction in expression at this point.

5.5.2. Experimental issues/future improvements

5.5.2.1. Extent of data variance

Due to a desire to monitor the expression of genes over the 11 day time course, this meant, due to limited growing space, that a reduced level of biological replication (3 per cohort) was possible. However, in the future, and based on the primary data of this experimentation, a more temporally focused methodology may be adopted. For example, in knowing that those genes, such as *TDF1* and *MYB80*, have little, to no, expression after 6 days of booting, if there was a desire to study these particular genes in the future, perhaps those replicates used in the later stages (days 7-11) could be reallocated over the earlier stages. A greater level of biological replication, within a cohort, would inevitably result in greater levels of clarity/confidence, which would be epitomised by much reduced standard error bars.

Upon examining the source of such, at times, high intra-cohort variance, it is apparent that there are two potential explanations. Firstly, upon observing the, at times, low levels of inter-sample consistency, in intra-sample levels of expression, amongst the five tapetal genes, this could indicate that the maturity of the material differed. Due to the transient nature of many of the genes presented within the results (e.g. *TDF1*, *MYB80* & *MS1*), half a day's difference in maturity, within a cohort, may make a great deal of difference in expression levels between biological replicates. Secondly, upon observing the, at times, high levels of inter-sample consistency, in intra-sample levels of expression, amongst the five genes, this variance could be attributed to something other than the maturity of plant/anther material. For example, the

health of material or relative biological composition of material (e.g. ratio of anther to perianth parts). Within a cohort, both of these possibilities are likely, and the best way to avoid such anomalies from distorting averages is to increase the number of biological replicates.

5.5.2.2. Design of primers for housekeeping gene

In order to gain greater clarity on relative levels of target gene expression change, not only due to heat stress, but also across the temporal/developmental time course, future improvements in the design of the primers for the housekeeping gene (*Actin*) may be needed. Such an improvement in design would be to have at least one primer spanning an intron, in the housekeeping gene, to prevent potential genomic DNA amplification, which would distort the relative expression levels of the target genes. However, this phenomenon is unlikely to have had any major impact on the results of this research. The reasons for this are twofold; the robustness of the digestion of genomic DNA, and the low number of qRT-PCR cycles (approx. 17 cycles) needed for *Actin* expression to be detected.

This spanning was done for each of the target genes, upon seeing, via RT-PCR, that when at least one primer was not designed spanning an intron, at high numbers of cycles (35 cycles), relatively faint bands, in keeping with the size expected for genomic DNA contamination, were produced. However, due to an oversight, not realising that the *Actin* primer was designed within an exon, intron spanning primer(s) were not designed for *Actin*.

5.5.2.3. Clarification of gene orthogonality

From the results of this research, there was little to dispute these genes being orthologues of those studied in other species in the past, especially due to their expression profiles being analogous. However, further work to support these being true orthologues may be needed. This work would most likely come as part of the continuing endeavours to translate the principles, derived from model species, to economically important crops, such as cereals, in order to help facility improved agricultural outputs (e.g. Wilson & Zhang, 2009; Fernández Gómez *et al.*, 2015).

5.5.2.3. Poor anther/microspore preservation

The ability to determine key anther/pollen/microspore stages was possible, based upon microscopic analysis of samples, and this information was subsequently added to Figures 5.4 & 5.5. However, this does not negate the fact that, in general, especially at the latter stages of microspore development, the preservation of the anther components, including the reproductive cells, was poor. At which stage, or stages, the protocol presented was

inadequate, is not apparent. However, since future, more temporally focused research, would invariably benefit from being able to view phenotypic changes in anthers, the changing of anther fixation/embedding protocols to aid this would be advised.

5.6. Conclusions

In contrast to the initial outputs of genetic engineering being largely only able to achieve yield stability improvements, in relation to changes in monogenic traits such as herbicide tolerance (Castle *et al.*, 2006; Century *et al.*, 2008), those traits resulting from comparatively more complex, polygenic controls, such as pollen development, will need to be based upon the results of a wide range of experiential investigations. However, perhaps foremost amongst these investigations, at least initially, will be the identification of genes of most importance to such traits, and their response to heat stress. Potentially, the results of this chapter have at least taken the initial steps in identifying a few of these genes in wheat, in order to work towards greater yield stability.

One potential candidate for such a further investigation, based not only upon the results of this primary experimentation, but also previously established knowledge, is *DTY1*. The principal reason for perhaps focusing on this gene, beyond its primary role of contributing towards normal tapetum development, is that of 32 genes examined, within a *DYT1* mutant, 21 had '*significantly reduced*' levels of expression, due to its apparent secondary roles (Zhang *et al*., 2006). Additionally, in relation to any future genotype screening, a comparative analysis of how different genotypes respond to early booting heat stress, in relation to the depressions seen in their *MYB80* peaks, would also prove to be a valuable data set, knowing the importance of this gene in preventing premature tapetal PCD (Phan *et al*., 2011).

Overall, from the results of this experimentation, it is clear that heat stress (35^{o}C for 24 hours) causes the dramatic change in both the expression levels, and patterns, of 6 anther related genes. As previously discussed, these findings are potentially the basis for future investigations, to achieve the long term goal of developing/finding germplasm less susceptible to heat stress. However, such future gene expressional investigations may, in light of studies such as Giorno *et al.* (2013), benefit from a more holistic appreciation of the challenges facing microspore/pollen development, including hormonal and heat shock protein analyses. Additionally, the analysis of other genes, that have been shown to be crucial for key stages of microspore/pollen development, may also be worth investigating, in relation to their responses to heat stress. One such gene may be RAFTIN, an anther specific gene that has been identified as critical in late pollen wall development in wheat (Wang *et al*., 2003), due to it perhaps being involved in the proper integration of sporopollenin polymers, from their

precursors of tapetal origin, into the developing pollen walls. Another may be *CalS5*, a gene essential in the early stages of exine formation (Ma *et al*., 2013).

Chapter 6

General Conclusions

6.1 Overview of findings

In many ways, the research contained within this thesis, and the academic papers that will subsequently be published, has very much 'picked up the mantle' of that work conducted by Saini & Aspinall in the early to mid-1980's (e.g. Saini & Aspinall, 1981; Saini & Aspinall, 1982; Saini *et al*., 1984). Despite, in the interim, there having been other noteworthy works related to pollen development, wheat and/or abiotic stress, few have so concentrated upon all three in the same way that Saini & Aspinall, and now this research has. However, the work continues.

Despite making progress in understanding the effects that any future heat stress events will have on the pollen development of field grown wheat, including the impact upon different wheat genotypes, there are still gaps in the collective knowledge, and requirements for clarification. Therefore, a discussion under three sub-headings will be undertaken.

6.1.1 The presence of data that consolidates previous understanding

A number of the results of this experimentation consolidated previous findings.

Firstly, heat stress during booting (39-47ZS) has a significant effect on the resulting grain set of wheat ears (Chapters 2, 3 & 4 and Saini & Aspinall (1982)). Secondly, the development of pollen is dramatically affected by the presence of heat stress (Chapters 2 & 3 and Saini *et al.* (1984)). Thirdly, of all the differing component parts of the anther, the tapetum, with its pollen wall biosynthetic capabilities and associated programmed cell death pathway, is particularly sensitive to heat stress (Chapters 2 & 3 and Ku *et al*. (2003)). Fourthly, even though there are exceptions, wheat physiology and anatomy tend to lend themselves to more intra-floral pollination, as opposed to any other pollination strategy (Chapter 4 and De Vries, 1971).

6.1.2 The presence of data that suggests the need for the continuation of research

Like much research, this thesis has resulted in as many questions as it has provided answers. Four further research areas have been identified as follows.

6.1.2.1 Clarifying inter-varietal avoidance of, and/or increased inter-varietal resilience to, heat stress.

Based upon two years of data, there is one winter wheat variety (KWS Sterling) which has the ability to avoid the detrimental effects of early booting heat stress on grain set, over and above another (Cordiale), due to its pollen stage during this time (Chapter 3). However, more work may be needed.

Perhaps bringing this research into the field may be the next logical step. Are the results outlined in Chapter 3 the same in the field as they were in the growth cabinets? The results of the field experiment in Chapter 2 may suggest that there is intra-varietal consistency between pollen maturity and the gross development of the plant. However, whether this is consistent across seasons has yet to be established. Is the approximately three day discrepancy in pollen stage, between the two varieties, when at 39ZS, still present in the field over multiple seasons?

It would be interesting to determine the resilience to heat stress, in KWS Sterling, if the stress was delivered at the corresponding stage of anther development as that seen in Cordiale. This could be achieved by testing both KWS Sterling and Cordiale over a time course, much like that outlined in Chapter 2, to determine whether similar grain sets, and anther wellbeings, are maintained under heat stresses directed towards identical pollen stages (e.g. meiosis). Based upon their similar genetic backgrounds (Appendix 5), the expectation is that they would.

6.1.2.2 Expanding research objectives, based upon the molecular results

One of the interesting new developments to come out of this research is the use of well-established molecular techniques to quantify the effects that heat stress has on the expression levels of six anther/pollen related genes (Chapter 5). However, due in part to relatively low biological replication, this data, even though valued, should be considered as somewhat preliminary, in that it should be primarily the catalyst for further research. Perhaps, based upon the findings of this preliminary data, as well as other findings throughout this thesis, such future research could only focus in on one gene and/or a specific stage in pollen development, in order to obtain greater clarity. For example, perhaps a greater focus on the analysis of the expression levels of the *MS1* gene, whilst under heat stress, may offer some

insights into why the latter stages of pollen development seem to be the most sensitive to heat stress (Chapter 2). This is due to *MS1* potentially playing a role in the co-ordination of incorporating tapetal cell debris left after tapetal programmed cell death (e.g. pollenkitt and tryphine) into the walls of maturing pollen grains (Vizcay-Barrena & Wilson, 2006).

6.1.2.3 Examining the possibility of resource reallocation due to heat stress

One phenomenon that consistently appeared across years, and experiments, was that when heat stress was imposed upon the first three days (72 hours) of booting, and in contrast to reductions in the grain set of more proximal spikelet florets, in floret 'e', across both primary and secondary tillers, there was an increase in grain number due to heat stress, and this increase was almost always significant. Similar phenomenon were also observed in relation to the number of both grains and florets above position 'e', due to heat stress (Chapter 3). As more expansively discussed in Chapter 2, and to a lesser extent in Chapter 3, one line of enquiry that may help explain this is the possibility of the reallocation of resources (e.g. sugars), after a period of heat stress, in order to try and mitigate grain loss.

Perhaps one feature of future research dedicated to clarifying whether resource reallocation is present after heat stress is the examination of anthers from floret 'e', to see whether there are differences between stressed and control plants. Do the anthers, from these florets of stressed plants, possess more microspores/pollen grains? Are their dimensions (e.g. length, breadth) different? Do stressed plants' floret 'e' anthers dehisce, when their unstressed counterparts do not?

Even though it would be of interest to agronomists, for now, this phenomenon would, in many ways, primarily interest plant biologists, who study resource reallocation within plants. This is due to the diminutive size of the compensatory grains, and the slight, if any, impact this would have in anyway restoring yields, which would be at the forefront of agronomists concerns. However, in light of a renewed (e.g. Gaju *et al*., 2009; Foulkes *et al*., 2011; Reynolds *et al*., 2012; Gaju *et al.*, 2014) desire to ensure that a large proportion of the resources, available to wheat plants, are indeed directed towards grain production, any findings associated with the possibility of yield increase/stabilisation would be of interest to those working in agronomy.

