INVESTIGATION OF THE PHYSIOLOGICAL BASIS OF MALTING QUALITY OF GRAIN DEVELOPING UNDER HIGH TEMPERATURE CONDITIONS

by

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DECLARATION

I HEREBY DECLARE that the thesis presented here has been carried out by myself and does not incorporate any material previously submitted for another degree in any university. To the best of my knowledge and belief, it does not contain any material previously published or written, except where due reference is made in the text.

If accepted for the award of Doctor of Philosophy, this thesis will be available for loan and photocopy.

Meredith Anne Blesing Wallwork

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LIST OF ABBREVIATIONS

ABARE	Australian Bureau of Agriculture and Resource Economics
ABB	Australian Barley Board
α-amylase	alpha amylase
ADPG	adenosine diphospho glucose
ADPGp	adenosine diphospho glucose pyrophosphorylase
ANOVA	analysis of variance
β-amylase	beta-amylase
β-glucan	beta-glucan
β-glucanase	beta-glucanase
BE	branching enzyme
CCL	crushed cell layer
°C	degrees Celsius
°Cd	degree day
CLSM	confocal laser scanning microscope
cP	centipoise
daa	days after anthesis
DP	diastatic power
et al.	and others
FAN	free amino nitrogen
Fig.	figure
GBSS	granule bound starch synthase
h	hour
HWE	Hot Water Extract
K	potassium
λ	wavelength
LM	light microscope
MBIBTC	Malting and Brewing Industry Barley Technical Committee
min	minute
N	nitrogen
nm	nanometre
P	phosphorus
PAS	periodic acid/Schiffs
SEM	scanning electron microscope
SSS	soluble starch synthase
TBO	Toluidine blue O
μm	micrometre
UDPG	uridine 5'-diphospho glucose
UDPGp	uridine 5'-diphospho glucose pyrophosphorylase
WBMQEL	Waite Barley and Malt Quality Evaluation Laboratory

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Abstract

Short periods (up to 5 days) of high temperatures have been shown to reduce grain weight in barley although the specific effects on malting quality are still not clearly understood. Controlled environment conditions were used in this study to investigate the physiological and biochemical responses in Schooner barley to 5 days of elevated temperatures during grain filling. Control plants were maintained at 21/16°C (14h day) from anthesis to harvest ripemess. Plants undergoing heat treatment experienced increasing temperatures for two days (from 16 days after anthesis) followed by 3 days at high temperatures (35°C day /25°C night). The period of high temperature reduced individual grain dry weight by 18%. Schooner barley endosperm (the grain storage tissue) exhibited greater sensitivity to high temperatures than the whole grain (25% reduction in dry weight) in response to high temperatures. Grain development was accelerated by exposure to high temperatures and a reduction of approximately 8% in the duration of grain filling was observed in heat treated compared with control grain.

Changes in endosperm composition provided evidence that exposure to high temperatures altered overall grain metabolism. Reduction in starch, the single most important grain component contributing to final grain weight was the major factor lowering final grain dry weight. b-glucan deposition was also reduced following high temperature exposure. Little change was observed in the absolute amount of nitrogen accumulated per endosperm, although nitrogen concentration was higher in heat treated grain, due mainly to reduced grain size and lower starch content.

The results of this study confirmed other reports that the conversion of sucrose to starch was limiting in grain exposed to a period of high temperature. The amount of substrate, sucrose, within heat treated endosperms was not found to be limiting starch accumulation. The reduction in starch synthesis appeared to result from the combined effects of diminished catalytic activity of several enzymes in the committed pathway of starch synthesis and/or delayed recovery of enzyme activity during the cooler conditions which followed the heating period. Soluble starch synthase (SSS) showed an

immediate loss of catalytic activity, even at moderate temperatures, while sucrose synthase and uridine 5'-diphosphoglucose pyrophosphorylase showed greatest reduction in activity only after plants were returned to cooler conditions. Individual enzymes showed variation in the level of recovery under the cooler temperature conditions which followed the heating period.

The high temperature conditions which induced changes in the metabolism of grains following high temperature exposure, were associated with alterations to the appearance of the starchy endosperm cells, including the distribution of starch granules and their growth. Under elevated temperatures the synthesis of endosperm starch did not appear to keep pace with cell division and cell enlargement and peripheral endosperm cells remained relatively empty of starch granules. A reduction in Calcofluor fluorescent cell wall material was observed in both the endosperm and the crushed cell layer of heat treated grains. Microscopic observations revealed that the lower levels of starch observed in heat treated barley grains were not only associated with reduced accumulation of starch, but also localised degradation of the barley endosperm. Increased embryo growth, which was observed to take place in heat treated grains, may have been due to the nutrients thus generated although further investigations are required to elucidate the mechanisms involved. Structural observations of harvest ripe grain developing under control conditions also revealed changes in the grain associated with early germination events, and the possibility of a continuum between development and germination has been put forward.

High temperature exposure resulted in an overall decrease in malt extract potential, but there was evidence that the effects of heat on individual malt parameters were both beneficial and detrimental. While DP, β-amylase deposition, β-glucan and viscosity were improved, heat treatment was associated with a reduced quantity of starch and increased protein concentration. Grain and malt β-glucan levels were reduced as a result of high temperature exposure and β-glucan degradation was enhanced in these grains. The overall reduction in the amount of 'maltable' grain (due to reduced grain size) represented one of the most significant effects of heat treatment.

The effects of a period of high temperature on grain growth and subsequent malting quality were examined for barley varieties Schooner and Arapiles and advanced breeding

line WI-2875*22. All showed reduced final grain dry weight in heat treated grains but the response to high temperature exposure with respect to starch, protein and β-glucan accumulation varied. The continued synthesis of starch by Arapiles and WI-2875*22 under high temperature conditions warrants further investigation. Evidence has also been presented indicating that water loss may not have a decisive role in the termination of grain filling.

The effect of high temperatures on the non-endosperm component of the grain showed both genotypic and environmental variation. Changes in the contribution made by the non-endosperm component to final grain weight was observed to lead to alterations in the relative concentration of important grain storage components. This was found to be crucial where potentially important heat stable quality characteristics, such as reduced accumulation of nitrogen, were masked by changes in the contribution to final grain dry weight by the non-endosperm component of the grain.

Chapter 1

Introduction

Australia is well placed to produce malting barley in excess of domestic requirements and to generate export income. Large areas in the south and south-east of the continent are capable of producing barley and other winter cereals (Nix, 1975). Barley has the additional advantage that it can be grown in more degraded soils and generally in more marginal areas than wheat. Barley produced by Australian growers is generally clean, free of chemical residues, low in moisture and free of detritus, all desirable qualities to the maltster. Significantly lower grower input costs than those of our major competitors mean that the production of malting barley can be achieved relatively efficiently (Anonymous, 1995).

Australia produces around 4.3 million tonnes of barley per annum, of which half is segregated for malting (Australian Barley Board, 1995/96). By world standards the Australian barley/malt industry is relatively small, although in 1992/93 it was worth $550 million and generated $290 million in export income (Anonymous, 1995). While the total production of malting barley in Australia is small compared with Canada (with an annual production of 12.9 million tonnes of barley (Home Grown Cereals Association, 1995), more than 50% of Australia's malting barley contributes to the export trade. Australia usually supplies between 25 and 45% of the world's import malting barley needs and rates as the fourth largest exporter of malt in the world (Anonymous, 1995). Sales to domestic maltsters in general account for approximately 40% of Australia's malting barley production; 16% is used in the domestic market and 24% is value-added and exported as malt (ABARE, 1995).

With the trend towards a relatively static, or even decreasing consumption per capita of beer within Australia, supplying the export market is currently perceived as the only opportunity for significant growth in this industry. China represents the most significant of the malting barley markets and has enormous growth potential. However, the export of barley as malt offers the greatest financial benefit to the Australian economy in the

form of value-adding to this commodity. Currently Japan and Brazil are the largest malt importing markets and Australia supplies a small proportion of their requirements (Anonymous, 1995). In spite of our 'clean and green' image, Australia has been overtaken in the market-place by international competitors from the European Union, USA and Canada. These producers continue to receive the benefit of financial subsidies. While this is obviously an important factor, the loss of market share by Australia has been attributed to at least two other significant factors. Firstly, the quality of current Australian malting barley varieties limits the ability of maltsters to supply malt to meet some buyer specifications (Armitt, 1991). This deficiency is being addressed by barley breeders, who now select for specific quality attributes, in particular increased diastatic power and malt extract potential, both acknowledged deficiencies in most of the current malting barley varieties (Anonymous, 1995). Secondly, loss of market share has also been attributed to the inability to consistently meet specific quality requirements (Anonymous, 1995). Seasonal variations in the size of the malting barley pool and in the quality of the malt represent the greatest limitation to Australia sustaining a successful malting barley export industry in the long term. Segregation of the crop according to market requirements would improve quality stability and is proposed by maltsters as one method to reduce variability within and between export malt parcels (Gill, pers. comm.). A greater understanding of barley malting quality and the interaction between barley genotype and environment are required at all levels in the industry as the market becomes increasingly discerning. In order to continue to invest confidently in this crop and ensure a large pool of malting quality barley each season, growers require varieties which will exhibit both consistent agronomic characteristics and stable quality attributes under a range of environmental conditions.

Both wheat and barley, as temperate species, achieve most of their vegetative growth in cooler weather, with grain development occurring as temperatures rise. The environmental boundaries for successful cereal cultivation in Australia have been determined largely by the water and temperature regime of the continent (Nix, 1975). Farrer, as early as 1898, rated heat and drought tolerance as qualities of primary importance in the breeding of successful Australian cereal varieties (Farrer, 1898). He sought early flowering and early maturity in new varieties as adaptations to these climatic factors. Breeding and selection for increased yield potential has occurred since that time and produced modern varieties with a shorter duration from sowing to maturity than

older varieties (Loss *et al.*, 1989; Austin *et al.*, 1989) but with a relatively long grain growth period which confers the advantage of extended grain filling capacity under favourable conditions.

In addition to absolute limits of climate and terrain, the successful cultivation of winter cereals such as malting barley may be influenced by a series of environmental restrictions, including;

(1) timing of opening rains
(2) soil moisture prior to sowing
(3) duration of midwinter low temperatures and reduced daylength
(4) likelihood of frost at ear emergence
(5) increasing evaporation during late spring and early summer
(6) increasing temperatures during late spring and early summer

Plants may still grow and produce seed under quite severe environmental conditions. While it is axiomatic that the water regime plays a dominant role in defining successful cereal cultivation, temperature during the grain filling phase sets further limits, imposing constraints on crop cultivation and yield. Factors 1-4 may all influence overall crop yield, while environmental conditions during grain filling (Factors 5 and 6) have the greatest potential to alter the final composition and quality of the grain even when anticipated yield is high (Blumenthal *et al.*, 1991). It is important to understand the physiological and biochemical bases of the response to adverse climatic conditions by barley in order to develop new malting varieties which retain acceptable quality attributes under adverse environmental conditions. High temperatures and drought conditions are often experienced together in the field. However, combinations of high temperature and drought have been found to have additive as well as interactive effects and results are difficult to interpret (Nicolas *et al.*, 1984; Savin and Nicolas, 1996). For this reason, attention has been focused on the effects of high temperature alone on barley grain and malting quality in this study.

Temperature during the grain filling stage has already been established as an important yield limiting factor for temperate cereals such as wheat and barley (Sofield *et al.*, 1977a; Wardlaw *et al.*, 1989), especially over 30°C (Jenner, 1994), while temperatures as high as 40°C are considered deleterious to plant growth (Al-Khatib and Paulsen, 1989). High

temperatures, above 30°C, and on occasions higher than 35°C, are experienced during grain filling, specifically during the months of October and November, in all cereal growing regions in southern Australia (Australian Bureau of Meteorology, 1996). For example, in October marginal cereal growing regions in South Australia record on average between 5 and 7 days above 30°C (Minnipa and Kyancutta, South Australia) (Table 1.1).

Table 1.1 Mean number of days above 30°C and 35°C recorded by the Australian Bureau of Meteorology for 5 cereal growing locations in South Australia, calculated from (minimum) 26 years of climatological data.

Location	**Region**	**Latitude**	**Mean no. days above 30°C**		**Mean no. days above 35°C**	
			October	November	October	November
Wanbi	Mallee	34:26S	3.1	6.7	0.3	2.3
Maitland	Yorke Pen.	34:23S	1.9	6.1	0.1	1.4
Elliston	Eyre Pen.	33:39S	4.5	8.6	1.0	3.7
Kyancutta	Eyre Pen.	33:08S	6.8	11.6	1.6	5.5
Minnipa	Eyre Pen.	32:51S	5.3	9.7	1.2	3.8

In addition to yield, high temperature has adverse effects on quality in both wheat (Blumenthal *et al.*, 1991, 1993) and barley (Savin *et al.*, 1997a; Logue pers. comm.). Without detailed knowledge of the effects of environmental conditions on the accumulation of the principal storage components of the endosperm, the selection of characters to increase yield and malting quality is a highly empirical process.

The aims of this research program have been to obtain detailed knowledge on the effects of a period of high temperature on the accumulation of grain dry matter and endosperm starch, protein and β-glucan in the developing grain of the malting barley variety Schooner. In this study, barley plants were exposed to high temperatures during mid grain filling for 5 days. Grain growth characteristics were measured prior to, during and following the high temperature period with the aim of characterising the high temperature response in developing grain. The synthesis (from sucrose) of starch

was of particular interest, since starch represents the most abundant endosperm storage component of the grain (MacGregor and Fincher, 1993) and is an important source of fermentable sugars in malt. The activities of several enzymes and metabolites of the pathway of starch synthesis were monitored and compared to those in grains maintained at a lower temperature. In addition, grain structure was also compared between control and heat treated grain during development, at maturity and following malting.

Harvest ripe grains were collected, micromalted and assessed for malting quality in order to identify attributes contributing to malting quality which are affected by high temperature during grain development. Finally, a comparative study was undertaken comparing Schooner with two other potential malting quality barleys, to characterise the interaction between genotype and high temperature during grain development. Grain filling characteristics of dry weight, starch, protein and β-glucan accumulation were monitored and harvest ripe grain from this experiment was micromalted to identify important quality attributes affected by interactions between genotype and growth environment (high temperature conditions).

Chapter 2

Literature review

The conversion of barley grain into malt is a complex process involving the germination of harvest-ripe grain under controlled conditions. Numerous biochemical and physiological changes occur within the germinating grain. In commercial malthouses to achieve a successful malt, germination conditions are carefully controlled in order to maximise endosperm cell wall degradation with minimal starch hydrolysis within the storage cells of the barley endosperm. In addition, sufficient hydrolytic enzymes must be produced within the grain during malting to facilitate starch and protein hydrolysis later during mashing, the first stage of beer production. Knowledge of grain development and the formation of the various grain tissues and the way in which they interact during germination and early seedling growth forms the basis for understanding and optimising the malting process. The composition and structure of the barley grain is therefore of vital importance to the malting process.

This review reports on current knowledge of the development of the barley grain beginning at anthesis and fertilisation, and continuing through to harvest ripeness. While genetic factors influence grain yield and composition, climatic conditions also play an important role in determining final grain structure and composition. The influence of adverse climatic conditions (in particular high temperature) will also be discussed in the context of grain development and subsequent malting quality.

2.1 **Grain structure**

The tissues of the mature grain (Fig 2.1) comprise the embryo, which is located at the proximal end of the grain, and the endosperm which is the storage organ of the grain. The outermost cells of the endosperm become differentiated into the aleurone cells, which in contrast to the rest of the endosperm, remain metabolically active throughout development. Several cells thick in the mature grain, the aleurone appears to extend to the base of the grain, although reduced to a single layer where it passes over the tissues of the embryo (Smart and O'Brien, 1979). In this region it is known as the germ aleurone. The germ aleurone differs in appearance to the rest and seems to play a role in preventing micro-

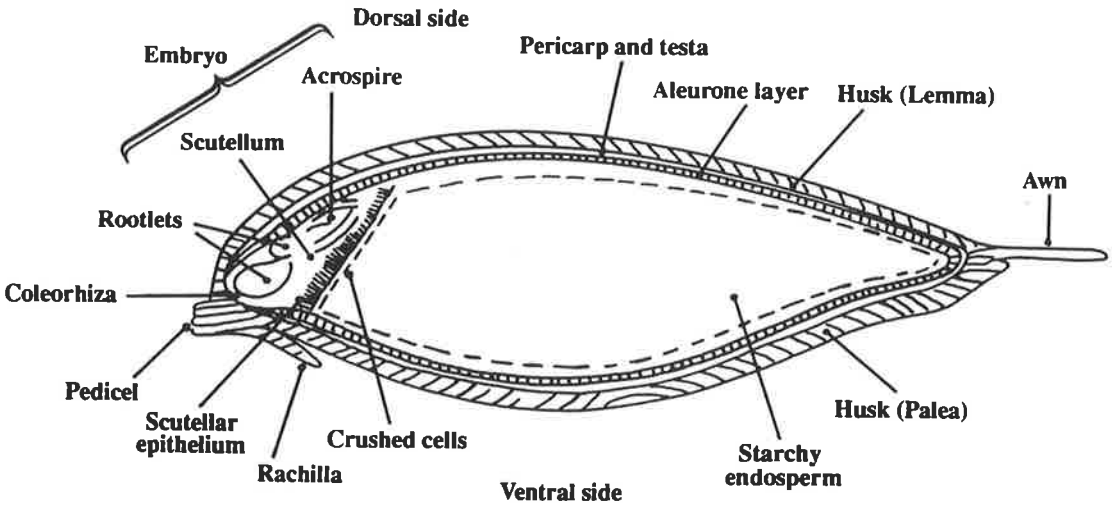


Fig 2.1 Longitudinal section through a barley grain.

organisms from attacking the embryo, both during development and germination (Cochrane, 1994). The starchy endosperm cells contain the carbohydrate reserves of the grain together with some protein. The tissues surrounding the exterior of the grain are collectively called the husk. The structure of the single cotyledon is reduced to the scutellum, which lies adjacent to the endosperm and separated from it by a layer of crushed or depleted cells.

2.2 **Grain development between anthesis and harvest-ripeness**

2.2.1 **Early development following fertilisation**

The seed develops from the fertilized ovule. Following fertilization of the female ovum (oospore) by the male nucleus, the first division produces an apical cell, which divides and differentiates to form the embryo, and a basal cell. The endosperm is produced as a result of the fusion between the second male generative nucleus and the two polar nuclei of the ovule (Bewley and Black, 1983).