6.1.2.4 Clarifying which stage of pollen development is most sensitive to heat stress

Perhaps the most paradigm altering finding of this thesis is that found in Chapter 2. In Chapter 2, 3 & 4, heat stress, imposed around pollen mother cell (PMC) meiosis, resulted in abnormal

microspore development, prematurely degraded tapetal cells, and hence significantly reduced grain set. However, unlike previous research (e.g. Saini & Aspinall, (1982)), it was not during PMC meiosis that the lowest grain sets, nor greatest retardation of microspore/pollen grain development, due to heat stress, was seen. Instead, it was during the latter stages of microspore/pollen grain development that heat stress had its most negative effect on both of these areas (Chapter 2).

However, with just one reported observation of this phenomenon not warranting a somewhat new approach to future research relating to the challenges affecting global wheat production from pre-anthesis heat stress, more research, to clarify, is needed. Perhaps such research could include the repetition of the methodologies found in Chapter 2, both on the same, and different, wheat genotypes. Are the final stages of tapetal presence, and its role in pollen wall development (Shi *et al*., 2015), as, if not more, vital in determining eventual wheat yields, when compared to the tapetum's role in and around meiosis?

6.1.3 The possible need to amend methodologies in future research endeavours

Upon looking back across Chapters 2-5, there are four principal areas that may require amendments in any future, associated, research endeavours. These suggested amendments would better facilitate the answering of some of the questions resulting from this thesis, and some questions not successfully answered by this thesis.

Firstly, as more eruditely expressed within the 'Discussions' section of Chapter 4, if wanting to better assess the ability of inter-ear pollen movement to mitigate the detrimental effect of early booting heat stress on grain number, more suitable methodologies may be needed. Such improvement to methodologies would primarily involve improved means of facilitating both pollen movement through the aerial environment, and pollen exclusion from ears.

Secondly, light microscopy, despite offering valuable data sets (Chapters 2-5), at certain stages of microspore and anther development; generally between microspore release and early anther dehiscence, the differing methods of cellular cessation and preservation all resulted in a noticeably poor presentation of the reproductive cells (Figure 6.1). This prevented a complete determination of the effect of heat stress on pollen development during these stages, and whether they related to changes in grain set, or gene expression.

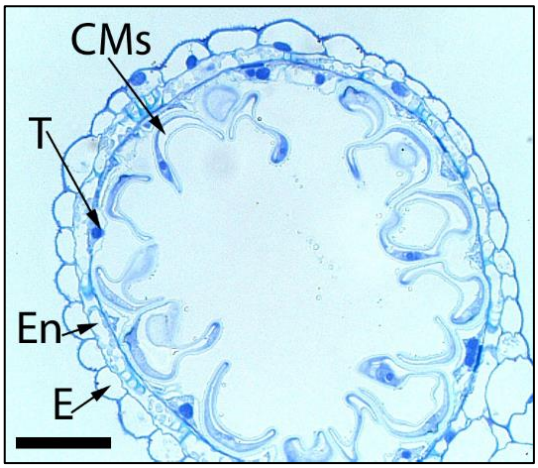


Figure 6.1: Un-stressed anther (20/20°C) from the centre of a Paragon ear, 9 days post 39ZS. The methodologies of 'Chapter 2' were used. CMs= collapsed microspores, E=epidermis, En= endothecium, T= tapetum. Scale bar 50µm.

Due to each of these protocols being, from the collection of anthers to final embedding in resin, multi-faceted, where these procedures were ineffective was not determined. However, it is worth noting that the methodology, in which only paraformaldehyde was used to cease cellular progress (Chapter 5), instead of a mixture of paraformaldehyde and glutaraldehyde (Chapters 2 & 3), gave poorer results. Additionally, the protocol of Chapter 5 was unique in that it included the medium-term storage of material in 70% (v/v) ethanol, rather than phosphate buffer (Chapters 2 & 3). It may be that the quicker the process can be, between collection and embedment in resin, the better.

Thirdly, the vast majority of the research contained within this thesis was conducted within controlled environmental facilities. Therefore, despite benefiting from the ability to exclude additional factors, which may have added to, or interacted with, the damage inflicted by heat stress, there must be an appreciation that controlled environmental facilities do not allow for the natural occurrence of factors which may affect heat stress tolerance mechanisms in the field. An example of such a factor is rooting structure. The structure of roots is conceivably very different when grown in a field, than when grown within the limited capacity, and unnatural shape, of a pot. Therefore, even though environmental factors (e.g. humidity, light, temperature) will be harder to control within the field, there is much merit in eventually scaling a lot of this fundamental research up to the field level.

Fourthly, despite 35°C, uninterrupted for three days (72 hours), offering good conditions for establishing principles, it is undoubtable that such conditions of stress are not within even the long term future of global, let alone British, agronomy. Therefore, in the future, if desiring to

obtain an appreciation for the effects of future 'real world' scenarios (e.g. such as those outlined by IPCC (2014_a)) on pollen development, in addition to bringing research into the field, it may be prudent to reduce the magnitude, and length, of heat stresses. One means of amending the temperature treatments, in order to make them more realistic, may be having differing day and night temperatures. This would be in order to reflect the almost universal phenomenon of day temperatures being higher than those of the night.

6.2 The contextualisation of findings

Despite this being a plant biology thesis, it would be remiss to forgo contextualising the findings contained, in light of current, and future, geopolitics. This is not least because climate change, and the associated temperature increases (Kang & Banga, 2013) are, as well as being one of the major geopolitical issue facing humanity, very much a major reason for conducting this research in the first place.

6.2.1 Continued changes in global climate

Despite global yearly greenhouse gas emissions having risen from 27Gt in 1970 to 49Gt in 2010, over this time the percentage of these gases, comprised of carbon dioxide (CO_2), has remained relatively constant at around 74% (IPCC, 2014_b). CO_2 emissions levels are strongly, positively correlated with both levels of GDP (Figure 6.2a) and human population (Figure 6.2b). Therefore, in a desire to see GDP continue to increase, especially within the least developed nations on earth, knowing the global human population is expected to grow further as the 21st century progresses, and in light of the CO_2's very strong historic correlations with elevations in temperature (Petit *et al.*, 1999), concerted efforts must be made to make sure CO_2 levels do not continue to rise. Whether this is done through further encouraging economies to be less carbon dependent, that therefore disassociate economic growth from increases in CO_2 emissions, or introducing measures that would curtail human population growth, something must be done.

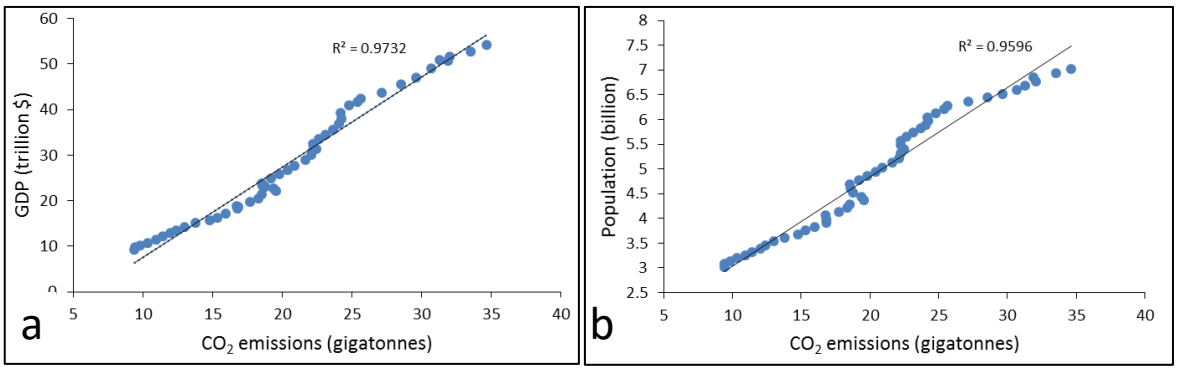


Figure 6.2: Correlations between global carbon dioxide (CO_2) emissions and a) global GDP (at market prices (constant 2005 US$)) (The World Bank, 2015), and b) global human population (FAOSTAT, 2015; The World Bank, 2015).

6.2.2 Other, non-agricultural, geopolitical issues effecting food security

Currently, sufficient food is produced to feed our existing global population (WFP, 2015). However, about 795 million people globally still remain undernourished (FAO *et al.*, 2015). There are, therefore, a plethora of sociological and economic factors that must be considered regarding food security, over and above purely agricultural factors. Two such areas are global conflict, and post-harvest losses.

6.2.2.1 Conflict/political instability

Conflict (e.g. war, rioting and general civil unrest) can be both a cause, and a consequence, of food insecurity (Brinkman & Hendrix, 2011). However, even though at times characterised by conflict, political uncertainty, even without conflict, can greatly reduce agronomic productivity. For example, crop production requires farmers to make numerous medium to long term investments, not least committing costly seeds to the ground. Therefore, knowing that any profit will only be attained after a number of months, the confidence in the political stability of the region, in which they work, will be instrumental in farmers' decisions, in relation to how much, if any, capital (e.g. seeds) they chose to invest (Deaton & Lipka, 2015).

An example of how politics can affect food security, and conversely, food security effect politics, is that one of the major global issues of the last years, the more pronounced political instability in the Middle East and North Africa, was initially caused, if only partially, by a rise in food prices (Brinkman & Hendrix, 2011).

6.2.2.2 The non-consumption of food after harvest

In many regions around the world, the many challenges of producing adequate amounts of food are followed by challenges in getting this food from 'farm to fork'. Especially in those countries were climatic conditions (e.g. elevated temperatures) and the speed of the supply chain are not conducive with the movements of perishable harvests (Hodges *et al.*, 2011), large amounts of food never make it to the consumer.

The total losses of food grains in India, pre-consumer purchase, is thought to be around 11-15% of total production (Basavaraja *et al.*, 2007). With most of this loss, at least in the rice and wheat grown in Karnataka state, experienced at the 'farm level', and especially during the storage of the grains at the 'farm level' (Figure 6.3). Basavaraja *et al.* (2007) attribute this high level of losses, during farm level storage, to failures in storage facilities in preventing harvest degradation due to biotic (rodents and insects) and abiotic (dampness) factors. Upon

observing the considerable levels of losses observed in both rice (37%) and wheat (37%) during the collection/preparation phases (harvesting, threshing, cleaning/winnowing & drying) (Figure 6.3), it is worth noting that the availability of more advanced machinery may reduce such losses at this stage.

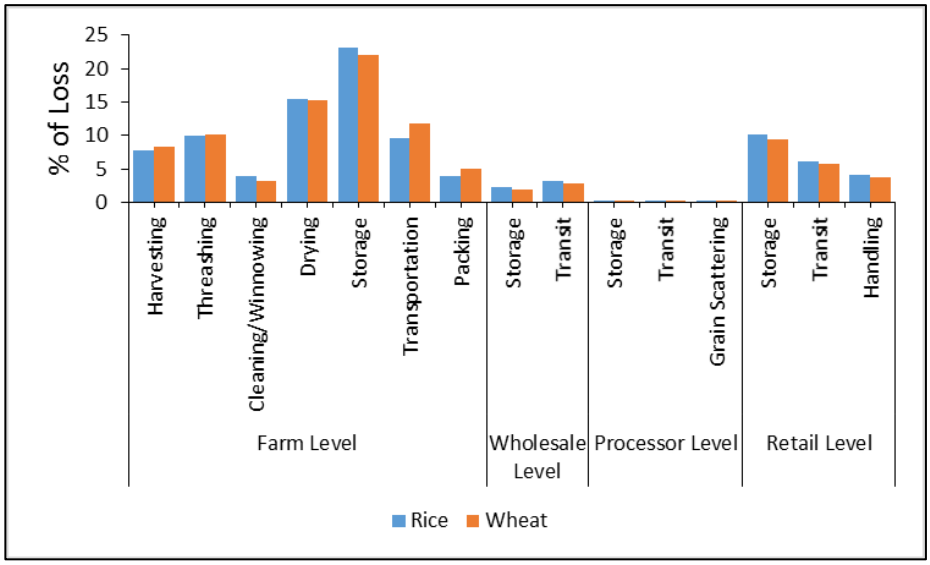


Figure 6.3: Estimated losses at different stages in rice and wheat processing, in Karnataka state, during 2003-2004 (Basavaraja *et al.*, 2007).

In addition to post-harvest losses, globally a varying amount of food goes wasted, in that once it is in the consumer's possession it is not actually consumed, but instead thrown away. With approximately one-third of food produced for human consumption being lost or wasted globally, this inevitably means that huge amounts of the resources used, and greenhouse gases emitted, in food production, are done so in vain (FAO, 2011).

In medium to high-income countries, food is lost, to a '*significant extent*', when in the consumer's possession, whereas in low-income countries, food is lost mostly during the early and middle stages of the food supply chain (FAO, 2011). Per capita food wastage by consumers in Europe and North-America is anticipated to be approximately 95-115 kg/year, while this figure in sub-Saharan Africa and South/Southeast Asia is only 6-11 kg/year (FAO, 2011).

6.3 Concluding remarks

I think that the results contained within this thesis will make a meaningful contribution to increasing global food security in one of the world's staple crops, wheat. Even though this contribution may not primarily come directly from this research, which perhaps lacked depth in order to accomplish breadth, I would expect the work contained within this thesis to provide a firm foundation for further research into one or more of the aspects covered.

However, whilst not wishing, as a biologist, to descend into a defeatist attitude, I cannot help thinking that any progress we as biologists/agronomists make, in relation to increasing production, must be contextualised in light of factors such as politics, economics and philosophy. This thinking is predicated on the understanding that where, for example, famine is not the intrinsic lack of food, but instead the lack of available food, food security will never be primarily a biological issue. Are we, as a species, ready to take active steps in order to reduce our population number, which has nearly doubled in my lifetime? Are we in the developed world ready to amend our consumption habits, which put a great strain on the global environment, and encourage those in the developing world to never adopt these same habits?

References

- Agar O., Tan D. K. Y., Barton D. A., Trethowan R. M., 2015, Genetic variation in wheat pollen heat tolerance, *Proceedings of the 17th ASA Conference*, Hobart.
- Ahmed F. E., Hall A. E., DeMason D. A., 1992, Heat injury during floral development in cowpea (*Vigna unguiculata*, Fabaceae), *American Journal of Botany*, **79** (7), 784-791.
- Alexandratos N., Bruinsma J., 2012. *World agriculture towards 2030/2050: the 2012 revision*. ESA Working paper No. 12-03. Rome, FAO.
- Alghabari F., Lukac M., Jones H. E., Gooding, M. J., 2014, Effect of Rht alleles on the tolerance of wheat grain set to high temperature and drought stress during booting and anthesis, *Journal of Agronomy and Crop Science*, **20** (1), 36-45.
- Armstrong L., 2014, Epigenetics, London/New York, Garland Scientific.
- Avnery S., Mauzerall D. L., Liu J., Horowitz L. W., 2011, Global crop yield reductions due to surface ozone exposure: 2. Year 2030 potential crop production losses and economic damage under two scenarios of O3 pollution. *Atmospheric Environment*, **45** (13), 2,297-2,309.
- Banga S. S., Kang M. S., 2013, Developing climate resilient crops: a conceptual framework, In: *Combating climate change, An agricultural perspective*, Kang M. S., Banga S. S, Eds., USA, CrC Press.
- Barnabás B., Jäger K., Feher A., 2008, The effect of drought and heat stress on reproductive processes in cereals, *Plant, Cell & Environment*, **31** (1), 11-38.
- Barnabás B., Kovács G., 1997, Storage of pollen, In: *Pollen Biotechnology for Crop Production and Improvement*, Shivanna K. R., Sawhney V. K., Eds., Cambridge University Press, UK.
- Basavaraja H., Mahajanashetti S. B., Udagatti N. C., 2007, Economic analysis of post-harvest losses in food grains in India: a case study of Karnataka, *Agricultural Economics Research Review*, **20** (1), 117-126.
- Basnet B. R., Glover K. D., Ibrahim A. M. H., Yen Y., Chao S., 2012, A QTL on chromosome 2DS of 'Sumai 3' increases susceptibility to Fusarium head blight in wheat, *Euphytica*, **186** (1), 91–101.
- Bennett M. D., Finch R. A., Smith J. B., Rao M. K., 1973, The time and duration of female meiosis in wheat, rye and barley, *Proceedings of the Royal Society London, Biological Sciences*, **183** (1072), 301-319.
- Bennington C. C., McGraw J. B., 1995, Natural selection and ecotypic differentiation in *Impatiens pallida*, *Ecological Monographaphs*, **65** (3), 303-324.
- Beri S. M., Anand S. C., 1971, Factors affecting pollen shedding capacity in wheat, *Euphytica*, **20** (2), 327-332.
- Bingham J., 1966, Varietal response in wheat to water supply in the field, and male sterility caused by a period of drought in a glasshouse experiment, *Annals of Applied Biology*, **57** (3), 365-377.
- Bita C. E., Gerats T., 2013, Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops, *Frontiers in Plant Science*, **4**, 273-291.
- Boden S. A., Cavanagh C., Cullis B. R., Ramm K., Greenwood J., Finnegan E. J., Trevaskis B., Swain S. M., 2015, *Ppd-1* is a key regulator of inflorescence architecture and paired spikelet development in wheat, *Nature Plants*, **1** (14,016), 1-6.

- Brinkman H.-J., Hendrix C. S., 2011, *Food Insecurity and Violent Conflict: Causes, Consequences, and Addressing the Challenges*, Rome, World Food Program.
- Burke M. B., Lobell D. B., Guarino L., 2009, Shifts in African crop climate by 2050, and the implications for crop improvement and genetic resoucres conservation, *Global Environment Change*, **19** (3), 317-325.
- Burney J. A., Davis S. J., Lobell D. B., 2010, Greenhouse gas mitigation by agricultural intensification, *Proceedings of the National Academy of the United Sciences of America*, **107** (26), 12,052-12,057.
- Cagauan A. G., 1995, The impact of pesticides on ricefield vertebrates with emphasis on fish, In: Impact of pesticides on farmer health and rice environment, Pingali P. L., Roger P. A., Eds., USA, Kluwer Academic Publishers.
- Castle L. A., Wu G., McElroy D., 2006, Agricultural input traits: past, present and future, *Current Opinion in Biotechnology*, **17** (2), 105-112.
- Cattivelli L., Rizza F., Badeck F.-W., Mazzucotelli E., Mastrangelo A. M., Francia E., Marè, Tondelli A., Stanca A. M., 2008, Drought tolerance improvement in crop plants: An integrated view from breeding to genomics, *Field Crops Research*, **105** (1-2), 1-14.
- Century K. Reuber T. L., Ratcliffe O. J., 2008, Regulating the regulators: The future prospects for transcription-factor based agricultural biotechnology products, *Plant Physiology*, **147** (1), 20-29.
- CerealCentral, 2015, Cereal Staging, [online], Available at: <http://www.cerealcentral.ca/crop-management_cereal-staging.aspx>, [Accessed 15/4/15].
- Cerović R., Ružić Đ., Mićić N., 2000, Viability of plum ovules at different temperatures, *Annals of Applied Biology*, **137** (1), 53-59.
- Chasan R., 1992, Breaching the callous wall, *The Plant Cell*, **4** (7), 745-746.
- Chen C. B., Xu Y. Y., Ma H., Chong K., 2005, Cell biological characteristics of male meiosis and pollen development in rice, *Journal of Integrative Plant Biology*, **47** (6), 734-744.
- Chrispeels M. J., Sadava D., 2003, Development, productivity, and sustainability of crop production, In: *Plants, Genes and Crop Biotechnology*, Second Edition, Chrispeels M. J., Sadava D. E., Eds., USA, Jones and Bartlett Publishers.
- Christiaensen L., Demery L., Kuhl J., 2011, The (evolving) role of agriculture in poverty reduction – an empirical perspective, *Journal of Development Economics*, **96** (2), 239-254.
- CIMMYT, 2015, China's Wheat Production Critical to Global Food Security, [online] Available at: <http://www.cimmyt.org/en/wheat-matters/item/china-s-wheat-production-critical-to-global-food-security> [Accessed 16/1/2015].
- Clément C., Chavant L., Burrus M., Audran J. C., 1994, Anther starch variation in *Lilium* during pollen development, *Sexual Plant Reproduction*, **7** (6), 347-356.
- Coghlan A., Marchall M., 2012, Africa's Underused Breadbasket, *NewScientist*, **2886**, 7.
- Cook O. F., 1913, Wild wheat in Palestine, Washington, USDA.
- Coumou D., Rahmstorf S., 2012, A decade of weather extremes, *Nature Climate Change*, **2**, 491-496.
- Craufurd P. Q., Vadz V., Jagadish S. V. K., Prasad P. V. V., Zaman-Allah M., 2013, Crop science experiments designed to inform crop modelling, *Agricultural and Forest Meteorology*, **170**, 8-18.
- Culley T. M., Klooster M. R., 2007, The cleistogamous breeding systems: a review of its