2.2.2 **Embryo development**

During the first 6 days following germination, Pope (1943) recorded only one cell division of embryo nuclei for every three in endosperm tissue. Subsequent development of the embryo becomes quite rapid between 20 and 40 days after anthesis (daa) (Duffus and Rosie, 1975), when endosperm development is well advanced. During this time the embryo increases in dry weight and moisture, and accumulates protein and carbohydrate reserves (Duffus and Rosie, 1975). At this time the axial organs are initiated (Smart and O'Brien, 1983) and the developing scutellum grows in length and thickness with a clearly defined scutellar epithelial layer (Merry, 1941). During development the embryo is a powerful sink for nutrients, obtaining sucrose from both the parent plant and from the developing endosperm (Smart and O'Brien, 1983). This is supported by evidence that sucrose, via the phloem, passes the embryo before entering the endosperm cavity (Lingle and Chevalier, 1984) and evidence that the embryo exhibits an increasing rate of sucrose uptake (active and passive transport) during development (Cameron-Mills and Duffus, 1979).

The mature embryo consists of (1) the root system, with up to 5 rootlets covered by the coleorhiza; (2) the acrospire (coleoptile enclosing the foliar shoot); (3) the point of origin of procambial strands which enter the root and shoot (scutellar node) and (4) the scutellum (Palmer 1989). In harvest-ripe grain it represents about 3-4% of the dry weight of the grain and contains lipids (approx. 25%), proteins (approx. 10%) and sucrose (approx. 10%), although generally no starch (Briggs, 1987a).

2.2.3 **Endosperm development**

The barley endosperm can be distinguished from the surrounding pericarp from 5 days after anthesis (daa) (Buttrose, 1960). Cell division occurs in both a radial (hence the columnar arrangement) and a tangential direction (indicated by the smaller peripheral cells) so that the interior cells remain the largest and oldest (Evers, 1970). Cell enlargement follows cell division from about half way through the grain filling period in barley (Cochrane and Duffus, 1981), a development pattern similar to wheat (Evers, 1970).

The formation of the crease on the ventral side of the grain occurs from about 14 daa (Evers, 1970). The cells in the crease region play a role in the movement of solutes into the endosperm cavity and water out of it as the grain matures (Cochrane and Duffus, 1980). The observed increase in dry weight of the cereal endosperm during this time is principally the result of cell expansion to accommodate such endosperm components as starch, protein and β–glucan. At harvest ripeness the starchy endosperm cells are no longer metabolically active, and are packed with starch granules embedded in a protein matrix (Briggs, 1987a).

2.2.3.1 **Differentiation of the aleurone**

Hoshikawa (1984) described the existence of a proaleurone layer and an inner, subaleurone layer of cells 9-10 daa and Cochrane and Duffus (1981) clearly distinguished an aleurone layer in barley 15 daa. Cell divisions, both anticlinally and periclinally result in an aleurone layer of between one and three (and up to four) layers at harvest-ripeness and in the quiescent grain (Palmer, 1989). Aleurone cells are characteristically small, regular (isodiametric) cells, with thick cell walls, large nuclei and dense granular contents (Evers, 1970). Unlike the bulk of the endosperm, aleurone cells do not synthesise starch during development and they remain metabolically active through to harvest-ripeness. They play an important role during early germination as a site of hydrolytic enzyme synthesis for the breakdown of endosperm reserves to nourish the growing embryo.

2.2.3.2 **Endosperm cell wall network**

A continuous network of thin cell walls has been observed throughout the endosperm six days after anthesis (MacGregor and Dushnicky, 1989b). The aleurone cell walls contain 65-67% arabinoxylans and 26-29% β–glucans (MacGregor and Fincher, 1993) while those isolated from mature starchy endosperm tissue have been found to contain 75% β–glucan and 25% arabinoxylan (Fincher, 1975). The structure of the principal β–glucan component of starchy endosperm cell walls, (1-3,1-4)-β–glucan, is variable as these molecules comprise

a group of heterogeneous polysaccharides made up of essentially linear chains of β–glucosyl residues polymerised through both (1-3) and (1-4) linkages. Total β–glucan content of the grain may vary from approximately 2-11% depending on the barley variety and growing conditions (Bourne and Wheeler, 1984; Aman, 1986; Smith *et al.*, 1987; Miller and Fulcher, 1994). The process of cell wall biosynthesis, in particular the precise distribution of β–glucan, other cell wall polysaccharides, proteins and glycoproteins is still not clearly understood (Delmer and Stone, 1988).

2.2.3.3 **Development of the crushed cell layer (CCL)**

Endosperm cells adjacent to the embryo and abutting the scutellum are crushed shortly after anthesis and form a β–glucan rich layer between the starchy endosperm cells and the embryo. Loss of cell contents and crushing of these cells has been observed as early as 10 daa (MacGregor and Dushnicky, 1989b), and continues during the grain filling period. The role of the crushed cell layer in the developing grain remains unclear, although its formation, following hydrolysis of cell contents, occurs while adjacent endosperm cells are synthesising starch, protein and β–glucan as well as other storage compounds. The formation of the crushed cell layer (CCL) has been associated with the period of embryo enlargement (Smart and O'Brien, 1983). In the germinating grain, hydrolytic enzymes from the scutellum pass through the CCL before penetrating the starchy endosperm (MacGregor *et al.*, 1994). Initial water uptake into the endosperm region adjacent to the scutellum (Collins, 1918; Davies, 1991) suggests that the CCL may influence water distribution within the grain during imbibition and early germination.

2.2.4 **Synthesis of endosperm storage components**

Precursors for the important endosperm storage products of starch, protein and cell wall material, are supplied by the rest of the plant and transported into the grain via the phloem (Lingle and Chevalier, 1985). The deposition of protein is mainly a nitrogen (source)-limited process (Perez *et al.*, 1989) while starch synthesis appears to be limited by processes within the endosperm (sink) rather than through the availability of sucrose (Lingle and Chevalier, 1984). Protein and starch accumulation, and cell wall deposition during grain filling do not commence simultaneously (MacGregor *et al.*, 1971) and their initiation under field conditions tends to occur in the following order; protein, followed by starch and finally β–glucan (MacGregor *et al.*, 1971; Coles, 1979; Aman *et al.*, 1989).

2.2.4.1 **Starch synthesis**

Carbohydrates constitute about 80% by weight of the barley grain, of which starch generally accounts for between 63-65% and is therefore the most abundant component (Harris, 1962). Starch comprises a mixture of two molecules, amylose and amylopectin, each with glucose as the building block. Amylose, the minor component of most cereal starches, consists of relatively long chains of α–(1-4)-linked D-glucose residues. Amylopectin, the major component of most starches, is a branched molecule composed of α–(1-4)-linked glucose residues, the branches arising through α–(1-6) bonds. The unit chains in amylopectin are relatively small and the resultant molecule is highly branched and compact in structure. Barley starch usually contains between 22-26% amylose and 74-78% amylopectin (Briggs, 1978). The relative contribution of amylose and amylopectin to total starch is, however, known to vary widely between genotypes, and this is also influenced by environment. In waxy barleys, starch approaches 100% amylopectin, while a (high amylose) mutant line of Glacier (CI9676) has been found with an amylose level of 44% (Walker and Merritt, 1969).

Starch, in nature, is found in discrete bodies called starch granules, whose appearance depends largely on the plant source. Purified starch granules are also known to contain low, but varying levels of protein (0.25-0.56%) which appear to be the remains of the enzymes responsible for starch synthesis. The enzyme associated with the synthesis of amylose, granule-bound starch synthase, has been identified (Goldner and Boyer, 1989) as one of these.

Starch granules are deposited within amyloplasts in endosperm cells of barley in two distinct populations (Bathgate *et al.*, 1973; MacLeod and Duffus, 1988b) which have been classified as A-type and B-type starch granules. Although there appears to be no abrupt change in size between the two populations (Bathgate *et al.*, 1973), A-type granules are characterised as large and lenticular in shape, while B-granules are smaller and spherical, although they may be distorted and polygonal in shape within close-packed endosperm cells (May and Buttrose, 1959; MacGregor *et al.*, 1971; Bathgate *et al.*, 1973; MacLeod and Duffus, 1988b). There is some evidence to suggest that the B-granules may develop from projections within the amyloplast following A-type granule initiation and growth (Buttrose, 1963; Parker, 1985).

The presence of starch granules has been recorded in amyloplasts of barley endosperm as early as 4 daa (May and Buttrose, 1959). A-type starch granules are initiated until 15 daa and they continue to increase in size throughout grain growth (May and Buttrose, 1959)

achieving final diameters between 10 and 35µm and up to 45µm (Goering *et al.*, 1973). B-type granules, with diameters from 1 to 10µm, are initiated and grow during the phase of endosperm enlargement, from about 14 daa. At maturity B-type granules represent approximately 90% of granules in number, but only 10% of the total volume (May and Buttrose, 1959; McDonald *et al.,* 1991). It appears that starch granules situated in cell layers produced late in development (i.e. sub-aleurone) never reach the size of the granules in the oldest cells (Briarty *et al.,* 1979). The potential for accumulating starch within the starchy endosperm may be closely related to amyloplast number as well as the total cell number (Chojecki *et al.*, 1986).

Starch deposition appears to follow a diurnal pattern with starch granules exhibiting rings within a three dimensional shell structure (Buttrose, 1960). The synthesis of individual starch components, amylose and amylopectin, varies throughout development (MacGregor *et al.*, 1971; Morrison and Gadan, 1987) so that within a given population of starch granules, small granules contain less total amylose than do large granules (Bathgate *et al.*, 1973; MacGregor and Fincher, 1993). McDonald *et al.* (1991) also found that the composition of starch in B-type granules differed from A-type granules throughout development. Changes in starch composition may also give rise to changes in the final starch granule population profile. For example, high amylose starch granules tend to have a more uniform size distribution, with A-type granules smaller and B-type granules larger than corresponding granule types in normal barley starch (Walker and Merritt, 1969). Overall, a sophisticated regulation of starch synthesis is operating to produce different A- and B-type starch granules concurrently within the same amyloplast and to give rise to starch granule populations with specific size distributions.

2.2.4.2 **Protein synthesis**

Synthesis of storage protein within membrane-bound protein bodies, has been reported to begin in the cells of the barley endosperm soon after the formation of the first endosperm starch granules (Duffus and Cochrane, 1993). Initial detection of protein in barley endosperm has been as small uniform deposits in very large vacuoles (Cameron-Mills and von Wettstein, 1980). With age, the protein aggregates have been found to become heterogenous in size and content (Duffus and Cochrane, 1992), eventually losing the integrity of the enclosing membranes and forming a matrix in which the starch granules are embedded. The greatest concentration of protein has been observed in the cells of the sub-aleurone region (Millet *et al.*, 1991), and is reduced in cells in the central endosperm.

The proteins found in the aleurone layer, accounting for 20% of the total grain protein (Wallace and Lance, 1988), differ from those in the starchy endosperm tissue. The proteins in aleurone grains function principally in synthetic and secretory roles. Not only does deposition of this protein occur later in development but it takes place in aleurone grains, which are vacuolar in origin and maintain their integrity through maturation and dehydration and into early stages of germination.

2.2.4.3 β–glucan deposition

Initiation of β–glucan synthesis occurs following that of starch although the pattern of accumulation appears to differ between varieties (Coles, 1979) and the average content of β–glucan also varies between cultivars (Bourne and Wheeler, 1984; Henry, 1985a; Aman, 1986; Smith *et al.*, 1987; MacGregor and Fincher, 1993). β–glucan accumulation has been correlated with the rate of post-anthesis transpiration (Coles *et al.*, 1991). Final distribution of β–glucan appears to be relatively uniform throughout the body of the starchy endosperm (Miller and Fulcher, 1994).

2.2.5 Grain maturation

Physiological maturity of the developing barley grain is generally accepted as the point at which the maximum grain weight is attained (Egli, 1994 and references therein). This is followed by the dehydration phase, during which the moisture content of the grain declines (less than 14%) and the grain attains harvest-ripeness (also known as harvest maturity) (Te Krony *et al.*, 1979) . The relationship between grain dry matter accumulation and grain moisture content has been well described, with the onset of rapid grain water loss coinciding with maximum grain weight (Sofield *et al.*, 1977b; Schnyder and Baum, 1992).

Starch deposition in the barley grain mirrors the increase in grain dry matter (MacGregor *et al.*, 1971). Cessation of starch accumulation within the maturing grain appears to be influenced by the level of hydration of the grain, but is not related to loss of starch synthetic enzyme activity (Caley *et al.*, 1990) nor to the supply of sucrose (Jenner and Rathjen, 1975; Cochrane, 1985). Cochrane (1985) postulated that grain dehydration was controlled by maternal tissues through restriction of water supply to the grain as a result of progressive blockage of the xylem with pectic substances.

2.3 Germination

The germination of a seed represents a series of events beginning with water uptake and leading to the resumption of full metabolic activity, which then results in the growth of the embryo. Germination is defined as having occurred when the radicle (the embryonic root) has emerged through the testa (Bewley and Black, 1983). The endosperm reserves of the barley grain are mobilised and consumed by the embryo in order to sustain growth during the final stages of germination and early seedling development until it becomes autonomous.

2.3.1 Structural changes during germination

2.3.1.1 Imbibition

The uptake and distribution of water during imbibition is crucial to the activation of the embryo and to the production of hydrolytic enzymes that will later degrade the endosperm. The testa, which acts as a semi-permeable membrane, is modified at the micropyle region to allow the passage of water. During the initial stages of water uptake the proximal (embryo) half of the grain has been observed to absorb water faster than the distal half (Collins, 1918; Axcell *et al.*, 1983). At later stages in the germination process, water has been found to pass from the endosperm to the embryo (Axcell *et al.*, 1983). According to Axcell *et al.* (1983) observations of water uptake patterns within grains provide an indication of subsequent enzyme hydrolysis during endosperm modification.

2.3.1.2 Hormonal control of germination

Plant hormones, particularly gibberellins, are known to have an important role in germination by stimulating the production of large amounts of hydrolytic enzymes (Palmer, 1971). Endosperm breakdown does not occur in barley seeds unless the embryo is present (MacLeod and Palmer, 1966), or exogenous gibberellic acid (GA) is supplied (Ashford and Gubler, 1984). This has led to the hypothesis that gibberellins are produced by the scutellum and/or embryo and move to their site of action, the aleurone where they induce the synthesis of specific enzymes which break down the endosperm reserves (Ashford and Gubler, 1984). Much of the evidence for this hypothesis is indirect and it continues to be disputed (Trewavas, 1982; Atzorn and Weiler, 1983; Lenton and Appleford, 1991). However, it does appear that an embryo-produced stimulus is required for endosperm mobilisation.

The mechanism by which GA acts on aleurone cells is variously thought to involve changes in membrane structure (Simon, 1974) and sensitivity (Trewavas, 1982). Trewavas (1982)

proposed that sensitivity (of receptors) rather than concentration of GA at the site of action was the important factor in producing the desired response. Progress is being made in defining the molecular mechanism of GA action. It is now known that binding of GA to its aleurone cell membrane receptor results in the production of a DNA-binding protein which in turn regulates the production of α–amylase mRNA (Taiz and Zeiger, 1991). GA does not cause major changes in the total amount of protein synthesised, but rather redirects the synthesis of certain proteins while depressing that of others (Ashford and Gubler, 1984).

2.3.2 **Mobilisation of endosperm reserves**

The cellular activity that takes place during germination and early seedling development is directed at synthesising and secreting hydrolytic enzymes, and the translocation of the products of hydrolysis back to the growing embryo. The reserves of the embryo are mobilised first, although this is a minor component of overall reserve mobilisation. Some enzymes are synthesised *de novo* and secreted into the endosperm (e.g. α–amylase), while there is clear evidence that others (e.g. β–amylase, 80% of which is synthesised within the starchy endosperm during development) are activated by being released from a bound form (Gibbons, 1979, Lauriere *et al.*, 1986).

Both the aleurone and the scutellar epithelium are actively involved in the production and secretion of hydrolases. The scutellar epithelium, placed between source and sink, is also responsible for the uptake of endosperm degradation products and their translocation to the growing seedling (Bewley and Black 1983; Fincher, 1989). Imbibition results in separation and elongation of the epithelial cells to about twice their previous length (Nieuwdorp and Buys, 1964). The increased surface area thus achieved is thought to contribute to both the secretory and absorptive roles attributed to this tissue (Fincher 1989).

Ultrastructural changes following germination begin at the embryo end, adjacent to the scutellum, as enzymes secreted there begin to penetrate and break down the endosperm cell wall material (Gibbons, 1979, 1980, 1981; Fretzdorf *et al.*, 1982; Briggs and MacDonald, 1983; MacGregor *et al.*, 1994). Not until this has commenced can the hydrolysis of the starch and protein contained therein take place (Briggs and MacDonald, 1983), so that the general sequence of modification within the endosperm is the breakdown of cell walls, followed by protein hydrolysis and starch hydrolysis (Fretzdorff *et al.*, 1982). Cell wall erosion begins soon (1-2 days) after imbibition and starch degradation begins a further 1-2 days later. Gibbons (1980) found indications that the pattern of cell wall breakdown

preceded but was similar to the synthesis of α–amylase during germination, i.e. up to day 3 the scutellum played the major role in disseminating enzymes, with the contribution from the aleurone increasing after this time. Using hybridisation histochemistry, genes for (1-3)(1-4)-β–glucanase, the enzyme which mediates starchy endosperm cell wall breakdown, were found to be expressed first in the scutellum and then the aleurone after a delay of only 1-2 days (McFadden *et al.*, 1988). Morrall and Briggs (1978) calculated that hydrolysis of endosperm cell wall material contributed the equivalent of 18.5% of the carbohydrate supply to the embryo, compared to 81.5% from starch (after 6 days), which indicates that the cell walls of barley provide a significant carbohydrate reserve for the embryo.

Structural changes produced by protein degradation become apparent indirectly through the increase in endosperm friability (Briggs, 1987b). The proteolytic activity of the germinating grain includes the hydrolysis of aleurone grains, the production of hydrolytic enzymes and hydrolysis of reserve proteins. Components of reserve protein are used variously for the synthesis of hydrolytic enzymes (e.g. α–amylase), the activation of endogenous enzymes (e.g. β–amylase), for the production and maintenance of carboxypeptidases and endopeptidases themselves and enzyme inhibitors (Wallace and Lance, 1988). Both *de novo* synthesis and activation of proteolytic enzymes have been demonstrated (Wallace and Lance, 1988), while the role of protease inhibitors is still under investigation (Briggs, 1992). Grain proteins also provide peptides and amino acids which pass into the growing embryo. Although the initial phase of protein hydrolysis is likely to be mediated by enzymes present in the quiescent grain and activated during hydration, rapid protein hydrolysis does not follow until approximately 2 days after imbibition (Wallace and Lance, 1988).