frequency, evolution, and ecology in angiosperms. *The Botanical Review*, **73** (1), 1-30.
- Dahm R., 2008, Discovering DNA: Friedrich Miescher and the early years of nucleic acid research, *Human Genetic*, **122** (6), 565-581.
- Dai A., 2013, Increasing drought under global warming in observations and models, *Nature Climate Change*, **3**, 52-53.
- Dale R. J., 1983, Temperature perturbations in the midwestern and southeastern United States important for corn production, In: *Crop reactions of water and temperature stresses in humid, temperate climates*, Raper C. D., Kramer P. J., Eds., USA, Westview Press.
- Dar W. D., Gowda C. L. L., 2013, Decline agricultural productivity and global food security, *Journal of Crop Security*, **27** (2), 242-254.
- Dawson J., Sözen E., Vizir I., Van Waeyenberge S., Wilson Z. A., Mulligan B. J., 1999, Characterization and genetic mapping of a mutation (ms35) which prevents anther dehiscence in Arabidopsis thaliana by affecting secondary wall thickening in the endothecium. *New Phytologist,* **144** (2), 213–222.
- Deaton B. J., Lipka B., 2015, Political instability and food security, *Journal of Food Security*, **3** (1), 29-33.
- Demotes-Mainard S., Doussinault G., Meynard J. M., 1995, Effects of low radiation and low temperature at meiosis on pollen viability and grain set in wheat, *Agronomie*, **15** (6), 357-365.
- De Vries A. P., 1971, Flowering biology of wheat, particularly in view of hybrid seed production – a review, *Euphytica*, **20** (2), 152-170.
- De Vries A. P., 1972, Some aspects of cross pollination in wheat (*Triticum aestivum* L.) 1. Pollen concentration in the field pattern, weather conditions and level as compared to the height of the pollen donor, *Euphytica*, **21** (2), 185-203.
- De Vries A. P., 1974_a, Some aspects of cross-pollination in wheat (*Triticum aestivum* L.). 3. Anther length and number of pollen grain per anther, *Euphytica*, **23** (1), 11-19.
- De Vries, A. P., 1974_b, Some aspects of cross-pollination in wheat (*Triticum aestivum* L.). 4. Seed set on male sterile plants as influenced by distance from the pollen source. Pollinator: Male sterile ratio and width of the male sterile strip. *Euphytica,* **23** (3), 601–622.
- Dolferus R., Ji X., Richards R. A., 2011, Abiotic stress and control of grain number in cereals, *Plant Science*, **181** (4), 331-341.
- Easterling W., 2005, Assessing the consequences of climate change for food security: a view from the intergovernmental panel on climate change, In: *Climate change and global food security*, Lal R., Uphoff N., Stewart B. A., Hansen D. O., Eds., USA, Taylor & Francis.
- Eastham K., Sweet J., 2002, *Genetically modified organisms (GMOs): the significance of gene flow through pollen transfer*, Environmental issue report No 28, Denmark, European Environment Agency.
- Echlin P., 1971, The role of the tapetum during microsporogenesis of Angiosperms, In: *Pollen: Development and Physiology*, Heslop-Harrison J., Eds., UK, Butterworth & Co.
- Eckardt N. A., 2010, Evolution of Domesticated Bread Wheat, *The Plant Cell*, **22** (4), 993.
- Elek H., Werner P., Smart L., Gordon-Weeks R., Nádasy M., Pickett J., 2009, Aphid resistance in wheat varieties, *Communications in Agriculture and Applied Biological Sciences*, **74** (1), 233-241.

- Ellen J., Spiertz J. H. J, 1980, Effects of rate and timing of nitrogen dressings on grain yield formation of winter wheat (*T. aestivum* L.), *Fertilizer Research*, **1** (3), 177-190.
- Endo M., Tsuchiya T., Hamada K., Kawamura S., Yano K., Ohshima M., Higashitani A., Watanabe M., Kawagishi-Kobayashi M., 2009, High tempertaure cause male sterility in rice plants with transcriptional alternatuons during pollen development, *Plant & Cell Physiology*, **50** (11), 1,911-1,922.
- Evans L. T., Wardlaw L. F., Fischer R. A., 1975, Wheat, In: Crop Physiology, Some Case Histories, UK, Cambridge University Press.
- Fan S., Hazell P., 2001, Returns to public investment in the less-favored areas of India and China, *American Journal of Agriculture Economics*, **83** (5), 1,217-1,222.
- FAOSTAT, 2015, Online Statistical Service, [ONLINE] Available at: http://faostat3.fao.org/home/E, [Accessed 09 January 15].
- Feng B., Lu D., Ma X., Peng Y., Sun Y., Ning G., Ma H., 2012, Regulation of the Arabodopsis anther transcription by *DYT1* for pollen development, *The Plant Journal*, **72** (4), 612-624.
- Fernández Gómez J., Talle B., Wilson Z. A., 2015, Anther and pollen development: a conserved developmental pathway, *Journal of Integrative Plant Biology*, **57** (11), 876-891.
- Fernández Gómez J., Wilson Z. A., 2012, Non-destructive staging of barley reproductive development for molecular analysis based upon external morphology*, Journal of Experimental Botany*, **63** (11), 4,085-4,094.
- Fernández Gómez J., Wilson Z. A., 2014, A barley PHD finger transcription factor that confers male sterility by affecting tapetal development, *Plant Biotechnology Journal*, **12** (6), 765-777.
- Food and Agriculture Organization, 1996. Rome Declaration on World Food Security and World Food Summit Plan of Action. World Food Summit 13-17 November 1996, Rome, Food and Agriculture Organization.
- Food and Agriculture Organization, 2002, *World Agriculture: towards 2015/2030, Summary Report*, Rome, Food and Agriculture Organization.
- Food and Agriculture Organization, 2009, *How to feed the world in 2050. High-level experts' forum*, Rome, Food and Agriculture Organization.
- Food and Agriculture Organization, 2011, *Global food losses and food waste – Extent, causes and prevention*, Rome, FAO.
- Food and Agriculture Organization, World Food Program, International Fund for Agricultural Development, 2012, The state of food insecurity in the world: economic growth is necessary but not sufficient to accelerate reduction of hunger and malnutrition, Rome, FAO/WFP/IFAD.
- Food and Agriculture Organisation, International Fund for Agricultural Development, World Food Program, 2015, *State of food insecurity in the world, in brief*, [ONLINE] Available at: http://www.fao.org/3/a-i4671e.pdf, [Accessed 14 December 15].
- Foulkes M. J., Slafer G. A, Davis W. J., Berry P. M., Sylvester-Bradley R., Martre P., Calderini D. F., Griffiths S., Reynolds M. P., 2011, Raising yield potential of wheat III. Optimizing partitioning to grain while maintaining lodging resistance, *Journal of Experimental Botany*, **62** (2), 469-486.
- Firon N., Shaked R., Peet M., Pharr D., Zamski E., Rosenfeld K., Althan L., Pressman E., 2006, Pollen grains of heat tolerant tomato cultivars retain higher carbohydrate concentration under heat stress conditions, *Scientia Horticulturae*, **109** (3), 212-217.
- Fischer R. A., Stockman Y. M., 1980, Kernel number per spike in wheat (*Triticum*

aestivum L.): responses to pre-anthesis shading, *Australian Journal of Plant Physiology*, **7** (2), 169-180.
- Ford K. E., Gregory P. J., Goodling M. J., Pepler S., 2006, Genotype and fungicide effects on late-season root growth of winter wheat, *Plant and Soil*, **284** (1), 33-44.
- Foulkes M. J., Sylvester-Bradley R., Weightman R., Snae J. W., 2007, Identifying phenolical traits associated with improved drought rristance in whinter wheat, *Field Crop Research*, **103** (1), 11-24.
- Gaju O., Reynolds M. P., Spakes D. L., Foulkes M. J., 2009, Relationships between large-spike phenotype, grain number and yield potential in spring wheat, *Crop Science*, **49**, 961-973.
- Gaju O., Reynolds M. P., Sparkes D. L., Mayes S., Ribas-Vargas G., Crossa J., Foulkes M. J., 2014, Relationships between physiological traits, grain number and yield potential in a wheat DH population of large spike phenotype, *Field Crop Research*, **164**, 126-135.
- Gale M. D., Youssefian S., 1985, Dwarfing genes in wheat. In: *Progress in plant breeding*, Russell G. E. (Ed), London, Butterworths and Co.
- Gesch R. W., King I. H., Gallo-Meagher M., Vu J. C. V., Boote K. J., Allen L. H., Bowes G., 2003, Rubisco expression in rice leaves is related to genotypic variation of photosynthesis under elevated growth CO_2 and temperature, *Plant Molecular Biology*, **48** (5), 667-681.
- Gilsinger J. Kong L., Shen X., Ohm H., DNA markers associated with low Fusarium head blight incidence and narrow flower opening in wheat, *Theoretical and Applied Genetics*, **110** (7), 1,218-1,225.
- Giorno F., Wolters-Arts M., Mariani C., Rieu I., 2013, Ensuring reproduction at high temperatures: the heat stress repronse during anther and pollen development, *Plants*, **2** (3), 489-506.
- Gu J. N., Zhu J., Yu Y., Teng X. D., Lou Y., Xu X. F., Liu J. L., Yang Z. N., 2014, *DTY1* directly regulates the expression of *TDF1* for tapetum development and pollen wall formation in Arabidopsis, *The Plant Journal*, **80** (6), 1,005-1,013.
- Gustafson P., Raskina O., Ma X. F., Nevo E., 2009, Wheat Evolution, Domestication, and Improvement, In: *Wheat Science and Trade*, Carver B. F. Ed., Wiley-Blackwell, USA.
- Hansen J., Sato M., Ruedy R., 2012, Perception of Climate Change, *Proceedings of the National Academy of Sciences*, **109** (37), 2,415-2,423.
- Harlan J. R., Zohary D., 1966, Distribution of wild wheat and barley, *Science*, **153** (3,740), 1,074-1,080.
- Hayase H., Satake T., Nishiyama I., Ito N., 1969, Male Sterility Caused by Cooling Treatment at the Meiotic Stage in Rice Plants : II. The most sensitive stage to cooling and the fertilizing ability of pistils, *Japanese Journal of Crop Science*, **38** (4), 706-711.
- Hazell, P.B.R., 2009, The Asian Green Revolution, Washington D.C, International Food Policy Research Institute.
- He Z., Xia X., Peng S., Lumpkin T. A., 2014, Meeting demands for increased cereal production in China, *Journal of Cereal Science*, **59** (3), 235-244.
- Hedhly A., Hormaza J. I., Herrero M., 2009, Global warming and sexual plant reproduction, *Trends in Plant Science*, **14** (1), 30-36.
- Heslop-Harrison J., Heslop-Harrison Y., 1992, Intracellular motility, the actin cytoskeleton and germinability in the pollen of wheat (*Triticum aestivum* L.), *Sexual Plant Reproduction*, **5** (4), 247-255.