Evidence of the commencement of starch hydrolysis appears in the form of dissolution of the small starch granules (Palmer, 1989). Large starch granules show pitting of the surface, followed by increased degradation in the region of the equatorial groove (Bathgate and Palmer, 1973). At later stages, the pits become a network of radial channels and eventually a hollow shell, reflecting the submicroscopic shell structure (Section 2.1.4.1). Starch granules can be converted to glucose by the combined action of α–amylase, β–amylase, limit dextrinase and α–glucosidase. The action of α–amylase produces oligosaccharides and branched dextrins and the action of β–amylase, limit-dextrinase and α–glucosidase completes the hydrolysis of these units. β–amylase cleaves successive maltose units from both amylose and amylopectin molecules and is apparently unable to attack native starch granules (Ashford and Gubler, 1984).

Sites of production of the starch degrading enzyme, α–amylase, are the scutellum and aleurone cells (Gibbons, 1980). There is evidence that during the first approximately 50 hours of the germination process, the scutellum is the dominant source of hydrolytic enzymes to degrade the barley endosperm (Brown and Morris, 1890; Munck *et al.*, 1981). However, the aleurone is also a source of hydrolytic enzyme activity (Jacobsen and Knox, 1973; Gubler *et al.*, 1987) and the relative importance of these two tissues in the production of endosperm degrading enzymes is still debated (Palmer 1983; Briggs, 1987a; Palmer, 1989).

2.4 **Malting**

Malting involves the controlled germination of the barley grain. During the first 24 hours grain moisture content is brought up to 35-45% (Burger and LaBerge, 1985) and germination is allowed to proceed for a further 4 days. This process is typically carried out at 12-25°C, with both air temperature and humidity carefully controlled. The grain germinates and the acrospire (the first shoot) and rootlets emerge and grow, however subsequent degradation of the endosperm is deliberately limited by the maltster. Growth of the embryo is terminated by drying the grain in a kiln at increasing temperatures over an 18 hour period. The kilning process halts germination while favouring the retention of enzyme activity required later in the brewing process (Hough, Briggs and Stevens, 1971). The product of this process is called malt.

The aim of this process is to produce a partially degraded endosperm, with maximum available carbohydrate (specifically starch) while ensuring adequate amounts of hydrolytic enzymes are activated or synthesised for starch and protein hydrolysis during mashing. During mashing (when ground malt is mixed with water) the enzymes present in the malt are able to attack the ground endosperm (and break down the starch) to yield a liquid called sweet wort. It is this wort, boiled to arrest further enzyme action and with the addition of hops as a flavour component, which provides the substrate upon which yeast grows during fermentation and ultimately results in beer production.

The process of germination and endosperm reserve mobilization is of great importance to the malting and brewing industry. An understanding of the fundamental processes involved in malting not only enables their management and control, but ultimately leads to opportunities to assess and select for qualities which may be incorporated in the development of successful varieties of malting barley.

2.4.1 **Endosperm modification and malting quality**

The term modification is used to include all the physical and chemical changes which occur when barley is malted (Briggs, 1987b). Changes in the barley endosperm result from the action of enzymes derived from or activated by the scutellum and the aleurone layer. Chemical indices of modification include extract yield (malt extract or Hot Water Extract (HWE)), which gives a measure of available carbohydrate brought into solution when a malt is mashed. Other examples can be grouped into (1) the nitrogen characteristics of malt (e.g. free amino nitrogen, Kolbach Index); (2) wort viscosity, the viscosity of the solution formed by adding hot water to ground malt; (3) cell wall material (e.g. malt β–glucan) and (4) hydrolytic enzyme levels (e.g. diastatic power, DP) (Briggs, 1987b). Physical indices of malt modification range from mechanical measures of friability and crushability of malted grain (Briggs, 1987b), to microscopic observation of cell wall breakdown in individual grains (Briggs and MacDonald, 1983). According to Munck *et al.* (1981), a combination of technological factors (such as wort viscosity and filtration) should be assessed in combination with both structural and chemical measures of malt modification in order to best ascertain and evaluate the degree of endosperm modification.

2.4.2 **Defining malting quality**

Analysis of grain and/or malt may be used to assess the potential malting quality of barley varieties. To date, barley attributes alone have not proved sufficient to satisfactorily define malting quality. Techniques such as whole grain Near Infra-red Reflectance Spectrometry are currently being investigated in a search for correlations between grain attributes and malting quality (Logue, pers.comm.). If successful, the use of this technology for malting quality assessment has the potential to avoid the time and expense of micromalting and subsequent biochemical analysis in the assessment of malting quality.

The Malting and Brewing Industry Barley Technical Committee (MBIBTC) in Australia has established a rating system for assessing malting barley varieties based on selected characterstics of malt (MBIBTC, 1991). Currently these parameters are; extract (HWE), DP, protein modification (Kolbach Index) and viscosity (MBIBTC, 1995) but these may be varied in order to best reflect current commercial malting quality requirements (MBIBTC, 1993). Despite the growing number of barley and malt analyses available and the increasingly detailed specifications required by the brewing industry (Gill, pers.comm.), the identity of specific attributes which most influence

malting quality have not yet been established (MacLeod *et al.*, 1993). This is due mainly to the complex interactions between components of the grain during the malting process. Industry generally uses an empirical combination of values for a number of physical and chemical characters, but even these do not always guarantee or accurately predict the performance of the malt during brewing (Palmer, 1989).

2.5 Effects of high temperature exposure during grain filling

2.5.1 Effects of high temperature on grain development

The temperature at which barley plants grow affects the rate at which morphological changes take place within the grain. Pope (1943) observed differences in the rate of cell division in response to elevated temperatures in both the embryo and endosperm during the first 6 days of grain development and Hoshikawa (1960) observed an association between temperature and faster pollen tube growth in wheat which resulted in more rapid penetration of the embryo sac. However, faster cell division under high temperature conditions may also be accompanied by a shortened time-frame of development (Hoshikawa, 1962; Nicolas *et al.*, 1984) so that final cell number varies little from that of grain grown under cooler conditions (Nicolas *et al.*, 1984). Anthesis represents a stage in development particularly sensitive to environmental stress, especially to high temperatures (Pope, 1943; MacLeod and Duffus 1988a). Applied to whole plants at anthesis, high temperatures may result in reduced fertility and grain set (Paulsen, 1994) and indeed, temperatures in excess of 40°C are often found to be lethal (Pope, 1943).

Temperatures of between 15°C and 30°C represent the normal temperature range in cereal growing areas in Australia and around the world. Short periods of temperatures in excess of 30°C, and up to 40°C, are quite commonplace in the Australian cereal belt during grainfilling (Australian Bureau of Meteorology, 1996; see Chapter 1). A number of studies have been made of the influence of elevated temperatures during grainfilling under both field and controlled environment conditions (Marcellos and Single, 1972; Sofield *et al.*, 1977a; Wardlaw and Moncur, 1995). Under field conditions drought often accompanies high temperatures. A number of researchers have investigated the effects, singly and combined, of drought and high temperature under controlled conditions, on the accumulation of grain dry matter (Nicolas *et al.*, 1984; Macnicol *et al.*, 1993; Savin and Nicolas, 1996) in order to determine the relative contribution of each. Drought alone, or in combination with high temperature, has been found to have the most significant effect on final grain weight in all cases, although varietal differences in sensitivity have

been identified (Savin and Nicolas, 1996). In field experiments where irrigation ensured that water was not limiting, MacDonald *et al.* (1983) found that temperature was the principal factor responsible for reduced grain weight. They found that each 1°C above 20°C resulted in up to 4% loss of yield.

Grain dry weight is determined by the rate of grain growth and its duration. This has been confirmed by field experiments (Meredith and Jenkins, 1976; MacDonald *et al.*, 1983) and under controlled environment conditions (Sofield *et al.*, 1977a; Chowdhury and Wardlaw, 1978; Wardlaw *et al.*, 1980), with the optimum temperature for grain development postulated to be 15/10°C (Chowdhury and Wardlaw, 1978). Increased rate of accumulation of grain dry matter has been observed at elevated temperatures (Sofield *et al.*, 1977a). However, as temperatures rise, a reduction in duration of grain growth has also been observed (Sofield *et al.*, 1977a). At temperatures above 21/16°C the reduction in duration of grain filling is not compensated for by an increase in growth rate, resulting in significantly reduced final grain weight (Marcellos and Single, 1972; Chowdhury and Wardlaw, 1978; Wardlaw *et al.*, 1980; Wardlaw and Moncur, 1995). Overall estimates of the fall in grain dry weight per 1°C rise in temperature in the range 12-31°C vary from 1.4 to 7.5 % of grain weight at the lower temperature (Wardlaw and Wrigley, 1994). These results may be confounded, however, by observations that enhanced sensitivity to high temperature has been reported at low irradiance under controlled environment conditions (Wardlaw *et al.*, 1989a; Cochrane *et al.*, 1996).

Many of these studies were carried out under controlled environment conditions and incorporated sustained elevated temperatures following anthesis and during grain filling. However, even when heat is experienced for only a brief period, some reduction in final grain dry weight may still be observed (Nicolas *et al.*, 1984; Stone and Nicolas, 1994; Savin and Nicolas., 1996). The magnitude of such a response appears most severe with exposure early in development (Nicolas *et al.*, 1984; Stone and Nicolas, 1995a; Savin *et al.*, 1997a) and with increased duration (Savin and Nicolas, 1996). Further, sudden high temperatures appear to have more severe detrimental effects on grain weight than conditions which allow some acclimatisation (Stone and Nicolas, 1995b) although this is not always the case (Savin *et al.*, 1997b).

Both wheat and barley show some genetic variability in tolerance to short periods of high temperature for both yield (Tester *et al.*, 1991 and Stone and Nicolas, 1994) and quality

(Tester *et al.*, 1991; Blumenthal *et al.*, 1995; Savin and Nicolas, 1996). Wardlaw and Moncur (1995), following analysis of seven cultivars, concluded that those most tolerant to high temperatures had the most enhanced rates of grain filling as a result of high temperature exposure.

2.5.2 Effects of high temperatures during grain filling on the accumulation of endosperm storage components

Starch accounts for most of the dry matter in the barley endosperm, so that a reduction in final endosperm dry matter is largely due to a reduction in starch (MacLeod and Duffus, 1988a). High temperatures during grain filling have been found to reduce starch accumulation (Bhullar and Jenner, 1986; MacLeod and Duffus, 1988a; Tester *et al.*, 1991; Shi *et al.*, 1994) and result in smaller A- and B-type starch granules (Shi *et al.*, 1994; Tester *et al.*, 1991; 1995). MacLeod and Duffus (1988b) found that high temperatures reduced the volume available for starch accumulation and the number rather than the relative size of starch granules. These observations have been supported by evidence of fewer amyloplasts in grain following heat treatment (Tester *et al.*, 1995). Schnyder and Baum (1992) concluded that a lower water content at the termination of cell division provide physical (spatial) restriction within endosperm cells to subsequent deposition of storage compounds. The effects of high temperature on the movement of water into the grain have yet to be determined.

The reduction in accumulation of starch under high temperature conditions does not appear to be due to a reduction in the supply of photosynthate (MacLeod and Duffus, 1988a). Alterations to the supply (reducing photosynthetic capacity) or demand (removal of grain) for sucrose did not alter the course of reduction in grain weight due to high temperature (Wardlaw *et al.*, 1980). High import of photosynthate was generally balanced by increased respiration, which may have accounted for up to 25% of the reduction in dry weight under high temperature conditions.

Limitation to starch synthesis under these conditions appears to be due to the conversion of sucrose to starch (MacLeod and Duffus, 1988a) which is determined in turn by the temperature of the grain, independent of the rest of the plant (Jenner, 1991a). At temperatures above 30°C (even for short periods) the rate of starch synthesis has been recorded slower than at lower temperatures and this effect may be carried over after transfer from high to low temperatures (Jenner, 1994). The effects of heat on starch synthesis may

be due, at least in part, to the heat sensitivity of one or more enzymes in the starch synthetic pathway. The metabolic pathway of starch synthesis has been reproduced in Fig. 2.2. Although the effects of temperature on the movement of starch precursors from the vascular system into the endosperm cavity (Wardlaw *et al.*, 1995) and across the amyloplast membrane still remain to be examined, there is evidence that the conversion of sucrose to starch is reduced at high temperatures through changes in the catalytic activity of a number of the enzymes in this pathway (Jenner *et al.*, 1993). For example, in barley, sucrose synthase showed reduced activity in response to high temperature exposure (MacLeod and Duffus, 1988a). In wheat however, the catalytic activity of this enzyme still remained far in excess of the requirement for sucrose to starch conversion (Hawker and Jenner, 1993) and the activity of soluble starch synthase (SSS) has been highly correlated to conversion of ^{14}C to starch under high temperature conditions (Jenner *et al.*, 1993). Soluble starch synthase appears to be the principal flux-controlling enzyme in the pathway of starch synthesis in wheat under these conditions (Hawker and Jenner 1993; Jenner *et al.*, 1993; Keeling *et al.*, 1993).

ADPglucose pyrophosphorylase (ADPGp), which has been implicated in the control of starch synthesis in photosynthetic tissues (Preiss, 1991), appears to influence starch synthesis under high temperature conditions primarily through a reduction in duration rather than through changes to the rate of grain filling (Singletary *et al.*, 1994). Previously considered to be exclusively amyloplastic, ADPGp has recently been located both inside and outside the amyloplast in barley endosperm (Thorbjornsen *et al.*, 1996) so that its role in the existing pathway of starch synthesis remains in question. Nevertheless, the activity of endospermic ADPGp is far in excess of the rate of starch synthesis (Thorbjornsen *et al.*, 1996).

Other metabolic processes in the cereal endosperm do not appear to be as sensitive to high temperatures as starch synthesis. For example, nitrogen accumulation (Bhullar and Jenner, 1985; Savin and Nicolas, 1996) and the rate of respiration (Keeling *et al.*, 1993) are not as severely affected by high temperatures as starch accumulation.

2.5.3 **Effects of high temperatures during grain filling on grain quality**

High temperatures during grain filling have the potential to modify grain quality (Blumenthal *et al.*, 1995). Increasing temperatures reduce the quantity of starch in both wheat (Shi *et al.*, 1994) and barley (MacLeod and Duffus, 1988b), even when the period of exposure is reduced to several days during mid grain filling (Savin and

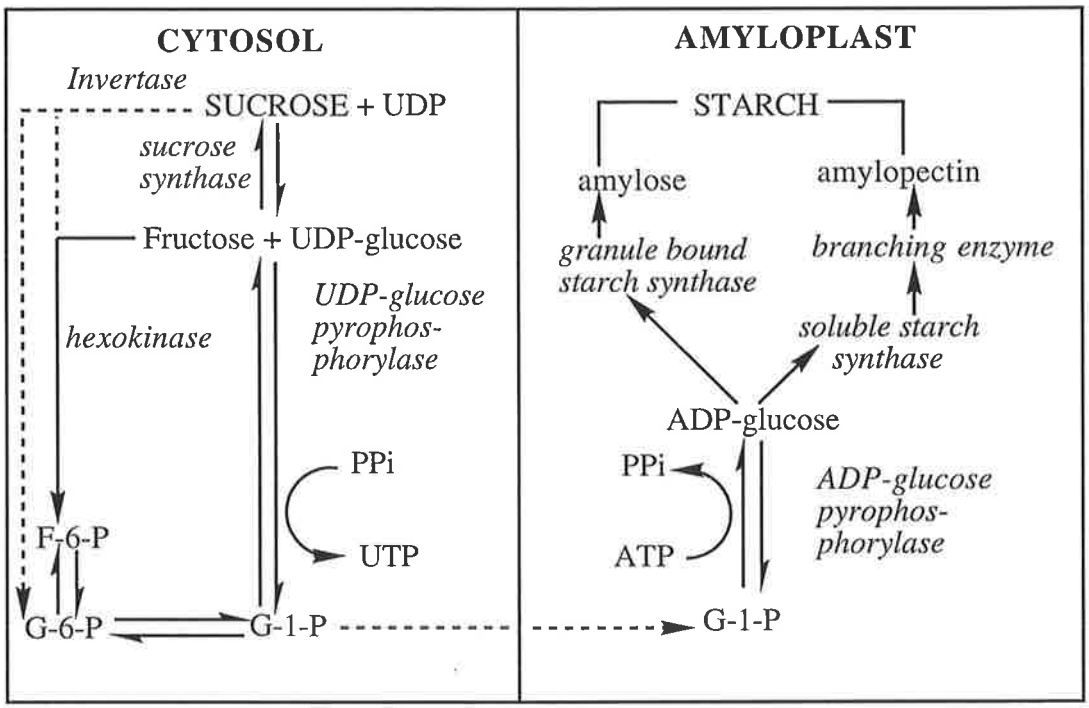


Fig 2.2 Biochemical pathway from sucrose to starch in developing wheat endosperm; source Keeling *et al.*, 1988.

and Nicolas, 1996). Changes in starch quality have also been associated with high temperatures. Increased amylose levels have been found with elevated ambient temperatures (Shi *et al.*, 1994; Tester *et al.*, 1995), although in the low temperature range (between 10-20°C) this appears little affected (Tester *et al.*, 1991). Lipid contents of starches showed a strong response with increased temperatures, even at temperatures as low as 15°C, compared with controls at 10°C (Tester *et al.*, 1991). Although the function of lipids in cereal starches is not clear (Morrison *et al.*, 1984), when complexed with amylose, lipid increases the peak temperature of gelatinisation (Morrison *et al.*, 1993). Experiments on wheat (Shi *et al.*, 1994; Tester *et al.*, 1995) and barley (Tester *et al.*, 1991; Cochrane *et al.*, 1996) have shown that the gelatinisation temperature of starch from grains grown at low temperatures is lower than that of starch from grains grown at higher temperatures. Thus, while higher lipid contents reduce the swelling potential of starch (Tester *et al.*, 1991) which may be important in some food processing applications (Shi *et al.*, 1994), the effects of temperature on starch gelatinisation in barley are important in relation to mashing temperatures in the brewing process.

In wheat, high temperatures have been associated with variations in the amount (Stone *et al.*, 1996) and also the composition of grain protein (Ciaffi *et al.*, 1996) in wheat. There is evidence that high temperatures during grain filling increase the concentration of protein in barley (Savin and Nicolas, 1996), and this is due in part to the dilution effect of starch (Henry, 1990).

2.5.4 **Effects of high temperatures during grain filling on malting quality**

It is known that malting quality varies from year to year (Anon., 1995) and environmental conditions are thought to contribute to low and variable quality (Henry, 1990; Macnicol *et al.*, 1993). A number of studies have recently been undertaken to investigate the effects of environmental conditions on malting quality (Macnicol *et al.*, 1993; Eagles *et al.*, 1995; Savin *et al.*, 1997a,b).

So far as the maltster and brewer are concerned, the yield of malt (and therefore the potential value of malt), is primarily a function of the potential extract, since this affects the amount of alcohol produced during brewing (Gromus, 1988). Under high temperature conditions, malt extract is generally reduced (Eagles *et al.*, 1995; Savin *et*

al., 1997b) although in other studies high temperature during grain filling had little effect on extract potential of the malt (Macnicol *et al.*, 1993; Savin *et al.*, 1997a).

Malting quality however may be assessed using a number of quality parameters (Section 2.4.2). High temperatures during grain development may affect individual attributes differently so that the effects of high temperature on malting quality may appear contradictory. For example, high temperature has been consistently associated with increased grain protein in barley (Correll *et al.*, 1994) which is an undesirable malting quality trait, while DP has also been found to increase under these conditions (Eagles *et al.*, 1995). DP provides a measure of the starch hydrolysing capacity of malt and this change represents an improvement in the potential for starch breakdown during mashing. No consistent response has been observed in malt β–glucan levels following high temperature exposure (Macnicol *et al.*, 1993; Savin *et al.*, 1997a,b), although Savin *et al.* (1997a,b) observed improved β–glucan degradation following high temperature treatment. An increase in the breakdown of endosperm cell wall material (principally β–glucan) increases the availability of cell contents to hydrolysis during mashing and therefore often leads to improved HWE potential. It is possible that high temperatures stimulated β–glucanase synthesis in malt, (Macnicol *et al.*, 1993), or in some way changed the structure or organisation of β–glucan in the endosperm cell walls to allow easier degradation (Savin *et al.*, 1997a,b).

While starch concentration and quality are not specified in definitions of malting quality (Palmer, 1989; Henry, 1990; MBIBTC, 1995), both may be affected by high temperatures and lead to reduced malting quality. A reduction in the overall amount of starch in grains grown under high temperature conditions is likely therefore to lower malt extract potential (Henry, 1990) by increasing the concentration of protein in the grain. Starch quality, the ratio of amylose to amylopectin, has been shown to be affected by temperature (Shi *et al.*, 1994). Gelatinisation temperatures may be elevated (Morrison *et al.*, 1993), reducing the yield of soluble carbohydrate in the mash and the potential malt extract (Cochrane *et al.*, 1996). Thus the contradictory nature of high temperature effects on malting quality highlights the need for more detailed information about the interactions between the various grain components during malting.

Chapter 3

Materials and methods

In order to investigate the effects of adverse conditions on grain growth and development, experiments were designed to apply a brief period of 5 days of elevated temperatures (up to 35°C), under controlled conditions. All experiments were conducted on potted plants grown between anthesis and maturity in growth rooms. Precise control was maintained over the timing of heat treatment and temperature and light conditions throughout this grain filling period.

3.1 **Plant material**

The barley variety Schooner was used throughout this study. Bred at the Waite Institute, Schooner has proven to be widely adapted to environmental conditions experienced throughout the cereal belt of southern Australia (ie South Australia, Victoria and southern New South Wales) and is widely grown in this region, averaging 35.6 and 63.1% of the area planted to barley between 1991 and 1995 in South Australia and Victoria respectively (Australian Barley Board, 1994/95). Schooner was released in 1983 as a malting barley variety and is used by the Malting and Brewing Industry Barley Technical Committee (MBIBTC) as a benchmark malting variety in evaluation procedures for assessing the malting quality of new barley varieties (MBIBTC, 1995). By international standards Schooner is characterised by average malt extract, low diastatic power (DP), low malt β-glucan and good grain size.

Arapiles is a Victorian malting barley variety released by Victorian Institute for Dryland Agriculture (VIDA) in 1993. Produced from a cross between Victorian breeding line 75031 and Domen (Moody, pers. comm.), Arapiles is recommended for the Wimmera district of Victoria, where it may be more specifically adapted to the typically cool, relatively high rainfall conditions. Arapiles is characterised by higher malt extract and DP than Schooner, but consistently higher malt β-glucan levels (WMBQEL, 1994, 1995, 1996).

WI-2875 has been bred by the Waite Barley Breeding Program. Potentially malting quality, WI-2875 is derived from a complex cross including Schooner and the Canadian variety Norbet, which is characterised by very high diastatic power. While agronomically similar to its Schooner parent, WI-2875 has improved straw strength and shattering resistance (A.Barr, pers.comm.). It has consistently higher DP than Schooner and extremely low malt and wort β-glucan (WBMQEL, 1995b, 1996). A re-selection of this line, WI-2875*22 has improved malt extract and indications of improved grain size (South Australian Field Crops Evaluation Program, 1995). WI-2875*22 is currently undergoing evaluation in commercial malting and brewing trials.

3.2 **Experimental growth conditions**

3.2.1 **Glasshouse conditions**

Experimental plants were grown in 25 cm pots of recycled soil (20 seed per pot) and with the exception of Experiment 3, placed in a glasshouse (temperature maximum 25°C; latitude 33° 43'S). For experiment 3, material was placed directly into a constant environment room. Pots were fertilised regularly with Aquasol™, an all purpose fertiliser (N:P:K ratio of 23:4:18), applied at 2.6 g per pot per fortnight from early seedling stage to anthesis. No fertiliser applications were made during grain filling.

Differences in sowing date resulted in variations in pre-anthesis temperature maxima and daylengths. These have been outlined in Table 3.1.

3.2.2 **Growth room conditions**

For Experiments 1,2,4 and 5 plants were transferred to a constant environment room set at 21/16°C, 14h day at least 1 week prior to anthesis. All plants remained in constant environment rooms until harvest ripeness. High pressure sodium lights, supplemented with fluorescent tubes provided a photosynthetic photon irradiance of 310-320 μE at ear level. Temperature probes were positioned at the level of the plant canopy. The growth rooms attained the set temperature within half an hour of the day/night change and maintained this for the prescribed period each day. Daily watering was maintained throughout growth and development and during heat treatment watering was carried out twice daily to ensure plants did not suffer any water deficit.

Table 3.1 Pre-anthesis growth conditions and the number of experimental pots subjected to control and high temperature conditions during Experiments 1-5.

Experimental growth conditions		**Experiment**				
Variable		**1**	**2**	**3**	**4**	**5**
Time of planting (month)		March	June	Nov	Sept	July
Number of pots	Control	7	7	9	11	5
	Heat treated	8	7	11	10	6
Sowing - anthesis (days)		72d	81d	49d	44d	71d
Average daylength (h)		10.0	10.5	14.0	12.3	10.3

Experiment 6 (varietal comparison) was also conducted in constant environment rooms. Seven pots each of Schooner, Arapiles and WI-2875*22 were sown following the schedule of previous experiments. Lighting in the constant environment room was provided by two banks of 5 metal halide lamps, adjusted to provide constant irradiance at canopy level of between 270-290 $\mu E.m^{-2}.sec^{-1}$. Pots were placed directly into a constant environment room and maintained at 18/13°C, 10h day, for 7 weeks. Conditions were then changed to 21/16°C, 14h day until grain maturity. Anthesis (Day 0) was recorded 85 days after sowing for both Schooner and Arapiles, and 7 days later for WI-2875*22. High temperature treatments were applied to 4 experimental pots of each variety in an adjacent room. All plants were returned to the original control growth room until maturity.

3.2.3 **High temperature treatment**

A standard heat treatment regime was applied throughout this study. Elevated temperatures were applied during grain filling from 16 days after anthesis, for a period of 5 days. Heat treatment was applied to the experimental pots in an adjacent, identical growth room before returning the pots to the original (control) growth room on day 21.

The high temperature schedule was as follows:

(a) High temperature treatment:

Anthesis - Day 15	21/16°C	14h day
Day 16	28/18°C	14h day
Day 17	32/22°C	14h day
Day 18,19,20	35/25°C	14h day
Day 21 - harvest-ripeness	21/16°C	14h day

(b) Control conditions

Anthesis - harvest-ripeness	21/16°C	14h day

All experiments were conducted using this high temperature schedule. Table 3.2 summarises the data collected/analyses performed during each experiment and the relevant Chapter in which it is presented.

3.3 **Sampling**

Main stem ears were tagged at anthesis, when mature pollen had dehisced from the anthers. Anthesis was established initially by dissection and was subsequently estimated on the basis of the degree to which the ear had emerged from the flagleaf. Anthesis normally ranged over a period of 7-10 days. Day 0 was calculated as the day on which the maximum number of main stem ears flowered.

3.3.1 **Sampling schedule**

Main stem ears were collected from individual pots, each representing a single sample replicate. Triplicate samples (ears) from individual pots were made on each sampling day and grains collected from the central region of each ear. Grain samples ~~was~~ were taken prior to heat treatment, on day 15. Ears were sampled every 3 days thereafter; at 18, 21, 24, 27, 30, 33, 36 and 39 daa to provide a developmental series through the grain filling period. In some experiments ears were collected 42 and 45 daa.

Whole grain was collected at harvest ripeness. All tagged ears were harvested from control pots; only ears tagged on day 0 (+/- 1 day) were harvested from heat treated pots.

Table 3.2: Summary of data presentation from Experiments 1-5 and the varietal comparison experiment.

Attribute measured/ analyses made	Experiment 1	2	3	4	5	Varietal comp.
Dry matter accumulation						
(endosperm)						
fresh weight	Chapt 4	Chapt 4	Chapt 4	Chapt 4	Chapt 4	Chapt 8
dry weight	Chapt 4	Chapt 4	Chapt 4	Chapt 4	Chapt 4	Chapt 8
moisture	Chapt 4	Chapt 4	Chapt 4	Chapt 4	Chapt 4	Chapt 8
(embryo)						
fresh weight			Chapt 6		Chapt 6	
dry weight			Chapt 6		Chapt 6	
moisture			Chapt 6		Chapt 6	
Endosperm storage products						
total starch	Chapt 4	Chapt 4	Chapt 4			Chapt 8
β-glucan$^+$	Chapt 4	Chapt 4	Chapt 4			Chapt 8
protein	Chapt 4	Chapt 4	Chapt 4			Chapt 8
Synthetic enzyme activities						
starch synthesis						
sucrose synthase	Chapt 5					
UDPGp$^+$	Chapt 5					
ADPGp$^+$	Chapt 5			Chapt 5	Chapt 5*	
SSS$^+$	Chapt 5			Chapt 5	Chapt 5*	
GBSS$^+$	Chapt 5					
Branching enzyme		Chapt 5				
Endosperm sugars						
sucrose		Chapt 5	Chapt 5			
maltose		Chapt 5	Chapt 5			
glucose		Chapt 5	Chapt 5			
RT 11.4 mins$^+$		Chapt 5	Chapt 5			
Endosperm sugar nucleotides						
UDPG$^+$					Chapt 5*	
ADPG$^+$					Chapt 5*	
Hydrolytic enzyme activities						
α-amylase$^+$		Chapt 5				
β-amylase$^+$		Chapt 5				
Microscopic observations						
SEM$^+$ (mature	Chapt 6*	Chapt 6*	Chapt 6*			
LM$^+$ observation	Chapt 6*			Chapt 6		
Crushed cell layer				Chapt 6		
Malting quality analysis	Chapt 7	Chapt 7	Chapt 7	Chapt 7		Chapt 8

* incomplete developmental series; selected sampling days only.

$^+$ see p iii for Abbreviations

3.3.2 **Endosperm dissection**

The endosperm, the principal storage organ of the barley grain, was dissected from each grain and used for subsequent analyses. This dissection was performed by carefully removing the outer layers of the husk and the pericarp, and the embryo. Up to 21 daa, the aleurone layer was separated from the rest of the endosperm tissue, but this was not possible as the grain matured beyond this stage. Dissections were carried out using gloves to prevent contact with skin contaminants (in particular, α-amylase).

In addition to endosperm tissue, whole grains were sampled during grain filling in Experiment 3. For Experiments 3 & 5 embryo tissue was also separated and collected.

3.4.1 **Dry weight accumulation**

Triplicate samples of 7-10 grain or endosperms were placed into pre-weighed foil boats, dried at 80°C for 24h and cooled over silica gel before weighing.

3.4.2 **Endosperm, grain and malt moisture**

Endosperm and grain moisture content was calculated from the difference between fresh and dry endosperm or whole grain weight and expressed as mg water per endosperm. Malt moisture measurements were made on flour samples. For Experiments 1-4, moisture was assessed by drying the flour at 104°C for 3 hours, cooled over silica gel and weighed. The larger quantities of malt produced from the varietal comparison experiment enabled moisture values to be determined using a Technicon 400 NIR (Near Infra-red Reflectance) Spectrophotometer, and based on a calibration checked and adjusted each season.

Endosperm fresh and dry weights and moisture contents were collected for each experiment. These attributes have been expressed on a per endosperm basis rather than as % dry weight to overcome differences between samples and treatments due to grain size. In the case of mature grain and malt samples when entire grain was analysed, a number of parameters have been expressed on % basis to allow comparison with appropriate quality attributes (MBIBTC, 1995).

3.4 **Grain filling**

Grain filling data, which included the accumulation of endosperm fresh and dry weight and changes in endosperm moisture content, were collected for each experiment. In order to overcome differences between samples and treatments due to grain size, these characteristics were routinely expressed on a per endosperm basis. In other instances, however, grain composition has been expressed on a concentration (percentage) basis for comparison with appropriate grain and malt quality attributes (MBIBTC, 1995).

3.4.1 **Rate and duration of grain filling**

Rate of grain filling was determined from the difference in dry weight between sequential harvest samples, divided by the number of days between harvests. The maximum rate of grain filling (Chowdhury and Wardlaw, 1978) was recorded as the greatest increase in dry matter of grains (endosperms) obtained from successive harvests.

Duration of grain filling represents the time between anthesis and physiological maturity (Egli, 1994). Because dry matter accumulation has not been found to follow a logistic curve when plants are subject to a brief period of heat (Savin and Nicolas, 1996), the duration of grain filling was determined less definitively. In this study the estimation of the duration of grain filling was made by assessing patterns of dry matter accumulation and changes in endosperm moisture (Sofield *et al.*, 1977b; Schnyder and Baum, 1992). Duration of grain filling was also determined from grain filling data presented in thermal time as accumulated temperature above a 7.1°C base temperature (Goyne *et al.*, 1996).

Under growth room conditions, the following calculation of thermal time (calculated for post anthesis development only) was used:

M = x (average daily temperature - Tb)

where M = accumulated degree days

x = number of days after anthesis

Tb = 7.1°C (base temperature)

average daily temperature = ((day temp*14) + (night temp*10))/24

3.4.2 **Endosperm components**

3.4.2.1 **Starch**

Starch determinations were carried out on triplicate samples of endosperm flour. Dried endosperm (or whole mature grain) tissue was crushed between sheets of glassine paper and ground to a fine powder using a mortar and pestle.

The Megazyme™ Total Starch method was used to determine the starch content of developing endosperm tissue as described by McCleary *et al.* (1994). This method was scaled down for a flour sample of 50 mg and standard starch preparations analysed to confirm the accuracy of this modified method.

The Megazyme™ Total Starch method involved the hydrolysis of starch to soluble fragments with minimal production of glucose, followed by the quantitative hydrolysis of starch dextrins to glucose. Dimethyl sulphoxide (DMSO) (laboratory grade, Sigma Chemical; cat.no.D5879) was used to solubilise the starch, followed by a five minute incubation in thermostable α-amylase at 100°C. Complete dextrinisation was achieved by further treatment with pullulanase and β-amylase, in which maltodextrins were converted to maltotriose and maltose. In the final assay step, amyloglucosidase released free glucose which was measured directly using glucose oxidase/peroxidase (GOPOD) reagent and provides a direct measure of total starch. In the presence of glucose oxidase, glucose was converted to gluconate and peroxide, which further reacted in the presence of peroxidase to release quinoneimine dye from 4-aminoantipyrine. The amount of colour was measured on the spectrophotometer with maximum absorbance at 510nm.

Total starch = DE x F x 1000 x 1/1000 x 100/W x 162/180

where DE = absorbance (read against reagent blank)

F = conversion factor from absorbance to mg

1000 = volume correction for aliquot assayed

1/1000 = µg to mg

100/W = express starch as % (w = weight in mg)

162/180= adjustment from free glucose to anhydroglucose

Calculation of flour moisture and endosperm dry weight enabled total starch to be expressed on a dry weight basis and as mg starch per endosperm. Starch standards (wheat and maize) were assayed with each batch of samples.

3.4.2.2 **Nitrogen**

The analysis of grain and endosperm nitrogen was made using the Dumas Total Combustion method (American Society of Brewing Chemists, 1996) utilising a Carlo Erba 1500 Nitrogen Analyser. Samples of flour (5 mg) were weighed, including standards of known nitrogen (oatmeal and alfalfa) and analysed for % nitrogen following complete combustion at 800°C. This was converted to percentage protein by multiplying by a factor of 6.25 (Tkachuk, 1969).

3.4.2.3 **β-glucan**

Triplicate samples of ground endosperm were assayed for β-glucan. The method developed by Megazyme™ (McCleary and Glennie-Holmes, 1985) was modified to accommodate a 50 mg sample. Checks were performed using standard flours.

After suspension and hydration in a buffer solution, samples were reacted with purified lichenase (specifically endo-(1-3),(1-4)-β-D-glucan 4-glucanohydrolase) to produce β-gluco-oligosaccharides. An aliquot of the filtrate was reacted to completion with excess β-glucosidase and the free glucose assayed using the GOPOD reagent in the standard glucose assay procedure (see total starch analysis, 3.4.4.1).

β-glucan = DE x F x V x1/1000 x 100/W x 162/180

where DE = absorbance (read against reagent blank)

F = conversion factor from absorbance to mg

V = volume correction factor (0.1ml aliquot sampled)

1/1000 = µg to mg

100/W = β-glucan as % (W = weight flour in mg)

162/180 = adjustment from free glucose to anhydroglucose

Standard flours were assayed with each set of experimental samples and results were expressed on a percent dry weight basis and as mg β-glucan per grain or endosperm.

3.4.2.4 **Endosperm sucrose and maltose**

Measurements of the major endosperm di-saccharide (sucrose) and disaccharide (maltose) were performed using water-elution high performance liquid chromatrography (HPLC). The HPLC system comprised a pump (Waters™ 510), a differential refractometer (Waters™ 410) and the Waters™ 840 control station. This was subsequently updated with Millenium™ software for data processing and quantification. Sugars were separated isocratically by a Waters™ 'Dextro-Pak' column.

Soluble sugars were extracted from ten endosperms in boiling 80% ethanol. The extract was concentrated in a Savant evaporator, redissolved in HPLC water (filtered through Millipore 45µm filters) and passed through a deionising resin column. The resin was prepared by combining cation exchange, Amberlite CG 120 (H^+ form) and anion exchange, Amberlite CG 4B (OH form) in the ratio 1:2. Mini resin columns were used for individual 2 ml samples which were filtered (Millipore 0.45µm) into vials and run through HPLC together with standards of sucrose, maltose and a mixture of the two (20µg of sugar per 100µl injection).

3.4.2.5 **Sugar nucleotides**

HPLC detection of sugar nucleotides was carried out using a 'Waters™ Associates' HPLC system as described by Jenner (1991a).