- Hodges R. J., Buzby J.C., Bennet B., 2011, Posthavest losses and waste in develped and less developed contries: opportunities to improve resource use, *Journal of Agrictural Science*, **149** (Supplment 1), 37-45.
- Honys D., Twell D., 2004, Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*, *Genome Biology*, **5** (11), R85.
- Howarth C. J., Ougham H. J., 1993, Tansley Review No. 51 Gene expression under temperature stress, *New Phytologist*, **125** (1), 1-26.
- Hucl P., 1996, Out crossing rates for 10 Canadian spring wheat cultivars, *Canadian Journal of Plant Science*, **76** (3), 423-427.
- Hurkman W. J., McCue K. F., Altenbach S. B., Korn A., Tanaka C. K., Kothari K. M., Johnson E. L., Bechtel D. B., Wilson J. D., Anderson O. D., DuPont F. M., 2003, Effect of temperature on expression of genes encoding enzymes for startch biosynthesis in developing wheat endosperm, *Plant Science*, **164** (5), 873-881.
- IPCC, 2007: *Summary for Policymakers,* In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Solomon, S., Qin D., Manning M., Marquis M, Averyt K., Tignor M. B., Miller H. L., Cheng Z., Eds., USA, Cambridge University Press.
- IPCC, 2014_a, Summary for Policymakers, In: *Climate Change 2014: Impacts, Adaptation, and Vulnerability*, Field C. B., Barros V. R., Dokken D. J., Mach K. J., Mastrandrea M. D., Bilir T. E., Chatterjee M., Ebi K. L., Estrada Y. O., Genova R. C., Girma B., Kissel E. S., Levy A. N., MacCracken S., Mastrandrea P. R., White L. L., Eds., Cambridge University Press, Cambridge/New York.
- IPCC, 2014_b, Technical Summary, In: *Climate Change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change,* Edenhofer, O., Pichs-Madruga R., Sokona Y., Farahani E., Kadner S., Seyboth K., Adler A., Baum I., Brunner S., Eickemeier P., Kriemann B., Savolainen J., Schlömer S., von Stechow C., Zwickel T., Minx J. C., Eds., Cambridge University Press, Cambridge/New York.
- IPCC, 2014_c, Food security and food production systems, In: *Climate Change 2014: Impacts, Adaptation, and Vulnerability*, Field C. B., Barros V. R., Dokken D. J., Mach K. J., Mastrandrea M. D., Bilir T. E., Chatterjee M., Ebi K. L., Estrada Y. O., Genova R. C., Girma B., Kissel E. S., Levy A. N., MacCracken S., Mastrandrea P. R., White L. L., Eds., Cambridge University Press, Cambridge/New York.
- Iwahori, S., 1966, High temperature injuries in tomato. V. Fertilization and development of embryo with special reference to the abnormalities caused by high temperature. *Journal of the Japanese Society for Horticultural Science*, **35** (4), 379–386.
- Jäger K., Fábián A., Barnabás B., 2008, Effect of water deficit and elevated temperature on pollen development of drought sensitive and tolerant winter wheat (*Triticum aestivum* L.) genotypes, *Acta Biologica Szegediensis*, **52** (1), 67-71.
- Ji X., Shiran B., Wan J., Lewis D. C., Jenkins C. L. D., Condon A. G., Richards R. A., Dolferus R., 2010, Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat, *Plant, Cell & Environment*, **33** (6), 926–942.
- Joppa L. R., McNeal I. H., Berg M. A., 1968, Pollen production and pollen shedding of hard red spring (*Triticum aestivum* L. em. Thell.) and durum (*T. durum* Desf.) wheat. *Crop Science*, **8** (4), 487-490.

- Kang M. S., Banga S. S., 2013, Global agricultural and climate change: a perspective, In: *Combating climate change, An agricultural perspective*, Kang M. S., Banga S. S, Eds., USA, CrC Press.
- Karakach T. K. Flight R. M., Douglas S. E., Wentzell P. D., 2010, An introduction to DNA microarrays for gene expression analysis, *Chemometrics and Intelligent Laboratory Systems*, **104** (1), 28-52.
- Kawanabe T., Ariizumi T., Kawai-Yamada M., Uchimiya H., Toriyama K., 2006. Abolition of the tapetum suicide program ruins microsporogenesis, *Plant and Cell Physiology,* **47** (6), 784–787.
- Kendy E., Gerard-Marchant P., Walter T., Zhang Y., Liu C., Steenhuis T. S., 2003, A soil-water-balance approach to quantify groundwater recharge from irrigated cropland in the North China Plain, *Hydrological Processes*, **17** (10), 2,001-2,031.
- Khan M. N. A., 1968, *Pollen production in twelve standard hard red winter wheat cultivars*, MSc. thesis, Kansas University.
- Khodadadi M., Fotokian M H., Miransari M., 2011, Genetic diversity of wheat (*Triticum aestivum* L.) genotypes based on cluster and principal component analyses for breeding strategies, *Australian Journal of Crop Science*, **5** (1), 17-24.
- Kirby E. J. M., Appleyard M., 1984, *Cereal Developmental Guide, 2nd edition*, UK, Arable Unit.
- Kohler G. O., 1944, The effect of stage of growth on the chemistry of grasses, *Journal of Biological Chemistry*, **152** (2), 215-223.
- Kokic P., Heaney A., Pechey L., Crimp S., Fisher B. S., 2005, Climate change – predicting the impacts on agriculture: a case study, *Australian Commodities*, **12** (1), 161-170.
- Komaki M. K., Tsunewaki K., 1981, Genetical studies on the difference of anther length among common wheat cultivars, *Euphytica*, **30** (1), 45-53.
- Krishnan M., Nguyen H. T., Burke J. J., 1989, Heat shock protein synthesis and thermal tolerance in wheat, *Plant Physiology*, **90** (1), 140-145.
- Ku S., Yoon H., Suh H. S., Chung Y.-Y., 2003, Male-sterility of thermosensitive genic male-sterile rice is associated with premature programmed cell death of the tapetum, *Planta*, **217** (4), 559–565.
- Langer S. M., Longin C. F. H., Würschum T., 2014, Phenotypic evaluation of floral and flowering traits with relevance for hybrid breeding in wheat (Triticum aestivum L.), *Plant Breeding*, **133** (4), 433-441.
- Lalonde S., Beebe D. U., Saini H. S., 1997, Early signs of disruption of wheat anther development associated with the induction of male sterility by meiotic-stage water deficit, *Sexual Plant Reproduction*, **10** (1), 40-48.
- Larkindae J., Mishkind M., Vierling E., 2005, Plant responses on high temperature, In: *Plant Abiotic Stress*, Jenks M. A., Hasegawa P. M., Eds., UK, Blackwell Publishing.
- Laser K. D., Lersten N. R., 1972, Anatomy and cytology of microsporogenesis in cytoplasmic male sterile Angiosperms, *Botanical Review*, **38** (3), 425-454.
- Law C. N., Snape J. W., Worland A. J., 1981, Reduced fertility of wheat associated with Rht3. Plant breeding institute annual report 1980, Cambridge, Plant Breeding Institute.
- Law C. N., Worland A. J., 1985, An effect of temperature on the fertility of wheat containing the dwarfing genes Rht1, Rht2 and Rht3, Plant breeding institute annual report 1984, Cambridge, Plant Breeding Institute.
- Leather S. R., 2010, Precise Knowledge of plant growth stages enhances applied and pure research, *Annals of Applied Biology*, **157** (2), 159-161.

- Li Q.-J., Xu B., Chen X.-Y., Wang L.-J., 2007, The effects of increased expression of an *Arabidopsis* HD-ZIP gene on leaf morphology and anther dehiscence, *Plant Science*, **173** (5), 567-576.
- Liu Y., Wang E., Yang X., Wang J., 2010, Contributions of climatic and crop varietal changes to crop production in the North China Plain, since 1980s, Global Change Biology, **16** (8), 2,287–2,299.
- Livak K. J., Schmittgen T. D., 2001, Analysis of relative gene expression dada using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method, *Methods*, **25** (4), 402-408.
- Lobell D. B., Asner G. P., 2003, Climate and management contributions to recent trends in US agriculture yields, *Science*, **299** (5,609), 1,032.
- Lobo I., 2008, Environmental influences on gene expression. *Nature Education,* **1** (1), 39.
- Lohmar B., Gale F., Tuan F., Hansen J., 2009, *China's ongoing agricultural modernization: challenges remain after 30 years of reform*, Economic Information Bulletin Number 51, USA, United States Department of Agriculture.
- Long S. P., Ort D. R., 2010, More than taking the heat: crops and global change, *Current Opinion in Plant Biology*, **13** (3), 241-248.
- Long S. P., 2012, Virtual special issue on food security – greater than anticipated impacts of near-term glabal atmospheric change on rice and wheat, *Glabal Change Biology*, **18** (5), 1,489 – 1,490.
- Lu P., Chai M., Yang J., Ning G., Wang G., Ma H., 2014, The Arabidopsis CALLOSE DEFECTIVE MICROSPORE1 gene is required for male fertility through regulating callose metabolism during microspore-sporogenesis, *Plant Physiology*, **164** (4), 1,893-1,904.
- Lukac M., Gooding M. J., Griffiths S., Jones H. E., 2012, Asynchronous flowering and within-plant flowering diversity in wheat and the implications for crop resilience to heat, *Annals of Botany*, **109** (4), 843-850.
- Ma L., Yang Z., Zhang S., 2013, Dex1, a plasma membrane-localized protein, functions in microspore development by affecting *CalS5* expression in Arabidopsis thaliana, *Chinese Science Bulletin*, **58** (23), 2,855-2,861.
- Maestri E., Klueva N. Perrotta C., Gulli M., Nguyen H. T., Marmiroli N., 2002, Molecular genetics of heat tolerance and heat shock protein in cereals, *Plant Molecular Biology*, **48** (5), 667-681.
- Mahan J. R., McMichael B. L., Wanjura D. F., 1995, Methods for reducing the adverse effects of temperatures stress on plants: a review, *Environmental and Experimental Botany*, **35** (3), 251-258.
- Malik A., Firoz A., Afzal O., Jha V., Choi I., 2013, Protein structure databases and 3D structure prediction tools, In: Advances in protein chemistry, Ishfaq G. M., Sheikh I. A. Eds., USA, OMICS Group.
- Martineau J. R., Specht J. E., Williams J. H., Sullivan C. Y., 1979, Temperature tolerance in soybeans. I. Evaluation of a technique for assessing cellular membrane thermostability, *Crop Science*, **19** (1), 75-81.
- Matsui T., Omasa K., 2002, Rice (*Oryza sativa* L.) Cultivars tolereant to high temperture at flowering: Anther Characteristics. *Annals of Botany*, **89** (6), 683-687.
- Matsui T., Omasa K., Horie T., 2001, Comparison between anthers of two rice (*Oryza sativa* L.) Cultivars with tolerance to high temperature at flowering or susceptibility, *Plant Production Science*, **4** (1), 36-40.

- Mearns L. O., Rosenzweig C., Goldberg R., 1997, Mean and variance change in climate scenarios: methods, agriculture applications, and measures of uncertainty, *Climate Change*, **35** (4), 367-396.
- Millet E, 1986, Relationship between grain weight and the size of floret cavity in the wheat spike, *Annals of Botany*, **58** (3), 417-423.
- Mizelle M. B., Sethi R., Ashton M. E., Jemen W. A., 1989, Development of the pollen grain and tapetum of wheat (Triticum aestivum) in untreated plants and plants treated with chemical hybridizing agent RH0007. *Sexual Plant Reproduction*, **2** (4), 231–253.
- Nabim, 2012_a, *Wheat Production*, [online] Available at: <http://www.nabim.org.uk/content/1/31/wheat-production.html> [Accessed 30 November 2012].
- Nabim, 2012_b, *Harvest Data*, [online] Available at: <http://www.nabim.org.uk/content/1/102/harvest-data.html> [Accessed 30 November 2012].
- Necchi A., Pogna N. E., Mapelli S., 1987, Early and late heat shock proteins in wheat and other cereal species*, Plant Physiology*, **84** (4), 1,378-1,384.
- Nelson G. C., Rosegrant M. W. Koo J., Robertson R., Sulser T., Zhu T., Ringler C., Msangi S., Palazzo A., Batka M., Magalhaes M., Valmonte-Santos R., Ewing M., Lee D., 2009, *Climate Change: Impact on agriculture and costs of adaptation*, Washington, International Food Policy Research Institute.
- Oliver S. N., van Dongen J. T., Alfred S. C., Manun E. A., Zhao X. C., Saini H. S., Fernandes S. F., Blanchard C. L., Sutton B. G., Geigenberger P., Dennis E. S., Dolferus R., 2005, Cold-induced repression of the rice anther-specific cell wall invertase gene OSINV4 is correlated with sucrose accumulation and pollen sterility, *Plant, Cell & Environment*, **28** (12), 1,534–1,551.
- Oshino T., Abiko M., Saito R., Ichiishi E., Endo M., Kawagishi-Kobayashi M., Higashitani A., 2007, Premature progression of anther early development programs accompanied by comprehensive alternations in transcription during high-temperature injury in barly plants, *Molecular Genetic and Genomics*, **278** (1), 31-42.
- Parish R. W., Li S. F., 2010, Death of a tapetum: a programme of developmental altruism, *Plant Science*, **178** (2), 73–89.
- Pearce S., Saville R., Vaughan S. P., Chandler P. M., Wilhelm E. P., Sparks C. A., Al-Kaff N., Koroley A., Boulton M. I., Phillips A. L., Hedden P., Nicholson P., Thomas S. G., 2011, Molecular Characterization of *Rht-1* Dwarfing Genes in Hexaploid Wheat, *Plant Physiology*, **157** (4), 1,820-1,831.
- Peet M. M., Sato S., Gardner R. G., 1998, Comparing heat stress effects on male-fertile and male-sterile tomatoes, *Plant, Cell & Environment*, **21** (2), 225–231.
- Pelletier G., Budar F., 2007, The molecular biology of cytoplasmically inherited male sterility and prospects for its engineering, *Current Opinion in Biotechnology*, **18** (2), 121–125.
- Percival J., 1921, *The wheat plant – a monograph*, London, Duchworth & Co.
- Peterson R. F., 1965, *Wheat – botany, cultivation and utilization*, London, Leonard Hill Books.
- Petit J. R., Jouzel J., Raynaud D., Barkov N. I., Barnola J. M., Basile I., Bender M., Chappellaz J., Davis M., Delaygue G., Delmotte M., Kotlyakov V. M., Legrand M., Lipenkov V. Y., Lorius C., Pépin L., Ritx C., Saltzman E., Stievenard M., 1999, Climate and atmospheric histroy of the past 420,000 years from the Vostok ice core, Antarctica, *Nature*, **399**, 429-436.