Three replicates of four endosperms were dissected from grain onto small pieces of glass fibre filter, freeze clamped immediately (clamps stored in liquid nitrogen) and transferred to cold perchloric acid (1.41M) for extraction. Extraction steps (for details see Jenner, 1991a) of grinding, washing with 1 ml water and spinning at 15,000 rpm, were repeated 3 times and then each sample neutralised with potassium carbonate (5M), taking care not to exceed pH 7. All steps were carried out on ice. The supernatant was adjusted to a final volume of 5 mls, filtered through a Sartorius filter (0.65µm) and stored at 0-4°C until analysis. Sugar nucleotide standards (Adenosine 5'-diphosphoglucose (ADPG) and Uridine 5'-diphosphoglucose (UDPG)) were injected for analysis with each batch of samples. At least one sample in each batch was spiked with a known quantity of sugar nucleotide, added prior to the first extraction step, in order to ascertain the overall yield of the assay. Data processing and analysis was carried out using Millenium™ software.

3.5 Starch synthetic enzyme analyses

3.5.1 Enzyme extraction

Starch synthetic enzymes were analysed in the crude supernatant of an extract of five endosperms (triplicate samples). The dissected endosperms were homogenised in an all-glass homogeniser with 1-2ml of 0.05M 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0) following the procedure described by Hawker and Jenner (1993). The homogenate was centrifuged and the supernatant retained on ice for immediate assay.

3.5.2 Sucrose synthase (EC 3.4.1.13)

Sucrose synthase activity in the crude supernatant was determined at 30°C in the direction of UDPG synthesis by measuring the increase in absorbance as β-Nicotinamide adenine dinucleotide (NAD) was reduced in the coupled reaction with commercial Uridine 5'-diphosphoglucose dehydrogenase, (EC 1.1.1.22) (Boehringer Mannheim) using the method of MacLeod and Duffus (1988a).

3.5.3 Uridine 5'-diphosphoglucose pyrophosphorylase (UDPGp) (EC 2.7.7.9)

UDPGp was assayed spectrophotometrically at 30°C by incubating UDPG and pyrophosphate (PPi) with 1 µl of crude extract and measuring the production of D-glucose-1-phosphate (G-1-P) in a coupled enzyme reaction (Entwistle *et al*, 1988).

3.5.4 Adenosine diphospho-glucose pyrophosphorylase (ADPGp) (EC 2.7.7.27)

ADPGp was assayed similarly to UDPGp using the substrate ADPG with pyrophosphate (PPi) (Entwistle *et al*, 1988).

3.5.5 Soluble starch synthase (SSS) (EC 2.4.1.21)

Soluble starch synthase was assayed using the method described by Hawker and Jenner (1993). In addition to blanks without the amylopectin primer, boiled enzyme blanks were also assayed.

3.5.6 **Branching enzyme (BE) (EC 2.4.1.18)**

Branching enzyme was measured using the decrease in absorption of the amylose-I_2 complex (Hawker *et al.*, 1974). Using this method, units of branching enzyme activity are glucose transferred, nmoles per minute.

3.5.7 **Granule-bound starch synthase (GBSS)**

A sample of 30µl of the original extract was removed prior to the original centrifugation step in the extract preparation and placed into 1.8ml of extraction buffer (Section 3.5.1) and centrifuged. The assay was performed on the washed precipitate using the method for GBSS described in Hawker and Jenner (1993).

3.5.8 **Alpha-amylase (α-amylase)**

The Megazyme™ α-amylase extract procedure (McCleary and Sheehan, 1987), was used to measure α-amylase activity in developing endosperm tissue. The method was scaled down to accommodate 50µl of extract. One unit of α-amylase activity was the amount of enzyme required to release one µM of *p*-nitrophenyl from BPNPG7 (blocked *p*-nitrophenyl maltoheptaoside) in one minute under the defined assay conditions.

3.5.9 **Beta-amylase (β-amylase)**

The Megazyme™ β-amylase assay method (McCleary and Codd, 1989) was used to measure soluble and total (soluble + bound) β-amylase in developing endosperm tissue. The latter was performed following additional 1h extraction with mercapto-ethanol. The assay was scaled down to accommodate 50 µl aliquots of extract. One unit of activity was defined as the amount of enzyme required to release one µM of *p*-nitrophenol from PNPG5 (*p*-nitrophpenyl maltopentaoside) in one minute under defined assay conditions.

3.6 **Micromalting procedure**

Micromalting was carried out by the Waite Barley and Malt Quality Evaluation Laboratory (WBMQEL), Univeristy of Adelaide (Waite Campus). The micromalting schedule developed by the WBMQEL employed a Phoenix^{TM} Automatic Micromalting System without the use of additives. The program comprised the following stages:

Steep and air rest	7h:8h:9h:6h:0.5h (wet:dry:wet:dry:wet)	
Germination	88.5h @15°C	
Kilning	30-40°C, 9h	
	40-60°C, 4h	
	60-70°C, 2h	
	70-80°C, 4.5h	
	Cool to 25°C, 0.5h	*Total time: 139h*

This program has been optimised to produce malts with a steep-out moisture of 42-46% and kilned moisture of 4-5%. Limited quantities of grain were produced from each experiment and this necessitated malting smaller than normal sample size of 30-60g. Micromalting was successfully performed on 15g samples which yielded enough malt for a complete standard malt analysis (see following methods).

3.7.1 **Malting loss**

Malting loss is the decrease (expressed as percent) in average grain weight of malt, following kilning, compared with mature grain (Hough *et al.*, 1971).

3.7 **Malt quality analysis**

3.7.1 **Hot Water Extract (HWE)**

Hot water extract (HWE) is a measure of soluble material (principally carbohydrate and protein) in malt. During the brewing process the carbohydrate is converted by yeast to alcohol. HWE was determined by a rapid small scale method (MacLeod *et al.*, 1991) developed by the WBMQEL, requiring 10g of finely ground malt (0.2mm Miag Mill). It has been closely correlated with the EBC (European Brewery Convention) fine grind extract method (Analysis Committee of EBC, 1975).

The malt flour was extracted with 40mls water at 47°C for 30 mins. The temperature of this extract was raised linearly over 27 mins to 74°C, 15 mls of water were added at this temperature and the sample allowed to extract for a further 57 mins at 74°C. The final extract volume, following further additions of 5 and 15 mls of water as the extract returned to room temperature, was 75mls. The specific gravity of the extract, measured using an Anton Paar digital densitometer, allowed HWE to be expressed as percent, on a dry weight basis, using the following calculations:

Hot Water Extract

E1 = P(M + D)/100-P

E2 = E1 x 100/100-M (units: % extract dry weight basis)

where P = wort extract content in g/100g wort

M = malt moisture

D = final mash dilution factor

3.7.2 **Diastatic power**

Diastatic Power (DP), also known as diastase, represents the combined activity of enzymes (including α-amylase, β-amylase, limit dextrinase and α-glucosidase) involved in the hydrolysis of starch to produce fermentable sugars. A 300mg sample (fine grind 0.5mm Cyclotec Mill) was used to determine DP. The method used was a rapid small scale variation of a standard starch digestion, followed by the measurement of reducing sugars with a neocuproine/copper sulphate reagent (WBMQEL laboratory methods, 1995). DP was determined by measuring the amount of enzyme activity present to break down starch into reducing sugars over a period of 10 minutes. After stopping the reaction, the level of reducing sugars present was measured spectrophotometrically (as above in Section 3.4.2.1). DP was expressed as micromoles of maltose equivalents released per min per gm dry weight.

3.7.3 **Malt α-amylase and β-amylase**

α- and β-amylase are the major starch degrading enzymes found in malt and therefore the principal components of DP. For the purpose of measuring DP in the WBMQEL , the levels of limit dextrinase and α-glucosidase, two other enzymes which also contribute to the breakdown of starch during mashing, were assumed to be negligible (Lance, pers. comm.). An aliquot of DP extract supernatant was reasssayed for DP following

denaturation of the heat labile β-amylase component by heating to 65°C for 15 minutes. This value represented the α–amylase activity component. β-amylase was then calculated from DP by difference. The results were expressed in the same units as DP.

3.8.4 **Malt β-glucan**

The Megazyme™ method (McCleary and Codd, 1991) was used to determine the amount of the cell wall material polysaccharide, β-glucan, remaining after malting. Initial washing with 50% aqueous ethanol ensured the removal of shorter chain sugars (glucose, sucrose and maltose) often present in germinated grain and which would result in an over-estimation of β-glucan present. The method for analysis was otherwise similar to grain β-glucan (Section 3.4.4.3). Megazyme™ provided standard flour of known β-glucan content which was assayed with each batch of samples.

3.8.5 **Malt protein**

Malt protein was measured using a Technicon 400 fixed filter Near Infra-red Reflectance (NIR) Spectrophotometer. This instrument was calibrated using a standard Kjeldahl nitrogen digestion (Institute of Brewing, 1991). Results were expressed as percentage malt protein on a dry weight basis.

3.8.6 **Free amino nitrogen (FAN)**

Free amino nitrogen (FAN) is important for yeast nutrition during fermentation, although very high levels can cause excessive yeast growth and promote the growth of contaminating microorganisms. FAN was measured on the wort prepared from HWE using a standard (Ninhydrin Colorimetric) EBC method (Analysis Committee of EBC, 1975). Results were expressed as mg/litre. This method gave an estimate of amino acids, ammonia and α-amino nitrogen groups on peptides and proteins in wort.

3.8.7 **Viscosity**

Viscosity is a measure of the degree of resistance to the flow of wort. It was measured on 0.5ml subsample of wort at 20°C, using a Wells-Brookfield cone/plate digital viscometer and the results expressed in centipoise (cP) (Ferris and McLeod, 1994).

3.8.8 **Malt beta-glucanase (malt β-glucanase)**

Malt β-glucanase was measured using the Megazyme™ malt β-glucanase assay procedure (McCleary and Shameer, 1987). An extract of malt was incubated with Azo-barley glucan under defined conditions. The malt β-glucanase present depolymerises the dye substrate to fragments which remain in solution in the presence of a precipitant solution. Absorbance at 590nm of the supernatant is directly related to the level of enzyme in the original extracted malt sample. Standard malt flours were analysed with each set of samples.

Malt β-glucanase $Y = MX + C$

where $X =$ absorbance (minus blank absorbance)

$M =$ slope of calibration graph

$C =$ Y intercept

M and C are values determined from the calibration curve for each batch of azo-dye.
Malt β-glucanase was expressed as units/kg malt (units = International Units of enzyme activity and equals one μmole glucose reducing-sugar equivalent released per minute at 30°C and pH 4.6).

3.9 **Light microscopy techniques**

3.9.1 **Tissue sampling, fixation and embedding**

Beween five and ten fresh grains were collected per sample day. Grains were halved longitudinally and fixed in a solution of 2.5% glutaraldehyde (EM grade, Probing and Structure) in 0.025M phosphate buffer, pH 7.2. Initial fixation (approximately 3h) was at room temperature and samples were then kept at 0-4°C for a minimum of 48h, and up to five days. After 2 rinses in phosphate buffer, the tissue was dehydrated through an alcohol series: methoxyethanol, ethanol, propanol, butanol. Successive daily changes of 1:1 and 1:2 of butanol:glycol methacrylate (GMA) (Sigma H-8633) (prepared by mixing 93 ml of 2-hydroxyethyl methacrylate polyethylene glycol, 7 ml polyethylene glycol 400 and 0.6g benzoyl peroxide). This was followed by several changes in 100% GMA. Infiltration with GMA continued over several weeks at 0-4°C.

Gelatin capsules (size '00', Panmedica, Sydney) were used for embedding half grains. Several drops of GMA were polymerised in the base of each capsule and provided a firm,

flat surface to ensure correct orientation of each half grain. Capsules containing half grains were labelled individually and polymerisation was achieved overnight at 60°C.

3.8.2 **Sectioning, staining and observation**

2.5μm median longitudinal sections through each barley grain were made using a Reichert Jung 2050 Supercut Microtome. Sections were collected onto droplets of water, six per slide. Small cuts were made through the perimeter of plastic with a scalpel blade to eliminate folding of sections as they dried. All slides were dried at 60°C for 1-2 days prior to staining. Following staining, sections were air-dried and mounted under coverslips in Micromount (Surgipath Medical Industries, USA) or in fluorescence-free immersion oil (Zeiss Immersionsoel 518C).

3.8.2.1 **Periodic acid-Schiffs /Toluidine blue**

The staining method used was based on O'Brien and McCully (1981), with reduced time (15-20 min) in Schiff's reagent (BDH) to avoid over-staining the large quantities of starch present in cereal endosperm. Sections were counter stained with a 0.05% solution of Toluidine blue in sodium benzoate buffer pH 4.5 (O'Brien and McCully, 1981), to provide detail of cell contents.

3.8.2.2 **Calcofluor**

The fluorescent stain Calcofluor M2R (Polysciences), which stains mixed-linkage β-glucans (Wood and Fulcher, 1978; Wood *et al.*, 1983), was used to highlight cell wall structure. Slides were stained 1-2 minutes in a 0.1% aqueous solution of Calcofluor (made fresh), rinsed in water and coverslipped. Sections were observed using UV fluorescence filters with barrier excitation λ 360nm and emission λ 415nm and Zeiss Axiophot fluorescence microscope.

3.8.2.3 **Congo red**

Congo red also stained mixed-link β-glucans (Wood and Fulcher, 1978; Wood *et al.*, 1983). Sections were stained 2-5 minutes with 0.01% aqueous solution of Congo Red (Grubler and Co., Leipzig), rinsed in water and coverslipped. Sections were observed in a fluorescence microscope using a filter combination: barrier excitation 5-600nm, emission filter 515-540nm.

3.8.3 **Confocal laser scanning microscopy**

The confocal laser scanning microscope (CLSM) offered improved resolution over that of the conventional fluorescence light microscope. A Bio-Rad MRC-1000 CLSM connected to a Krypton-Argon laser was used for observations of the developing CCL following staining with Congo Red (Section 3.8.2.3). Digital images were collected at excitation 448 nm (blue) and emission filter 605 nm (red). Observations were made using a Zeiss 60x oil immersion lens.

The improved resolution provided by the CLSM over that of the conventional fluorescence light microscope provided clear images of optical slices as thin as 0.5µm generated from 2.5mm sections prepared for conventional microscopy (Section 3.8.2). Imaged through the tissue sample without interference from out of focus regions, individual cell layers comprising the CCL structure were clearly distinguished. When viewed in the conventional fluorescence microscope, detail of the composition of the CCL was obscured by the high intensity of fluorescence.

3.9 **Scanning Electron Microscopy (SEM)**

Grain and malt were sampled for SEM by halving triplicate samples of the grain and fixing them to stubs with Supaglue (Selleys Chemical Company, Australia). A carbon and gold/palladium coating was applied and they were observed in a Cambridge Stereoscan S250 SEM. Photographic images, recorded using Ilford FP4 film, were collected from specific regions of the endosperm, namely in the region adjacent to the embryo (including scutellar epithelium, CCL and starchy endosperm cells) and in the central endosperm. Images were routinely collected at 250x and 650x magnification.

3.10 **Statistical analysis**

Analysis of variance (ANOVA) was performed using the GENSTAT 5 statistical package (Lawes Agricultural Trust, Rothamstead Experimental Station). Standard errors (SEM) were calculated and included in graphed results. Details of specific analyses have been included in the methods section of each chapter.

The Tukey method (Zar, 1984) was used for pairwise comparison of means.

Chapter 4

Effects of a period of heat during grain filling on the accumulation of endosperm storage products and on final grain quality.

4.1 Introduction

Periods of high temperature are quite common during the grain filling period in crops grown in the temperate cereal growing regions of Australia (Nix, 1987; Blumenthal *et al.,* 1991; Macnicol *et al.*, 1993; Stone and Nicolas, 1994) and the importance of high temperature stress in agricultural systems is expected to increase if predictions of general global warming eventuate (Adams *et al.*, 1990). Elevated temperatures during grain filling have been found to result in reduced grain weight and therefore yield in wheat (Chowdhury & Wardlaw, 1978; Wardlaw *et al.*, 1989b; Blumenthal *et al.*, 1991; Wardlaw and Moncur, 1995) and in barley (Chowdhury and Wardlaw, 1978; Savin and Nicolas, 1996). For example, in wheat as few as 50 hours accumulated temperatures above 35°C have been found to adversely affect yield (Blumenthal *et al.*, 1991). Elevated temperatures affect yield through changes in the rate and duration of grain filling (Sofield *et al.*, 1977a). Below 18-22°C any reduction in the period of grain filling is likely to be accompanied by a compensating increase in the rate of grain filling so that final grain weight may not be significantly reduced (Sofield *et al.*, 1977a; Jenner 1994). At higher ambient temperatures (above 30°C) this is generally not the case and lower final grain dry weight results from an overall reduction in the duration of grain filling (Sofield *et al.*, 1977a; Weigand and Cuellar, 1981; Stone and Nicolas, 1995a; Wardlaw and Moncur, 1995). Wardlaw and Moncur (1995), in an analysis of rate and duration of grain filling for several wheat cultivars, concluded that enhanced rate of grain filling and tolerance of high temperature (i.e. least reduction in final grain weight) were closely related. Loss *et al.* (1989) also correlated final dry matter per grain with maximum growth rate. A wide ranging survey of 28 cultivars of wheat grown under controlled conditions revealed a 3-4% reduction in final grain weight with each 1°C temperature rise between 24-30°C (Wardlaw *et al.*, 1989a).

The response of barley grain to high temperature during development has long been considered similar to that of wheat (Chowdbury and Wardlaw, 1978) but there are few reports of comparisons between wheat and barley under identical conditions. Also, much of the information available has been accumulated by exposing plants to sustained high temperatures after anthesis in artificial environments (Wardlaw and Wrigley, 1994). While providing valuable physiological information, this cannot be extrapolated to predict the response in cereals under conditions more commonly experienced in the field, where high temperature exposure is brief. In addition, a sudden rise in temperature has been found to cause a greater reduction in yield than a gradual elevation of temperature of equivalent thermal time (Stone and Nicolas, 1995b). These workers also found that in wheat there may be differences between varieties in response to high temperature, with various levels of heat tolerance being exhibited on the basis of growth response to high temperature (40°C).

Previous reports detailing the accumulation of grain dry matter during development, particularly in reponse to changes in ambient conditions, have been produced by analysing whole grain samples. Whole grain samples contain the endosperm, comprising approximately 85% of total grain dry matter with the remainder made up of other tissues including the husk and embryo. When considering any deleterious effects of environment on grain development it is important to realise that individual grain tissues may respond differently to changing ambient conditions. It is possible therefore, that minor changes in the accumulation of endosperm dry matter may be masked by opposing changes in other tissues of the developing grain.