- Pfeiffer T. W., 2003, From classical plant breeding to modern crop improvement, In: *Plants, Genes and Crop Biotechnology*, Second Edition, Chrispeels M. J., Sadava D. E., Eds., USA, Jones and Bartlett Publishers.
- Phan H. A., Iacuone S., Li S. F., Parish R. W., 2011, The *MYB80* transcription factor is required for pollen development and the regulation of tapetal progammed cell death in Arabodopsis thaliana, *The Plant Cell*, **23** (6), 2,209-2,224.
- Pingali P. L., Rosegrant M. W., 1994, *Confronting the environmental consequences of the Green Revolution in Asia*, USA, International Food Policy Research Institute.
- Porch T. G., Jahn M., 2011, Effects of high temperature stress on microsporogenesis in heat sensitive and heat tolerant genotypes of *Phaseolus vulgaris*, *Plant, Cell and Environment*, **24** (7), 723-731.
- Prasad P., Boote K., Allen L., Sheehy J., Thomas J., 2006, Species ecotype and cultivar differences in spikelets fertility and harvest index of rice in response to high temperature stress, *Field Crops Research*, **95** (2-3), 398-411.
- Rajki E., 1960, A nemesítésben használt búzafajtát nyitva és zárva virágzása, *Növénytermelés*, **9**(4), 309-320.
- Randolf L. F., 1932, Some effects of high temperature on polyploidy and other variations in maize, *Proceedings of the National Academy of Sciences of the United States*, **18** (3), 222-229.
- Reynolds M., Foulkes J., Furbank R., Griffiths S., King J., Murcchie E., Parry M., Slafer G., 2012, Achieve yield grain in wheat, *Plant, Cell & Environment*, **35** (10), 1799-1823.
- Rogers H. J., 2006, Programmed cell death in floral organs: how and why do flowers die?, *Annals of Botany*, **97** (3), 309–315.
- Rawson H. M., Baggs A. K., 1979, Influence of temperature between floral initiation and flag leaf emergence on grain number in wheat, *Australian Journal of Plant Physiology,* **6** (3), 391-400.
- Saini H. S., Aspinall D., 1981, Effect of water deficit on sporogenesis in wheat (Triticum aestivum L.)., *Annals of Botany*, **48** (5), 623-633.
- Saini H. S., Aspinall D., 1982, Abnormal Sporogenesis in Wheat (Triticum aestivum L.) Induced by Short Periods of High Temperature, *Annals of Botany*, **49** (6), 835-846.
- Saini H. S., Sedgley M., Aspinall D., 1984, Developmental anatomy in wheat of male sterility induced by heat stress, water deficit or abscisic acid, *Australian Journal of Plant Physiology*, **11** (4), 243-253.
- Sakata T., Takahashi H., Nishiyama I., Higashitani A., 2000, Effects of high temperature on the development of pollen mother cells and microspores in barley *Hordeum vulgare* L., *Journal of Plant Research*, **113** (4), 395-402.
- Sanders P. M., Bui A. Q., Weterings K., McIntire K. N., Hsu Y.-C., Lee P. Y., Truong M. T., Beals T. P., Goldberg R. B., 1999, Anther development defects in *Arabidopsis thaliana* male-sterile mutants, *Sexual Plant Reproduction*, **11** (6), 297-322.
- Sato S., Peet M. M., Thomas J. F., 2002, Determining critical pre- and post-anthesis periods and physiological processes in Lycopersicon esculentum Mill. Exposed to moderately elevated temperatures, *Journal of Experimental Botany*, **53** (371), 1,187-1,195.
- Sax K., 1937, Effect of variations in temperature on nuclear and cell division in *Tradescantia. American Journal of Botany,* **24** (4), 218-225.
- Sayed H. I., Godallah A. M., 1983, Variation in dry matter and grain filling characteristics in wheat cultivars, *Field Crops Research*, **7**, 61-71.

- Scott R. J., Spielman M., Dickinson H. G., 2004, Stamen structure and function, *The Plant Cell*, **16** (Supp. 1), 46-60.
- Semenov M. A, Shewry P. R., 2011, Modelling predicts that heat stress, not drought, will increase vulnerability of wheat in Europe, *Scientific Reports*, **1** (66), 1-5.
- Semenov M. A., Stratonovitch P., Alghabari F., Gooding M. J., 2014, Adapting wheat in Europe for climate change, *Journal of Cereal Science*, **59** (3), 245-256.
- Shi J., Cui M., Yang L., Kim Y.-J., Zhang D., 2015, Genetic and biochemical mechanisms of pollen wall development, *Trends in Plant Science,* **20** (11), 741-753.
- Shiferaw B., Smale M. Braun H.-J., Duveiller E., Reynolds M., Murichp G., 2013, Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security, *Food Security*, **5** (3), 291-317.
- Shivanna K. R., Cresti M., Ciampolini F., 1997, Pollen development and pollen-pistil interaction, In: *Pollen Biotechnology for Crop Production and Improvement*, Shivanna K. R., Sawhney V. K., Eds., UK, Cambridge University Press.
- Shivanna K. R., Sawhney V. K., 1997, Pollen biology and biotechnology: an introduction, In: *Pollen Biotechnology for Crop Production and Improvement*, Shivanna K. R., Sawhney V. K., Eds., UK, Cambridge University Press.
- Sillmann J., Roeckner E., 2008, Indices for extreme events in projections of anthropogenic climate change, *Climate Change*, **86** (1-2), 83-104.
- Sorensen A.-M., Kröber S., Unte U. S., Huijser P., Dekker K., Saedler H., 2003, The arabdopsis ABORTED MICROSPORES (*AMS*) gene encodes a MYC class transcription factors, *The Plant Journal*, **33** (2), 413-423.
- Stein L. D., 2010, The case for cloud computing in genome informatics, *Genome Biology*, **11** (5), 207-213.
- Steiner-Lange S., Unte U. S., Eckstein L., Yang C., Wilson Z. A., Schmelzer E., Dekker K., Saedler H., 2003, Disruption of Arabidopsis thaliana *MYB26* results in male sterility due to non-dehiscent anthers, *The Plant Journal*, **34** (4), 519-528.
- Steinmeyer F. T., Lukac M., Reynolds M. P., Jones H. E., 2013, Quantifying the relationship between temperature regulation in the ear and floret development stage in wheat (*Triticum aestivum* L.) under heat and drought stress, *Functional Plant Biology*, **40** (7), 700-707.
- Swindell W. R., Huebner M., Weber A. P., 2007, Plastic and adaptive expression patterns associated with temperature stress in *Arabidopsis thaliana*, *Heredity*, **99** (2), 143-150.
- Tao F., Yokozawa M., Xu Y., Hayashi Y., Zhang Z., 2006, Climate changes and trends in phenology and yields of field crops in China, 1981–2000, *Agricultural and Forest Meteorology*, **138** (1-4), 82–92
- Tashiro T., Wardlaw F., 1990, The response to high temperature shock and humidity changes prior to and during the early stages of grain development, *Australian Journal of Plant Physiology*, **17** (5), 551-561.
- The World Bank, 2015**,** World Bank Open Data, [ONLINE] Available at: http://data.worldbank.org/, [Accessed 09 January 15].
- Torlesse H., Kiess L., Bloem M. W., 2003, Association of household rice expenditure with child nutritional status indicates a role for macroeconomic food policy in combating malnutrition, *The Journal of Nutrition*, **133** (5), 1,320-1,325.
- Tribe D., 1994, *Feeding and greening the world: The role of international agricultural research*, Wallingford, CABI.

- United Nations, 2013, *World Population Prospects: The 2012 Revision,* [ONLINE] Available at: <http://esa.un.org/unpd/wpp/unpp/panel_population.htm> [Accessed 09 January 15].
- Uphof J. C. T., 1938, Cleistogamic flowers, *Botanical Review*, **4** (1), 21-49.
- Urzay J., Llewellyn S. G., Thompson E., Glover B. J., 2009, Wind gusts a plant aeroelasticity effect on the aerodynamics of pollen shedding: A hypothetical turbulence-initiated wind-pollination mechanism, *Journal of Theoretical Biology*, **259** (4), 785-792.
- Van Dingenen R., Dentener F. J., Raes F., Krol M. C., Emberson L., Cofala J., 2009, The global impact of ozone on agriculture crop yields under current and future air quality legislation, *Atmospheric Environment*, **43** (3), 604-618.
- Vizcay-Berrena G., Wilson Z. A., 2006, Altered tapetal PCD and pollen wall development in the Arabidopsis *MS1* mutant, *Journal of Experimental Botany*, **57** (11), 2,709-2,717.
- Wahid A., Gelani S., Ashraf M., Foolad M. R., 2007, Heat tolerance in plants: an overview, *Environmental and Experimental Botany*, **61** (3), 199-223.
- Wang A., Xia Q., Xie W., Datla R., Selvaraj G., 2003, The classical Ubisch bodies carry a sporophytically production structural protein (RAFTIN) that is essential for pollen development, *Proceedings of the National Academy of Sciences of the United States of America*, **100** (24), 14,487-14,492.
- Wei L. Q., Xu W. Y., Deng Z. Y., Su Z., Xue Y., Wang T., 2010, Genome-scale analysis and comparison of gene expression profiles in developing and germinated pollen in *Oryza sativa*, *BMC Genomics*, **11**, 338-357.
- Wiese M. V., 1987, *Compendium of Wheat Disease, Second Edition*, Minnesota, APS Press.
- Wilson Z. A., Song J., Taylor B., Yang C., 2011, The final split: the regulation of anther dehiscence, *Jounral of Experimetal Botany*, **62** (5), 1,633-1,649.
- Wilson Z. A., Zhang D. B., 2009, From Arabidopsis to rice: pathways in pollen development, *Journal of Experimental Botany*, **60** (5), 1,479-1,492.
- White M. A., Diffenbaugh N. S., Jones G. V., Pal J. S., Giorgi F., 2006, Extreme heat reduces and shifts United States premium wine production in the 21st century, *Proceedings of the National Academy of the United Sciences of America*, **103** (30), 11,217-11,222.
- Whitford R., Fleury D., Reif J. C., Garcia M., Okada T., Korzun V., Langridge P., 2013, Hybrid breeding in wheat: technologies to improve hybrid wheat seed production, *Journal of Experimental Botany*, **64** (18), 5,411-5,428.
- Wibberley E. J., 1989, *Cereal Husbandry*, UK, Farming Press Books.
- Willey R. W., Holliday R., 1971, Plant populations, shading and thinning in wheat, *The Journal of Agricultural Science*, **77** (3), 453-461.
- Worland A. J., Korzun V., Röder M. S., Ganal M. W., Law C. N., 1998, Genetic analysis of the dwarfing gene Rht8 in wheat. Part II. The distribution and adaptive significance of allelic variants at the Rht8 locus of wheat as revealed by microsatellite screening, *Theoretical and Applied Genetics*, **96** (8), 1,110-1,120.
- Worland A. J., Snape J. W., 2001, Genetic bases of worldwide wheat varital improvement, In: *The world wheat book: a history of wheat breeding*, Bonjean A. P., Angus W. J., Eds., Lavoisier Publishing, Paris.
- World Food Program, 2015, *What causes hunger?*, [ONLINE] http://www.wfp.org/hunger/causes, [Accessed 14 December 15].