The cereal endosperm is the major storage organ of the grain and represents the principal site of carbohydrate accumulation, with 65-70% by weight in the form of starch (MacGregor and Fincher, 1993). Any deleterious effects of high temperature on the synthesis of starch therefore have a profound effect not only on grain weight and therefore yield (MacLeod and Duffus, 1988a; Jenner,1994), but also on the carbohydrate composition of the grain and therefore its quality. Barley quality is dependent on the principal components of the cereal endosperm, namely carbohydrate and protein (MBIBTC, 1995) and for this reason the focus of this study has been the accumulation of the constituents of the developing barley endosperm.

In grain filling studies, yield data have sometimes been expressed on a whole plant basis (Savin and Nicolas, 1996) or as grain yield per ear (Wardlaw *et al.*, 1980; Nicolas *et al.*, 1984) under defined cultural conditions, but more commonly as average individual grain weight (Wardlaw, 1970; Sofield *et al.*, 1977a,b; Nicolas *et al.*, 1984: Bhullar and Jenner, 1985; Stone and Nicolas, 1995a; Wardlaw and Moncur, 1995). Once seed has set following anthesis, then average individual grain weight is related to yield and can therefore provide an indication of the effects of high temperature exposure on yield variability. This study was commenced following anthesis, using dissected endosperm tissue to provide an indication of both the yield and quality of barley grain developing under defined environmental conditions.

Evaluating temperature effects is important in assessing the overall response by plants to altered environmental conditions. Helpful predictors of yield and quality may thus be generated and breeders assisted in their endeavours to develop varieties with improved tolerance of high temperature. The objectives of this study were to determine the effect of exposure to a brief period of high temperature during mid grain filling on grain/endosperm dry weight and rate and duration of grainfilling, and to compare the composition of this grain with control grain developing under cooler conditions (21/16°C) between anthesis and harvest-ripeness.

4.2 **Experimental conditions and methodology**

4.2.1 **Experimental design**

Five experiments were conducted using the Australian malting barley variety Schooner, grown between anthesis and maturity under controlled environment conditions (growth rooms). Plants were germinated and grown in the glasshouse (temperature maximum 25°C) and transferred to the growth room prior to anthesis (see Chapter 3, Table 3.1). Regular watering ensured that plants were not subjected to water deficit during development.

Growth room conditions and heat treatments have been detailed in Chapter 3 (Materials and Methods). The temperature regime was designed to provide 2 days of increasing temperature followed by 3 days of high temperature (35°C) between 16-20 days after anthesis (daa), followed by return to cooler conditions (21/16°C, 14h day) until maturity and harvest ripeness. Control plants remained at 21/16°C (14h day) throughout

development following anthesis. Fig 4.1 shows the high temperature regime and the sampling schedule during this period.

Detailed descriptions of ear tagging, sampling schedules, endosperm dissection and measurement of fresh, dry weight and grain moisture appear in Chapter 3 (Materials and Methods). Table 3.1 shows the data collected and analyses performed for each experiment. Only main stem ears were used in the experiments and each was tagged at anthesis (Day 0). Triplicate samples were collected at 3 day intervals from 15 days after anthesis (daa), prior to the beginning of heat treatment, to 45 daa. Sampling took place during the first 4-5h of daylight on the designated day. Grain was harvested at the termination of each experiment for quality analysis (micromalting and malt quality analysis). Samples of whole grain were collected in Experiment 3, and in Experiments 3 and 5, the grains were dissected and endosperm and embryos collected separately.

4.2.2 **Rate and duration of grain filling**

The focus of this research was the grain filling stage of development, and although pre-anthesis conditions varied between experiments (see Table 3.1) no differences in fertility and seed set were apparent within each experimental unit (data not shown). Individual endosperm (or grain) weight provided an indication of yield and a record of differences resulting from post-anthesis conditions.

Accumulation of fresh weight and dry weight were determined over each 3 day period. The maximum rate of grain filling (Chowdhury and Wardlaw, 1978) was taken to be the greatest increase in endosperm dry matter obtained in successive samplings over each 3 day period. Rate of change of moisture was calculated as mg water per endosperm per day. Average rates of grain filling were calculated from data collected for individual experiments, not from pooled data from all experiments.

The duration of grain filling was calculated as the time between anthesis and physiological maturity. Grains achieved physiological maturity when no significant addition occurred in grain dry matter. The duration of grain filling was estimated from consideration of endosperm moisture, dry matter accumulation and grain filling expressed on the basis of thermal time (Russelle *et al.*, 1984). In this experiment, thermal time was calculated for post anthesis development only, assuming a base

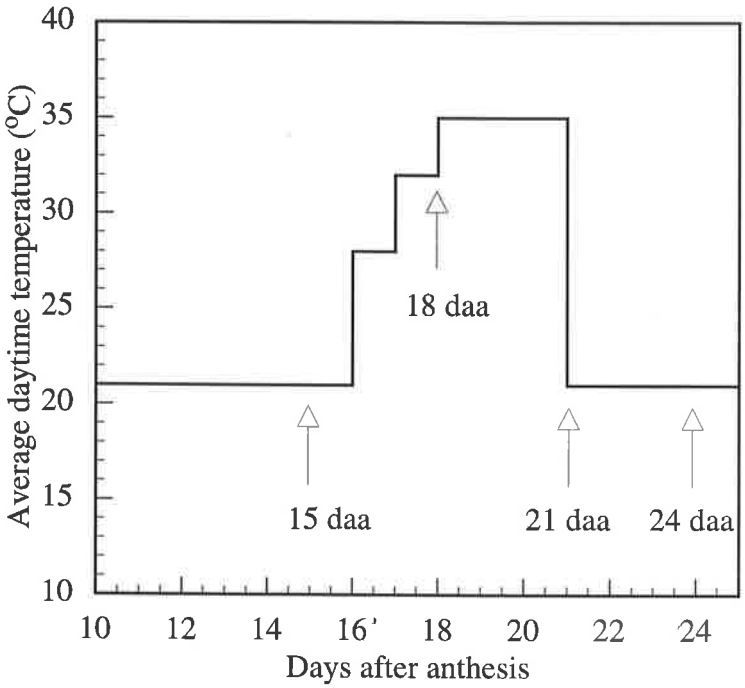


Fig 4.1 Temperature profile and sampling times 15-24 daa for heat treated plants.

temperature of 7.1°C (Goyne *et al.*, 1996). Details of calculations appear in Chapter 3 (Section 3.4.3).

Growth rooms were set at 14h day throughout. Temperature changes between day and night were achieved within approximately 30 minutes and deviation from set temperatures was generally within the range of +/- 2°C .

4.2.3 **Analysis of endosperm storage components**

Analyses of endosperm starch, nitrogen and β-glucan were carried out using methods described in Chapter 3. Dried endosperm material (from which grain filling data were derived) was used for these analyses. The material was ground to a fine powder with a mortar and pestle. Moisture measurements made on samples of heat treated and control flour were used to express endosperm component data on a dry weight basis.

4.2.4 **Statistical analysis**

Analysis of variance was applied to experimental results to determine the difference between treatments based on the interaction between time (after anthesis) and treatment. Data were analysed for each experiment and where significance was established, comparisons made on the basis of accumulated data from all (5) experiments.

The Tukey method was used to provide pairwise comparisons of means. Significance between (developmental) means occurred even where no significance was apparent in the overall F test (ANOVA). Error bars (standard error of means, SEM) have been included for individual data points.

4.3 **Results**

Statistical analysis of individual experiments revealed differences in the significance level between experiments due to exposure to a period of high temperature during mid grain filling (see Table 4.1). There was greater variation within than between experiments, due to the low number and the variability of replicates (3) within each experiment. Analysed collectively, data from Experiments 1-5 showed a higher percentage significance than the individual analyses due to the greater degrees of freedom (data not shown). Data have therefore been presented following analysis of pooled data, except where noted separately in the text, and average values for each attribute, including rates of

accumulation of endosperm components, calculated on the basis of individual experiments.

Table 4.1 Results of analysis of variance for attributes measured within each experiment comparing grain development between control and heat treated plants, analysed separately and collectively.
*** significantly different from control P< 0.001; ** P<0.01; * P<0.05; ns, not significant; nd, not determined.

	Attribute (mg per endosperm)					
Experiment	**Fresh wt**	**Dry wt**	**Moisture**	**Starch**	**Nitrogen**	**β-glucan**
1	***	**	***	**	ns	***
2	ns (0.083)	**	ns	***	*	***
3	**	**	*	***	***	ns
4	ns (0.06)	**	*	nd	nd	nd
5	***	***	***	nd	nd	nd
1-3	nd	nd	nd	***	*	***
1-5	***	***	***	nd	nd	nd

The accumulation of dry weight within the endosperm was observed to follow a similar pattern overall to that of dry matter accumulation of the whole grain (Fig 4.2). Endosperm tissue however, appeared to be more responsive to temperature changes than the whole barley grain. For example, an immediate increase in endosperm dry matter was observed (16-18 daa) in response to a moderate increase in temperature (Fig 4.2b). This was not observed in the whole grain.

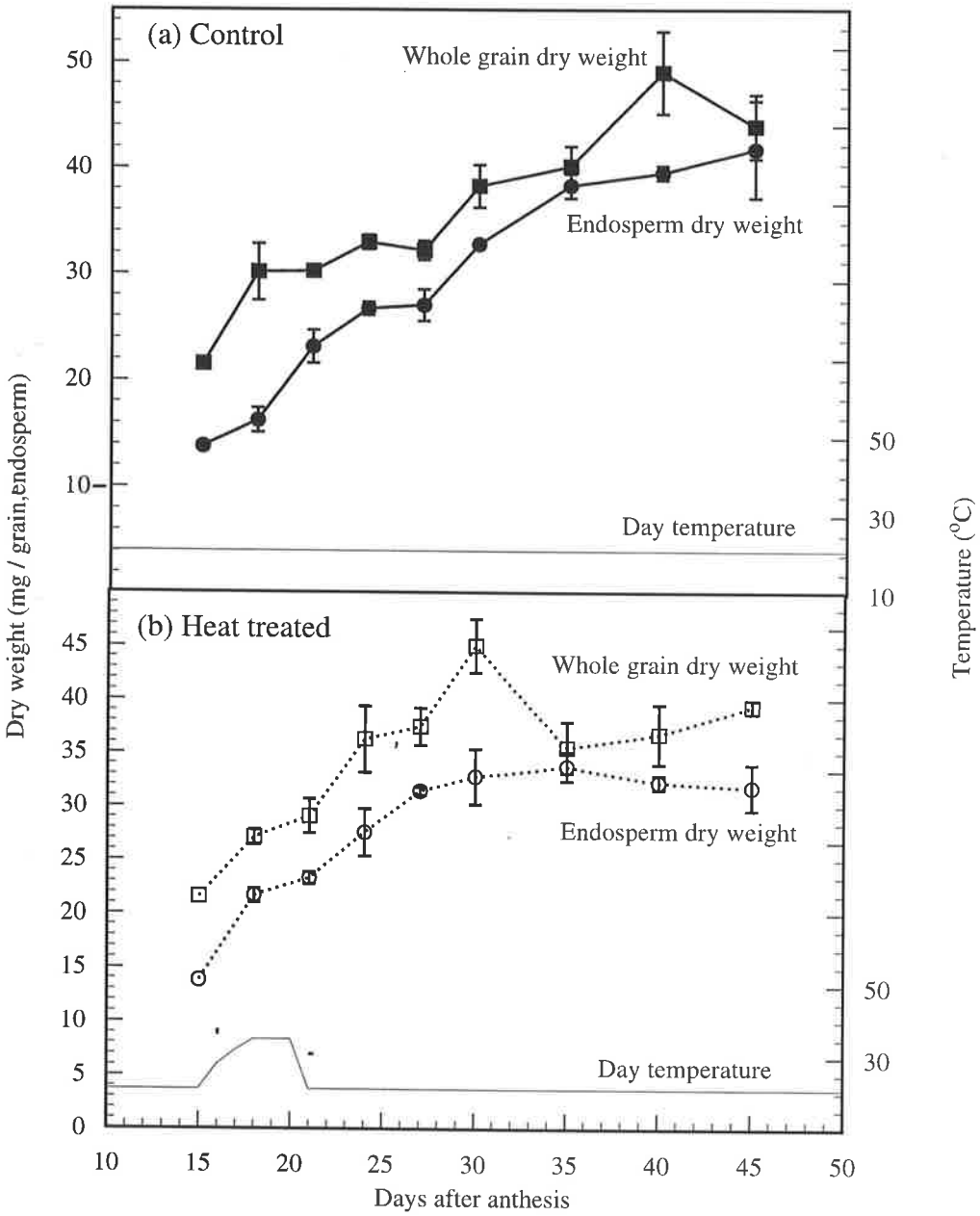


Fig 4.2 Changes in dry weight accumulation of whole grain and endosperms in grain developing under (a) control and (b) high temperature conditions (Experiment 3 data). Temperature profiles show the average daytime temperature. Vertical bars in this and all subsequent graphs represent 2x standard error of mean.

4.3.1 **Effect of high temperature exposure on final grain weight**

Final grain and endosperm dry weight were significantly reduced in Experiments 1-5 as a result of a period of high temperature during grain filling. Overall yield reductions were 18% in whole grain and 23% in endosperm tissue. The decrease in endosperm dry weight at 40 daa and in whole grain brought about by the heat treatment was highly significant. Although yield loss between experiments was somewhat variable, reductions were observed for whole grain and endosperm tissue in each case (Table 4.2).

The accumulation of whole grain dry matter appeared to show a number of fluctuations during development when compared to the more consistent accumulation of dry matter of endosperm tissue (Fig 4.2). All subsequent grain filling data have been derived and presented on a per endosperm basis, indicating the specific response of this grain storage organ to the effects of high temperature exposure during grain development.

Table 4.2 Yield reduction due to a period of high temperature during grain filling in endosperm and whole grain dry weight; summary of data from Experiments 1-5. Values in parentheses have not been included in mean.
* yield reduction in endosperm tissue significant difference from that in whole grain $P< 0.05$; nd, not determined.

	% Yield reduction	
Experiment	**Endosperm 40 daa**	**Whole grain**
1	24.8	20.9
2	(31.0)	nd
3	22.1	15.2
4	22.8	19.0
5	(24.9)	nd
Average	23.2*	18.4

4.3.2 **Effect of high temperature exposure on the accumulation of fresh and dry weight**

Control and heat treated grain accumulated an average of 74% and 65% respectively of their final endosperm dry matter between 15 and 40 daa in experiments 1-5 (data not shown). Data collected from all 5 experiments (Fig 4.3), revealed that the pattern of

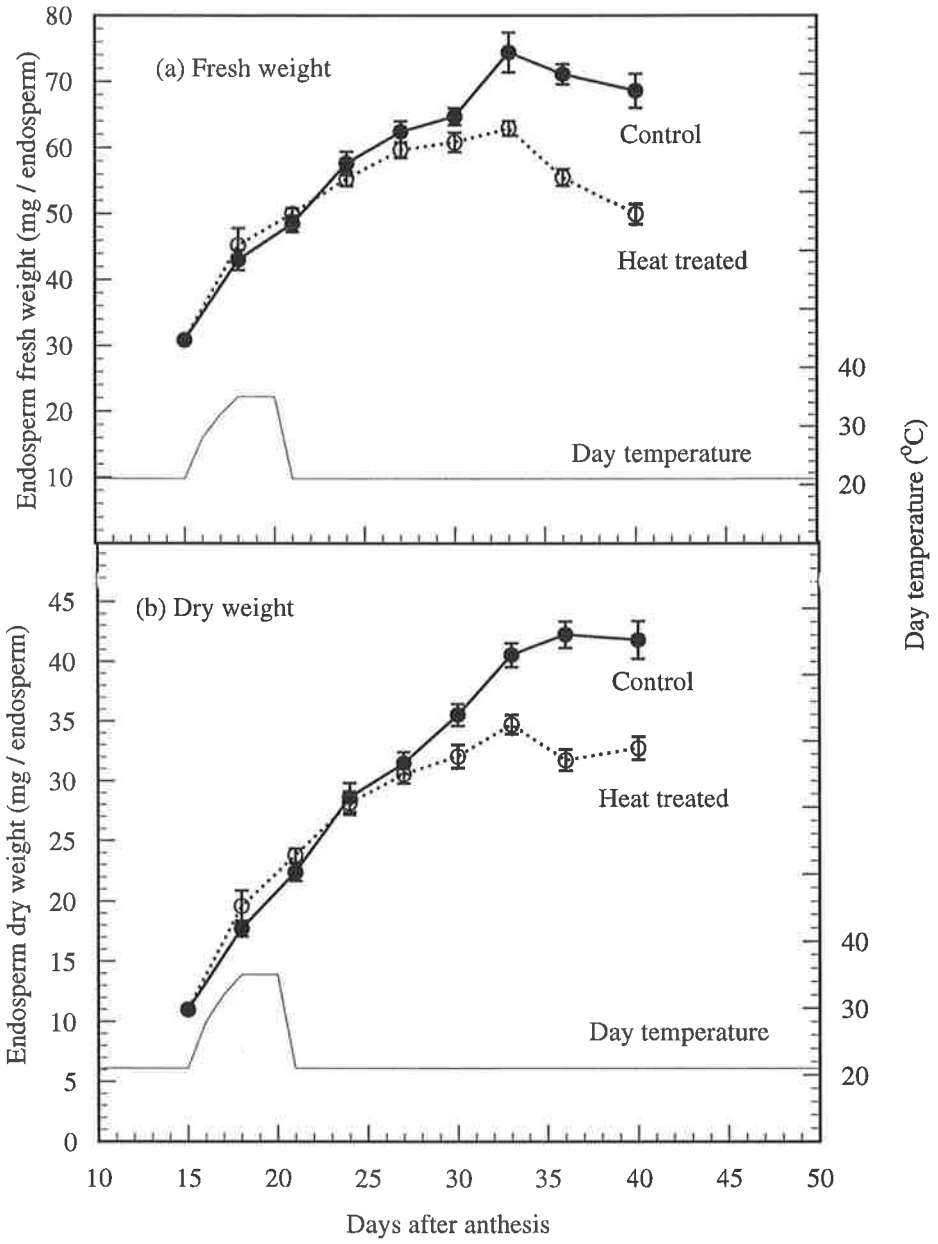


Fig 4.3 Endosperm fresh weight (a) and dry weight (b) accumulation under control and high temperature conditions in developing grains.

accumulation of endosperm dry matter showed two separate effects during the high temperature period. Initially, up to 18 daa, the accumulation of dry matter increased under moderate temperatures. An extended period at higher temperatures (35°C) caused this rate to slow (from 3.04 to 1.41 mg per endosperm per day) and by 24 daa heat treated endosperms were similar in size to those of control grain. From this time the pattern of dry weight gain in heat treated grain was altered and the rate of increase in endosperm dry weight tapered off until 40 daa. Heat treated endosperms were smaller than those of control grain from 30 daa (Fig 4.3).