- Xie, W., E. Nevo. 2008. Wild emmer: Genetic resources, gene mapping and potential for wheat improvement, *Euphytica*, **164** (3), 603–614.
- Xu J., Ding Z., Vizcay-Barrena G., Shi J., Liag W., Yuan Z., Werck-Reichhart D., Schreiber L., Wilosn Z. A., Zhang D., 2014, ARORTED MICROSPORE acts as a master regulator of pollen wall formation in *Arabidopsis*, *Plant Cell*, **26** (4), 1,544-1,556.
- Xu J., Yang C., Yuan Z., Zhang D., Gondwe M. Y., Ding Z., Liang W., Zhang D., Wilson Z. A., 2010, The ABORTED MICROSPORES regulatory network is required for postmeiotic male productive development in *Arabidopsis thaliana*, *The Plant Cell*, **22** (1), 91-107.
- Yan L., 2009, Wheat Evolution, The flowering pathway in wheat, In: *Wheat Science and Trade*, Carver B. F. Ed., Wiley-Blackwell, USA.
- Yan L., Loukoianov A., Tranquilli G., Helguera M., Fahima T., Dubcovsky J., 2003, Positional cloning of wheat vernalization gene *VRN-1*. *Proceedings of the National Academy of Sciences Of the United States of America*, **100** (10), 6,263–6,268.
- Yan L., Loukoianov A., Tranquilli G., Blechl A., Khan I. A., Ramakrishna W., San Miguel P., Bennetzen J. L., Echenique V., Lijavetzky D., Dubcovsky J.. 2004, The wheat *VRN-2* gene is a flowering repressor down-regulated by vernalization, *Science*, **303** (5664), 1,640– 1,644.
- Yan L., Fu D., Li C., Blechl A., Tranquilli G., Bonafede M., Sanchez A., Valarik M., Yasuda S., Dubcovsky J., 2006. The wheat and barley vernalization gene *VRN-3* is an orthologue of *FT.*, *Proceedings of the National Academy of Sciences Of the United States of America,* **103** (51), 19,581–19,586.
- Yang C. Y., Vizcay-Barrena G., Conner K., Wilson Z. A., 2007_a. MALE STERILITY1 is required for tapetal development and pollen wall biosynthesis, *The Plant Cell,* **19** (11), 3,530–3,548.
- Yang C., Xu Z., Song J., Conner K., Barrena G. V., Wilson Z. A., 2007_b, *Arabidopsis MYB26/MALE STERILE35* regulates secondary thickening in the endotecium and is essential for anther dehiscence, *The Plant Cell*, **19** (2), 534-548.
- Yang X. Y., Li J. G., Pei M., Gu H., Chen Z. L., Qu L. J., 2007_c, Overexpression of a flower-specific transcription factor gene *AtMYB24* causes aberrant anther development, *Plant Cell Reporter*, **26** (2), 219–228.
- Zadoks J. C., Chang T. T., Konzak C. F., 1974, A decimal code for the growth stages of cereals, *Weed Research*, **14** (6), 415-421.
- Zhang W., Sun Y., Timofejeya L., Chen C., Grossniklaus U., Ma H., 2006, Regulation of Arabodopsis tapetum development and function by *DYSFUNCTINAL TAPETUM 1* (*DYT1*) encoding a putative bHLH transcription factor, *Development*, **133** (16), 3,085-3,095.
- Zhang Z. B., Zhu J., Gao J. F., Wang C., Li H., Zhang H. Q., Zhang S., Wang D. M., Wang Q. X., Huang H., Xia H. J., Yang Z. N., 2007, Transcription factors *AtMYB103* is required for anther development by regulating tapetum development, callose dissolution and exine formation in Arabodopsis, *The Plant Cell*, **52** (3), 528-538.
- Zhu J., Chen H., Li H., Gao J. F., Jiang H., Wang C., Guan Y. F., Yang Z. N., 2008, Defective in tapetal development and function 1 is essential for anther development and tapetal function for microspore maturation in Arabidopsis, *The Plant Journal,* **55** (2), 266–277.
- Zhu X. G., Long S. P., Ort D. R., 2010, Improving photosynthesis efficiency for greater yield. *Annual Review of Plant Biology*, **61**, 235-261.
- Zhu J., Lou Y., Xu X., Yang Z. N., 2011, A genetic pathway for tapetum development and function in Arabidopsis, *Journal of Integrated Plant Biology*, **53** (11), 892-900.

- Zinn K. E., Tunc-Ozdemir M., Harper J. F., 2010, Temperature stress and plant sexual reproduction: uncovering the weakest links, *Journal of Experimental Botany*, **61** (7), 1,959-1,968.

Appendices

Appendix 1

Spikelets harvested for sectioning, in relation to the total number of spikelets

Number of un-retarded spikelets (excluding terminal spikelet)	Spikelet in the lower third of ear	Spikelet in the middle third of ear	Spikelet in the upper third of ear
15	3	9	15
16	3	9	16
17	3	10	17
18	3	10	18
19	3	11	19
20	3	11	20
21	3	12	21
22	3	12	22
23	3	13	23
24	3	13	24
25	3	14	25
26	3	14	26
27	3	15	27
28	3	15	28
29	3	16	29
30	3	16	30

Appendix 2

Number of spikelets in each third of the ear, in relation to the total number of spikelets

Total Number of Spikelets on Ear (including retarded spikelets)	**Spikelets in the lower third of ear**	**Spikelets in the middle third of ear**	**Spikelets in the upper third of ear**
15	5	5	5
16	6	5	5
17	6	5	6
18	6	6	6
19	7	6	6
20	7	6	7
21	7	7	7
22	8	7	7
23	8	7	8
24	8	8	8
25	9	8	8
26	9	8	9
27	9	9	9
28	10	9	9
29	10	9	10
30	10	10	10

Appendix 3

GLMM (spikelet number)

	n.d.f.	d.d.f.
Temperature Treatment	5	130

ANOVA (grain number (from the start of booting), floret number above 'e')

	d.f.
Temperature Treatment	5
Residual	130
Total	135

ANOVA (grain number (from the end of booting))

	d.f.
Temperature Treatment	14
Residual	94
Total	108

ANOVA (intra-ear/intra-spikelet grain set, grain number (from the start of booting, just the stresses))

	d.f.
Temperature Treatment	4
Residual	109
Total	113

Appendix 4

ANOVA (1-3 post 39ZS)

	d.f.
Maturity	3
Residual	96
Total	99

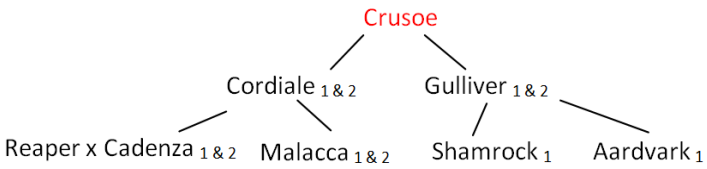
ANOVA (3 post 39ZS - anthesis)

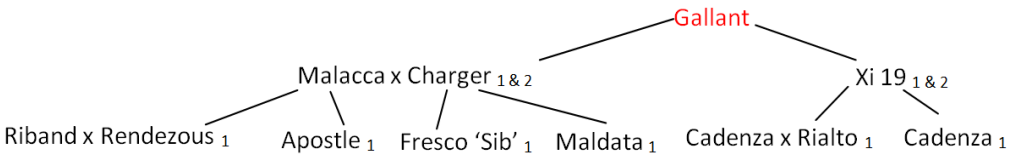
	d.f.
Maturity	2
Residual	92
Total	94

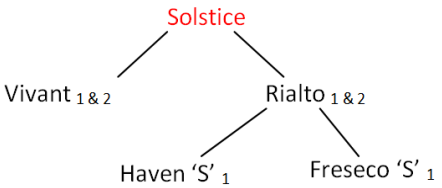
Appendix 5

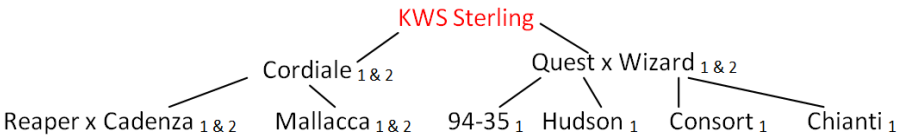
Parental background of seven winter wheat varieties.

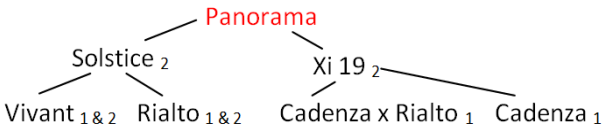
1 = The Scottish Wheat Variety Database, 2 = Home Grown Cereal Authority.