The maximum rate of fresh and dry matter accumulation within the developing endosperm occurred earlier in heat treated grains than under control conditions (data not shown). An average rate of 3 mg dry weight per endosperm per day was recorded between 18 and 21 daa during exposure to moderately high temperatures (up to 32°C but below 35°C). In control grain the maximum rate of dry matter accumulation occurred 21-24 daa (2.1 mg per endosperm per day).

The water content of the endosperm (Fig 4.4) increased most rapidly between 15-18 daa in both control and heat treated grains. Rapid water loss was observed to occur from control and heat treated grain after 33 daa, indicating that the onset of net water loss appeared not to be accelerated in heat treated grain.

4.3.3 Effects of high temperature exposure on the amount of endosperm storage products accumulated at maturity

The synthesis of grain endosperm constituents during development was investigated to establish if heat treatment had a direct affect on the composition of the developing grain. The accumulation of endosperm components has been recorded on a per endosperm basis in this study in order to take into account alterations to grain size resulting from treatments. The concentration (as percentage of dry weight) of these components is more often cited when discussing grain quality (Henry, 1990) and this will also be considered (see Fig 4.5).

Starch, nitrogen and β-glucan represent the principal components of the barley endosperm and were calculated to be 64-70% of total endosperm dry weight in these experiments. While this was somewhat lower than expected, it remained consistent

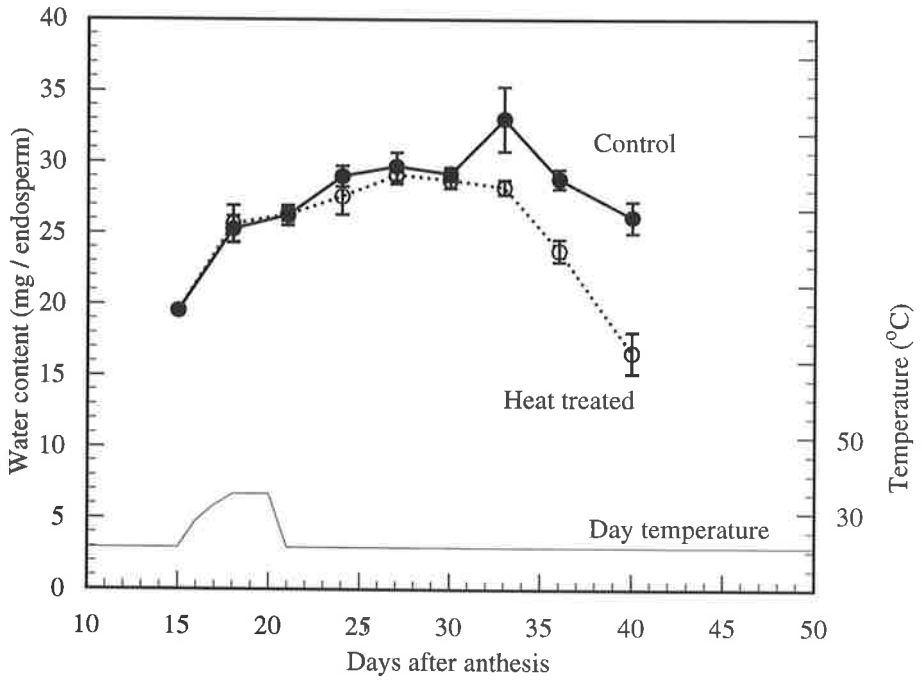


Fig 4.4 Changes in endosperm water content between control and heat treated grain during development.

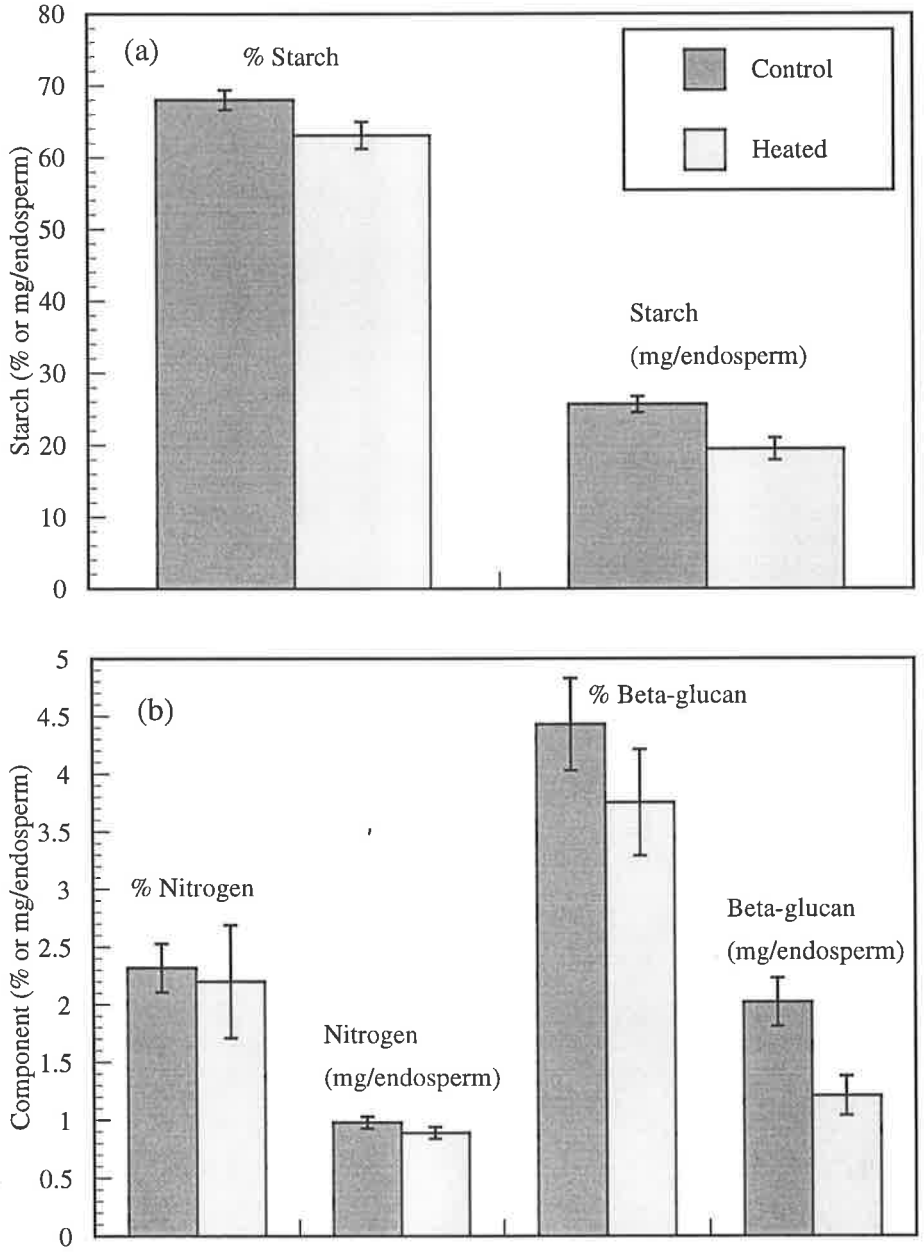


Fig 4.5 Endosperm component analysis from control and heat treated grain 40 daa, showing the composition of (a) starch and (b) nitrogen and beta-glucan, expressed as percent and as mg/endosperm on a dry weight basis.

between treatments and experiments. Limitations to the accuracy of assays may account for some of the shortfall, in addition to the presence of other compounds which could not be quantified.

Table 4.3 Mature grain endosperm composition as mg of component per endosperm (summary of data collected from Experiments 1-3).
*** significantly different from the control P<0.001; ns, not significant.

	Endosperm component mg/endosperm			
	Starch	**Nitrogen**	**β-glucan**	**Total**
Control	26.40	0.98	2.28	29.66
Heat treated	17.79***	0.89^{ns}	1.32***	20.00

The results of these experiments indicated that the 32% overall reduction in endosperm starch in heat treated grain accounted for at least 81% of the corresponding reduction in endosperm dry matter accumulation (Table 4.3). When measured on a per endosperm basis, β-glucan was reduced to a greater extent than starch as a result of exposure to high temperature, ie 42%, compared with 32%. Nevertheless, the reduction in starch synthesised in the endosperm until 40 daa provided the greatest contribution to the reduced endosperm dry weight observed between treatments (Table 4.3, Fig 4.5a). Less nitrogen was accumulated in heat treated endosperms to 40 daa than in control endosperms. However, the 9.2% decrease in the quantity of endosperm nitrogen due to heat treatment was not statistically significant and represented only a 5% decrease in this constituent as a percentage of endosperm dry weight (Fig 4.5b). This result was a consequence of the comparatively low contribution to endosperm dry weight made by nitrogen and the lower endosperm dry weight of heat treated grain.

4.3.4 **Effects of high temperature on the accumulation of endosperm storage products**

4.3.4.1 **Starch accumulation**

The pattern of endosperm starch accumulation followed that of dry matter during grain development in control grain (Fig 4.6). The rate of accumulation of dry matter and starch in control grain remained relatively steady until about 33 daa, after which the rate declined.

Starch deposition was accelerated in response to moderately high temperatures (up to 32°C) over a 2 day period (ie day 16 and 17 after anthesis) and more endosperm starch was accumulated in high temperature endosperm 18 daa (Fig 4.6) than in control grains. The longer period (3 days) at higher temperature (35°C) resulted in a reduction in the rate of starch accumulation so that by 21 daa heat treated grain contained less starch than control grain. Some recovery in starch synthetic activity was apparent between 21 and 24 daa (a rate of 1.2 mg starch per endosperm per day was recorded) almost matching the rate in control grain (1.21 mg per endosperm per day) at this time. However, this was not sustained and starch synthesis almost ceased in high temperature treated grain from 24 daa, and by 27daa they contained significantly less starch than control grains.

4.3.3.2 **Nitrogen accumulation**

Control grain accumulated nitrogen at an almost constant rate from 15 daa until 33 daa, when the rate slowed (Fig 4.7). Nitrogen accumulation showed an immediate stimulation in response to a period of heating. Enhanced by moderate temperatures (up to 32°C), the rate of nitrogen accumulation was sustained at these elevated rates at high temperatures and continued upon return to cooler conditions. The rate of nitrogen accumulation peaked at 0.12 mg per day in heat treated grain 24 daa, more than twice the maximum (0.05 mg per endosperm per day) recorded in developing control endosperm. The accumulation of endosperm nitrogen ceased after 30 daa in heat treated grain, so that by 40 daa there was no significant difference in nitrogen levels between the endosperms of control and heat treated grain.

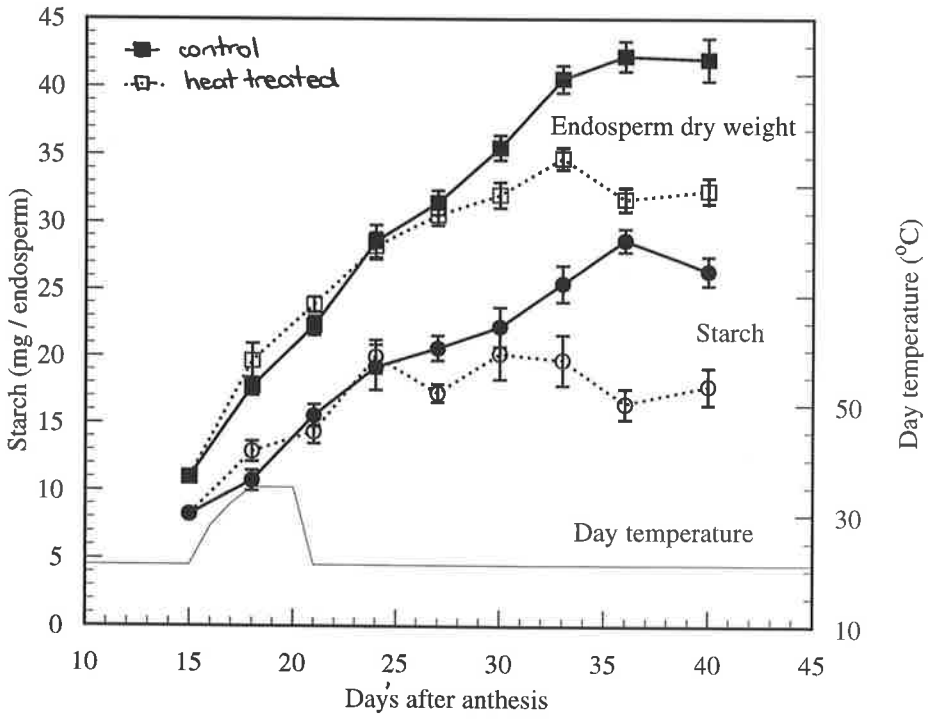


Fig 4.6 Endosperm dry matter and starch accumulation in the endosperm of developing control and heat treated grain. Starch data from Experiments 1-3, dry weight data from Experiments 1-5.

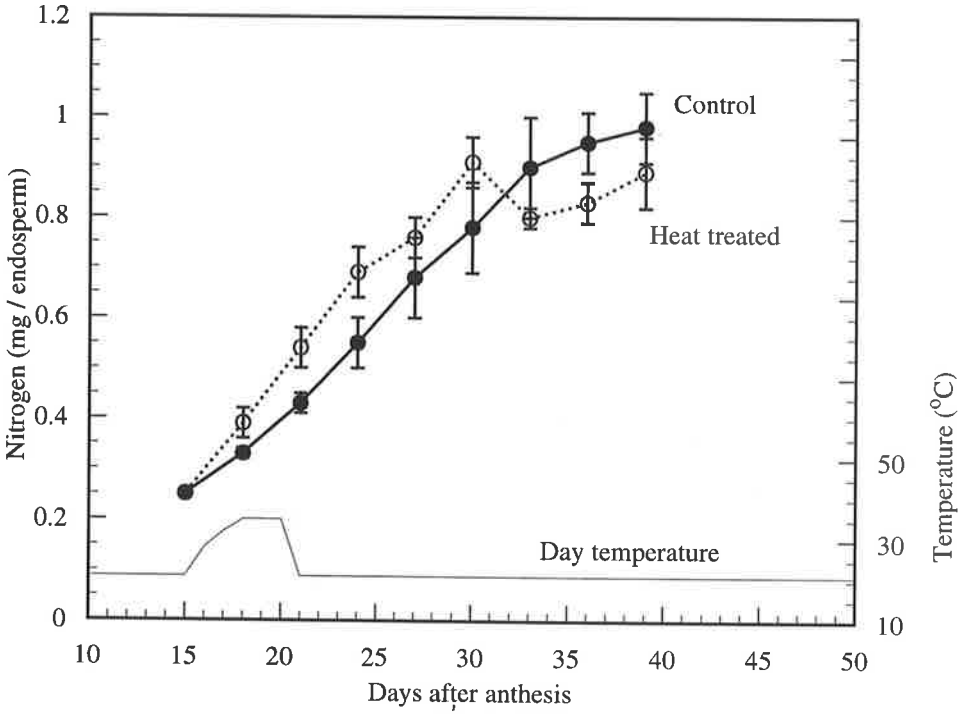


Fig 4.7 Nitrogen accumulation (on dry weight basis) in developing endosperms of control and heat treated barley grain.

4.3.4.3 β-glucan accumulation

Between 15 and 18 daa there was little if any β-glucan synthesis under control conditions (Fig 4.8). From 21 daa the rate had increased to 0.13 mg per day and similar rates were sustained until 33 daa. Under these experimental conditions, the maximum rate of accumulation of β-glucan was recorded between 36 and 39 daa.

Elevated temperatures stimulated an immediate increase in β-glucan in the endosperm. Indeed, 2 days of moderate heat (up to 32°C) resulted in the maximum rate of β-glucan synthesis recorded for any treatment, of 0.14 mg per day. Higher temperatures of 35°C reduced β-glucan synthesis between 18 and 21 daa, to a rate almost half the control rate. This rate was further suppressed 21-24 daa but some recovery took place after 24 daa, and control rates were almost achieved between 27 and 30 daa (0.8 c.f. 0.9 mg per day for control grain). Endosperm β-glucan in heat treated grain did not increase significantly beyond 30 daa.

4.3.3.4 Rate of accumulation of endosperm storage components

The effects of high temperature treatment on the rate of accumulation of the three endosperm components are compared in Table 4.4 (derived data not statistically analysed). A moderate rise in temperature stimulated the synthetic capacity of developing endosperm. Heat treated grain recorded higher rates of accumulation of starch, nitrogen and β-glucan at 18 daa when compared to control grain. Exposure to a prolonged period of higher temperature, however, eventually led to changes in the capacity and sustainability of the synthetic machinery for all three endosperm components of interest. The irreversible reduction in starch synthesis led to alterations in the overall concentration of nitrogen, and to a lesser extent β-glucan, in the developing endosperm from 24-40 daa.

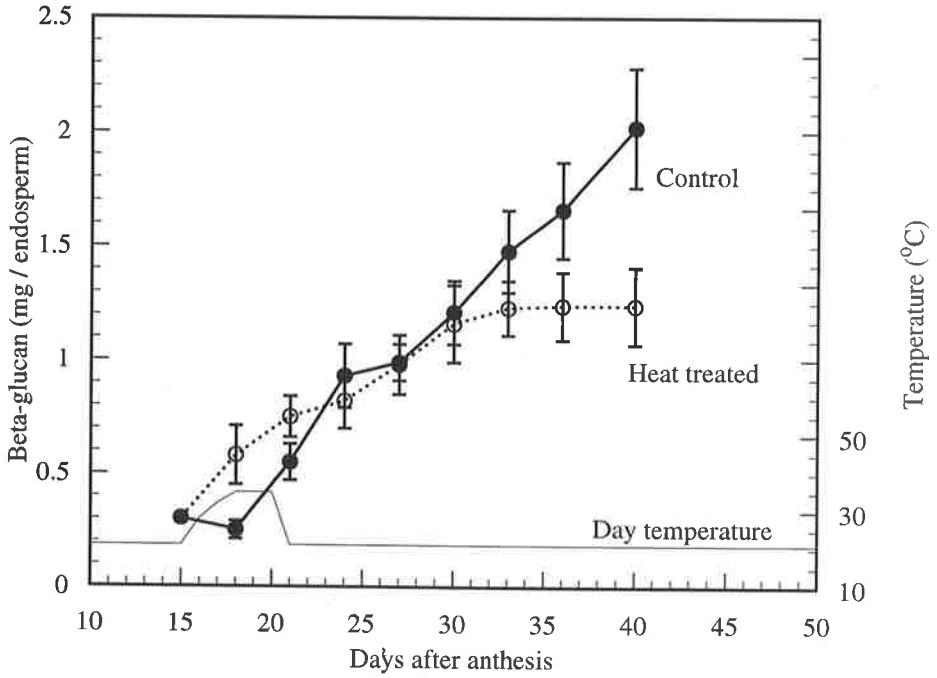


Fig 4.8 Accumulation of endosperm beta-glucan in control and heat treated grain during development.