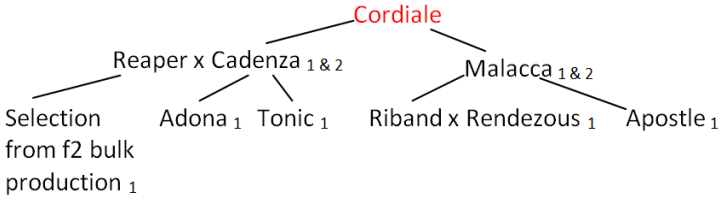


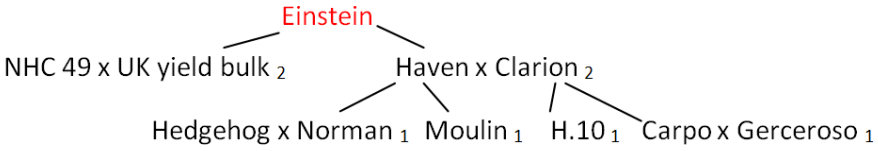








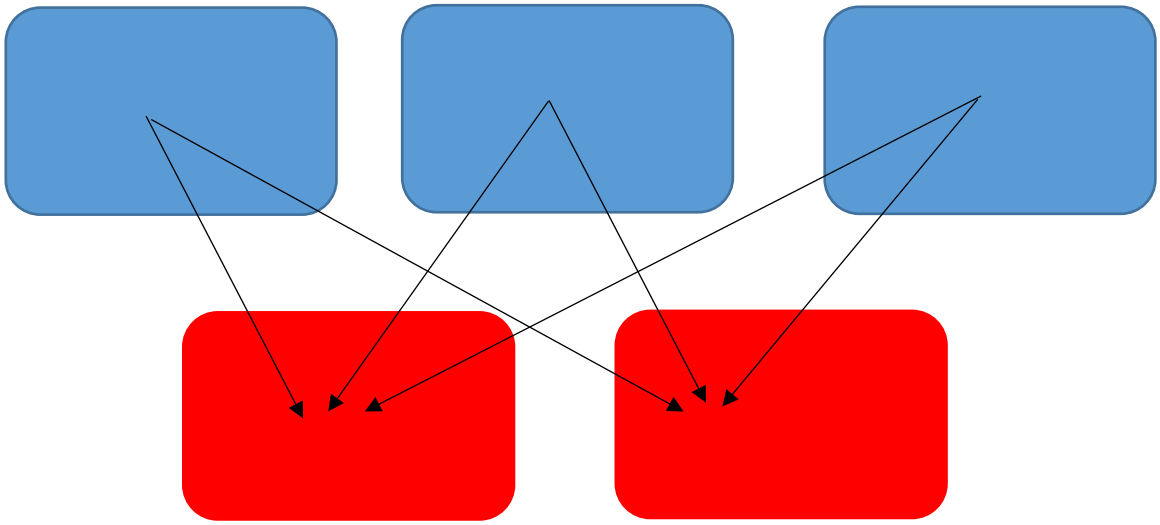




Appendix 6

Inter-cabinet movement dynamics of plants

(blue = 20/20°C cabinet, red = 35/35°C cabinet, arrow = approx. ¼ of plants from 20/20°C cabinet)



Appendix 7

GLMM (spikelet number)

	n.d.f.	d.d.f.
Variety	6	42
Temperature Treatment	1	42
V*TT	6	42

ANOVA (grain number)

	d.f.
Temperature Treatment	1
Variety	6
TT*V	6
Residual	42
Total	55

Appendix 8

ANOVA (ear & average spikelet dry weight)

	d.f.
Variety	5
Residual	46
Total	51

Appendix 9

GLMM (spikelet number, phenology)

	n.d.f.	d.d.f.
Variety	1	55
Temperature Treatment	1	55
V*TT	1	55

ANOVA (grain number, floret number above 'e', intra-ear/intra-spikelet grain set, weights)

	d.f.
Temperature Treatment	1
Variety	1
TT*V	1
Residual	55
Total	58

ANOVA (control cabinets)

	d.f.
Growth Cabinet	2
Variety	1
GC*V	2
Residual	23
Total	28

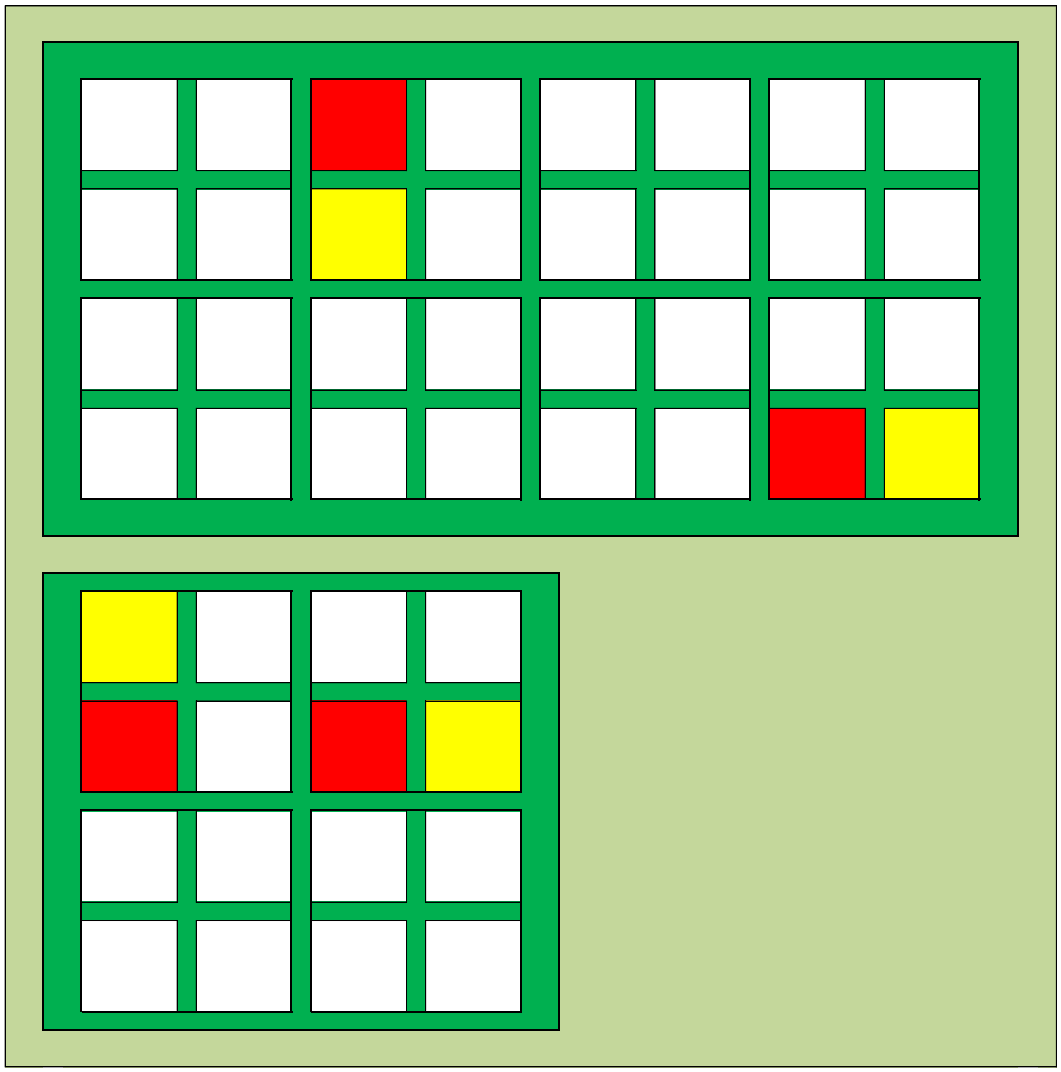
ANOVA (stress cabinets)

	d.f.
Growth Cabinet	1
Variety	1
GC*V	1
Residual	26
Total	29

Appendix 10

Layout of LIBERATION plots.

Yellow plots = Santiago, Red plots = Scout, Dark Green = Rye, Light green = grassland.
Each plot = 12x12m, each plot contained five 1.9x12m runs.
Diagram not to scale.



Appendix 11

GLMM (spikelet number)

	n.d.f.	d.d.f.
Temperature Treatment	1	44
Pollination Bag	1	44
TT*PB	1	44

ANOVA (grain number, intra-ear/intra-spikelet grain set, weights)

	d.f.
Temperature Treatment	1
Pollination Bag	1
TT*PB	1
Residual	44
Total	47

ANOVA (stress cabinets)

	d.f.
Growth Cabinet	1
Pollination Bag	1
GC*PB	1
Residual	19
Total	22

ANOVA (control cabinets)

	d.f.
Growth Cabinet	2
Pollination Bag	1
GC*PB	2
Residual	19
Total	24

Appendix 12

GLMM (spikelet number)

	n.d.f.	d.d.f.
Variety	1	128
Pollination Bag	1	128
V*PB	1	128

ANOVA (grains per spikelet (both total and regional), ear weight per spikelet (both total and regional), average grain weight (both total and regional))

	d.f.
Block	3
Variety	1
Pollination Bag	1
V*PB	1
Residual	125
Total	131

Appendix 13

ANOVA (temperature inside pollination bags)

	d.f.
Time of Day	7
Pollination Bag	1
ToD*PB	7
Residual	72
Total	87

Appendix 14

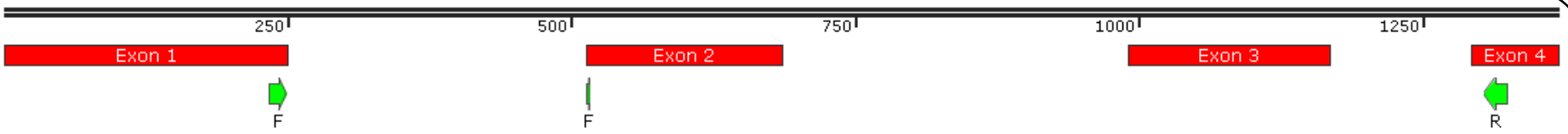
Areas of ears to be harvested

Number of Un-retarded Spikelets on Ear (excluding terminal)	Range of harvested spikelets (numbered from the base of the ear))	Spikelet for sectioning
24	10 to 15	11
25	11 to 16	12
26	11 to 16	12
27	12 to 17	13
28	12 to 17	13
29	13 to 18	14
30	13 to 18	14
31	14 to 19	15
32	14 to 19	15
33	15 to 20	16
34	15 to 20	16
35	16 to 21	17

Appendix 15

F= 3' tcagccgagcgggaaattgt 5'
R= 5' cctctctgcgccaatcgt 3'
(156bp)

Actin
1310 bp



F= 3' GCCCAACATCACAAAGATG 5'
R= 5' GCTCCACCATCACAACATCC 3'
(398 bp)

DYT1
1368 bp

F= 3' ATGCTGGGAAGCAGGTGGTC 5'

R= 5' GGATGTAGGAGGACGAGGAG 3'

(220 bp)

TDF1
1525 bp

F= 3' GGCTCTCTCCTGACCAGAGG 5'

R= 5' AGATTGGCTGGTTCGACAGT 3'

(162 bp)

AMS
1662 bp

F= 3' GGTGCTAGGGAACAGGTGGG 5'

R= 5' GGAAGGGATGTTACCGGAGT 3'

(145 bp)

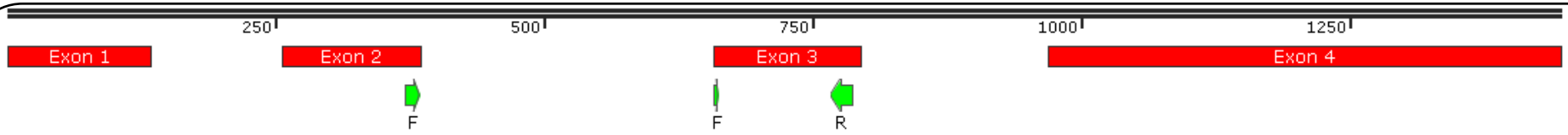
MYB80
1246 bp

F= 3' AGCCTTACACGTAAGGACGGT 5'

R= 5' CAGAGCGGTATGGACTGGAG 3'

(195 bp)

MS1
2249 bp



F= 3' GGCAACAGGTGGTCGGTGAT 5'

R= 5' GGTGGAGCATCTCGTCCTTG 3'

(232 bp)

MYB26
1445 bp