Table 4.4 Rate of accumulation of endosperm starch, nitrogen and β-glucan under control and heat treated conditions, during the heating period (16-20 daa) and the cooler recovery period (21-30 daa), expressed as mg dry matter per endosperm per day.

Days after	**Starch**		**Nitrogen**		**β-glucan**	
anthesis	control	heated	control	heated	control	heated
15-18	0.83	1.55	0.028	0.049	0.00	0.11
18-21	1.61	0.49	0.031	-0.004	0.13	0.07
21-30	0.74	0.65	0.044	0.058	0.09	0.06

4.3.5 **Effect of high temperature exposure on the duration of grain filling**

The logistic function has been found to be the most appropriate model to describe grain growth in field plots (Loss *et al.*, 1989). Fitting a model to describe grain growth of control plants under the cultural conditions of this study was tested. The control grain filling curve fitted to this data was; $y = 0.3449x^2 + 7.4696x + 3.7307$ (r^2=0.99).

However, the growth curve of heat treated grain departed from this logistic function and it was not possible to use a mathematical equation to describe it, so assessment of the duration of grain filling in heat treated grain had to be less definitive. It was determined from assessment of the pattern of dry weight accumulation making use of data expressed in thermal time (degree days above 7.1°C). Fig 4.9 shows the grain filling data from Experiments 1-5 plotted in this way. On this basis the effects of high temperature treatment did not become apparent for some time and it was more than 60 degree days (°Cd) after the onset of heating before the plots diverged. After 240°Cd the deleterious effects become progressively more pronounced although the course of development appeared not to have been affected. From these data, control grain attained maximum dry weight approximately 36 daa, while in heat treated grain this was achieved 33 daa. These points represent 414 and 429°Cd respectively, indicating that by this criterion physiological maturity almost coincided in thermal time for both heat treated and control grains.

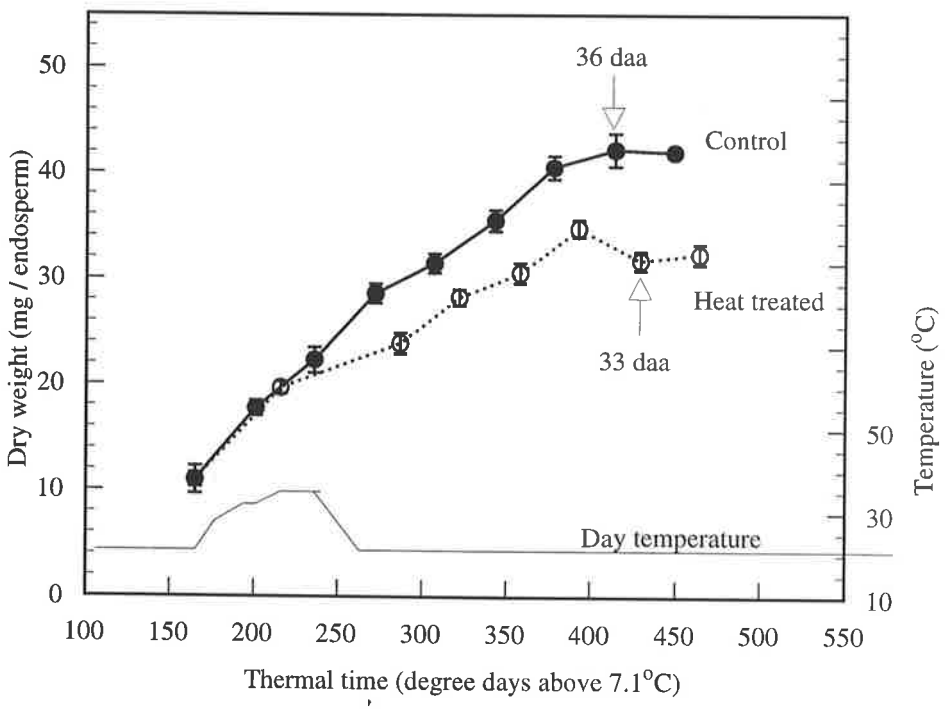


Fig 4.9 Accumulation of dry weight by control and heat treated endosperms, plotted in thermal time (degree days above base temperature of 7.1°C) after anthesis.

When plotted in thermal time, the pattern of moisture loss from endosperm tissue showed that rapid loss of water from the endosperm of control and heat treated grain corresponded closely (Fig 4.10). Heat treated grain lost water 33 daa, while the highest rate of water loss (1.57 mg per endosperm per day) was recorded in control grain from 36 daa.

4.4 **Discussion**

4.4.1 **Effects of high temperature on final grain weight**

While pre-anthesis conditions which result in the accumulation of insufficient reserves may limit grain filling potential (Russell and Ellis, 1988), this did not appear to be the case in the present study, despite differences in pre-anthesis conditions (see Table 3.1). Final grain weights of 47 mg compared favourably with field grown Schooner at 46 mg (Logue *et al.*, 1994) and other growth room grown material, 49 and 54 mg (Savin and Nicolas, 1996 and Savin *et al.*, 1997a), respectively. The high temperature regime was applied for 11% of the grain filling period and increased the average temperature during this time by 1.1°C (i.e. from 18.9°C to 20°C), adding 50°Cd to the control. In total, 6% of the grain filling period was spent at high temperatures, i.e. 35/25°C.

Elevated temperatures for 5 days caused a reduction of 17% in individual grain weight in this study. This falls in the mid-range of reductions in average grain dry weight recorded as a result of a brief period of high temperature during grain filling for Schooner barley (Savin and Nicolas, 1996; Savin *et al.*, 1997a,b). The greatest reduction in grain weight so far reported, of 35%, was recorded when the treatment was given early during grain filling (i.e. 15-20 daa) and temperatures were high (40°C) (Savin *et al.*, 1997a). In other studies with cereals, the magnitude of response was greatest with early exposure to heat (Nicolas *et al.*, 1984; Randall and Moss, 1990; Stone and Nicolas, 1995a; Savin *et al.*, 1997a) or when exposure time was increased (Jenner, 1991b). Reductions in final grain weights recorded by Macnicol *et al.* (1993) and Savin and Nicolas (1996), who exposed plants to 3 and 5 day heat treatments, respectively, were considerably lower than in the present study. In these instances the reduced effects on grain dry weight were probably due to the shorter duration of daily exposure to heat. In both studies the accumulated heat exposure represented comparatively less of the total grain filling period than in the present study (*ca.* 2.6%; Savin and Nicolas, 1996) so that even though the maximum temperature was higher (40°C), it was maintained for only 5 hours each day.

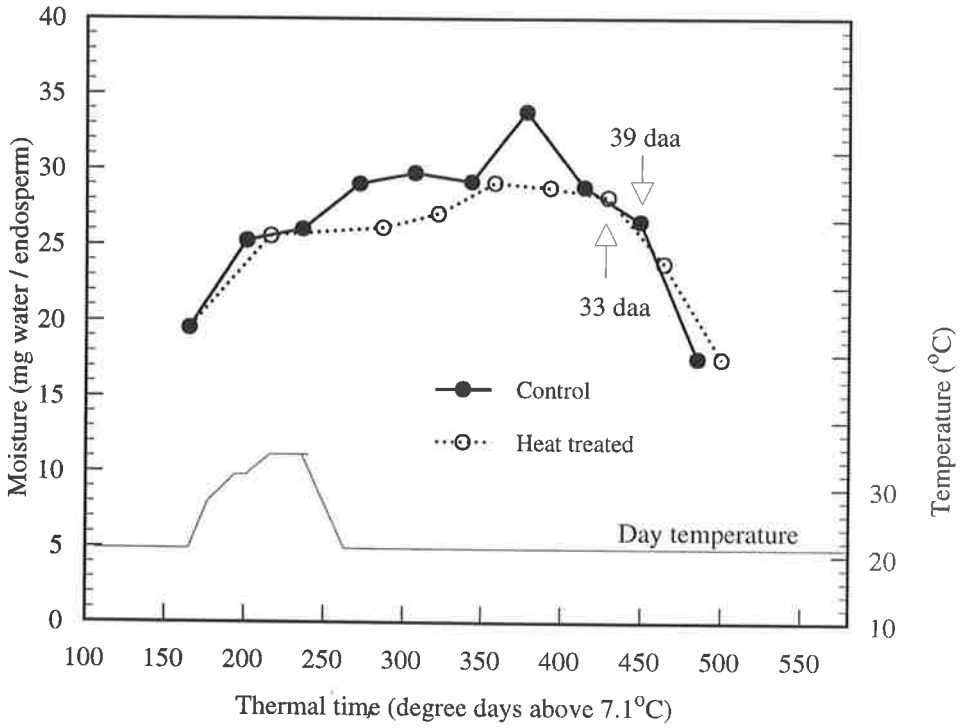


Fig 4.10 Changes in endosperm moisture between control and heat treated grain, plotted in thermal time (degree days above base temperature of 7.1°C).

Schooner barley endosperm tissue exhibited greater sensitivity to high temperatures than the whole grain. These results indicate that the non-endosperm portion of the grain suffered to a lesser extent than endosperm tissue as a result of exposure to heat treatment (Table 4.2). When plants were exposed to high temperature conditions during grain filling it is likely that the non-endosperm tissues of the grain, which includes the embryo and husk, may be important determinants of final individual grain weight. Under such conditions the non-endosperm portion of the grain has the potential to influence the apparent degree of high temperature tolerance in barley grain (Stone and Nicolas, 1995a). Data are required on the effects of high temperature on the growth behaviour of these tissues, which have largely been overlooked in definitions of barley grain quality (Palmer, 1989; MBIBITC, 1995).

4.4.2 **Rate and duration of grain filling**

Exposure to high temperatures during grain filling accelerated development of the grain, resulting in a reduction of 8% in the duration of grain filling compared to control grain which was considerably less than that recorded by Nicolas *et al.* (1984) and Savin and Nicolas (1996). In these studies the greater reduction in the grain filling period (23 and 33%) appeared to be related to the early application of heat treatment and the length of heating period. In wheat, early application of heat (Stone and Nicolas, 1995a) and temperatures up to 40°C (Sofield *et al.*, 1977a) also appear to have the greatest effect. Sudden exposure to high temperature also reduced the duration of grain filling to a greater extent than gradual exposure (Stone and Nicolas, 1995b).

It is possible that exposure to moderate temperatures (up to 32°C) may serve to acclimatise plants for subsequent higher temperature exposure (Stone and Nicolas, 1995b). For example, the rate of grain filling during the 3 day period at 35°C in the present study exceeded the maximum rate recorded by Savin and Nicolas (1996) during 5 days of temperatures up to 40°C (6h per day). Indeed, as little as 2h pre-exposure to intermediate temperatures (30-34°C) was found by Savin *et al.* (1997b) to reduce the effects of subsequent severe heat (40°C).

In the present study, grain filling rates varied with moderate and high temperatures and upon return to cooler recovery conditions. Normal, but possibly short lived, plant responses to changing temperatures may be overlooked where rate of grain filling is

simply considered as one of the parameters characterising the linear phase of grain growth. As in the present study, Savin and Nicolas (1996) observed deviations from the logistic pattern in the growth curve following brief exposure to high temperatures (Section 4.3.4). They recorded a delay between the cessation of heat treatment and the effects of high temperature on the rate of grain growth. Failure to detect an immediate response during the heating period may have been partly due to less frequent sampling (5d c.f. 3d in the current study). Jenner (1991b) and Stone *et al.* (1995) also observed a delay between the cessation of heat treatment and the effects of high temperature on endosperm dry weight accumulation indicative of changes in the rate of synthesis of endosperm storage components.

4.4.3 **Accumulation of endosperm starch, nitrogen and β-glucan**

Hastened development obviously contributed to the reduction in final grain weight but was not the only factor involved. Changes in endosperm composition provided evidence of alterations to metabolism as a consequence of exposure to high temperatures. Use of degree days (°Cd) to compare grain filling between treatments indicated physiological changes due to or associated with the high temperature treatment were apparent by 21 daa. This provided evidence of the inhibitory effects of high temperature exposure on grain growth.

Starch accumulation appeared to be irreversibly reduced following 5 days of 14h daily exposure to high temperatures. While 5 days of short periods of daily exposure to high temperature did not appear to reduce the accumulation of starch (Savin and Nicolas, 1996), when the high temperature period was longer, i.e. 10 days, the capacity to accumulate starch was diminished even upon return to cooler conditions. The results in the present study appear consistent with the view that the more severe the reduction in starch synthesis the slower the recovery of starch synthesis (Savin and Nicolas, 1996). A possible explanation for this may be provided by the response of enzymes to starch synthesis to high temperature exposure, particularly soluble starch synthase (Jenner *et al.*, 1993; Keeling *et al.*, 1993). For example, Hawker and Jenner (1993) observed a greater reduction in the recovery of soluble starch synthase activity following 7 days of heating to 35/25°C than in grain subjected to only 2 days of these high temperatures. In addition, in studies where the number of hours exposure to high temperature (35°C) was short, recovery of up to 100% of synthetic enzyme activity has been observed (Hawker

and Jenner, 1993) and starch accumulation following mild heat treatment appeared unaffected (Savin and Nicolas, 1996).

Compared with starch the accumulation of endosperm β-glucan appeared more sensitive to exposure to a period of high temperature, although the reduction in starch accumulation provided the greatest contribution to lower endosperm dry weight. Even though β-glucan accumulation occurs late in development (Coles, 1979; Aman *et al.*, 1989), high temperature exposure early in grain filling appeared to alter the pattern of deposition of this cell wall component. While the synthesis of mixed-link β-glucan has a broad optima around 20°C (Becker *et al.*, 1995), at temperatures above 20°C (1-3)(1-4)-β-glucan synthase is increasingly unstable. Two days of temperatures above 20°C resulted in an increase in β-glucan synthesis in this study. A very rapid increase in β-glucan synthesis has been associated with the completion of cell elongation in early seedling development in barley (Becker *et al.*, 1995). It is likely that moderate temperatures imposed between 16-18 daa increased the rate of endosperm cell division and expansion (Nicolas *et al.*, 1984) so that hastened endosperm development may have contributed to the increase in β-glucan synthesis observed during the early stages of the high temperature treatment. The apparently irreversible reduction in β-glucan synthesis which followed high temperature (35°C) exposure may have been due to the accelerated development of the endosperm and associated early termination of endosperm cell wall biosynthesis (Aman *et al.*, 1989), to increased instability of synthases at high temperatures (Becker *et al.*, 1995) and/or to changes in the status of membranes to which these enzymes are tightly bound (Raison *et al.*, 1980; Saadalla *et al.*, 1990).

Little change was observed in the amount of nitrogen accumulated per endosperm as a result of exposure to high temperatures. Similar findings have been reported previously in both barley and wheat (Sofield *et al.*, 1977b; Bhullar and Jenner, 1985). However, the use of the conversion factor to calculate protein content from nitrogen (Tkachuk, 1969), may not have provided a valid indication of the total protein content of the endosperm under all experimental conditions. Endosperm reserve protein is made up of a number of components, including β-amylase, enzyme inhibitors, protein Z, and protein associated with cell walls and starch (Wallace and Lance, 1988) and up to 10% of the nitrogen present may be present as non-protein nitrogen in the mature grain (Miflin and Shewry, 1979). Consequently, given that the effects of heat on non-protein nitrogen have not

been documented, it is not possible to determine whether these growth conditions altered overall grain protein levels in this study.

While there is no evidence that grain nitrogen levels in barley are significantly affected by a period of high ambient temperatures (Savin and Nicolas, 1996; Savin *et al.*, 1996, 1997a,b), a significant reduction in the quantity of grain protein has recently been reported in wheat (Stone *et al.*, 1996). An increase in grain nitrogen and protein concentration however, has been recorded in both wheat and barley under high temperature conditions (Sofield *et al.*, 1977b; Bhullar and Jenner, 1985; Savin and Nicholas, 1996). The association between high temperatures and high protein concentration under both field and controlled growth conditions (Jenner *et al.*, 1990; Bhullar and Jenner, 1985; Correll *et al.*, 1994) has largely been attributed to the complimentary relationship between protein and starch content of the grain (Henry, 1990; Jenner *et al.*, 1990) and the reduction in starch synthesis under high temperature conditions (MacLeod and Duffus, 1988a; Bhullar and Jenner 1986).

4.5 **Conclusions**

High temperature exposure for several days in mid grain filling reduced final grain weight. The endosperm exhibited greater sensitivity to high temperatures than the grain as a whole (including the non-endosperm component) and endosperm composition was altered as a result of high temperature exposure. Although β-glucan synthesis was affected more than either starch or nitrogen accumulation, the reduction in starch accumulation made the greatest contribution to dry weight reduction.

Chapter 5

Effect of high temperature during grain filling on starch synthesis in the developing barley grain

5.1 Introduction

Under high temperature conditions the ability of the grain to sustain an increased growth rate is important in minimising the effects of exposure to heat, in particular, to counterbalance the reduced duration of grain filling associated with high temperatures (Wardlaw and Moncur, 1995). Starch is the single most abundant component in the barley grain, accounting for 65-70% by weight (MacGregor and Fincher, 1993), and its synthesis is diminished under high temperature conditions (Jenner and Bhullar, 1985; MacLeod and Duffus, 1988a). Lower final starch content in cereal grains is largely responsible for the decrease in yield which has been associated with exposure to high temperatures. While the supply of sucrose has not been found to be limiting under these conditions (Lingle and Chevalier, 1984; Nicolas *et al.*, 1984; MacLeod and Duffus, 1988a), a reduction in the conversion of sucrose to starch has been associated with high temperature exposure in wheat (Bhullar and Jenner, 1986) and barley (MacLeod and Duffus, 1988a).

The metabolic pathway of starch synthesis from sucrose to starch has been established for developing sink organs, including the cereal endosperm (Keeling *et al.*, 1988; Okita, 1992), and is reproduced in Fig 2.2. However, this scheme remains incomplete as it does not include recent evidence of the presence of ADP-glucose pyrophosphorylase in the cytosol as well as in the amyloplast in barley endosperm (Thorbjornsen *et al.*, 1996). Regulation of starch synthesis is thought to involve the enzyme ADP-glucose pyrophosphorylase, based on biochemical and genetic studies of starch accumulation in a number of plant organs and tissues (Sivak and Priess, 1995). There is evidence however, that soluble starch synthase has a low temperature optimum (Keeling *et al.*, 1993) and is susceptible to heat inactivation (Rijven, 1986; Hawker and Jenner, 1993; Keeling *et al.*, 1993) so that under high temperature conditions this enzyme limits starch synthesis in

wheat (Keeling *et al.*, 1993; Denyer *et al.*, 1994). Previous studies in barley have focussed on sucrose synthase (MacLeod and Duffus, 1988a).

Much of what is known of the effects of high temperature on grain filling has been derived from research on wheat (Jenner, 1994), although preliminary comparisons suggest that barley responds to high temperature in a similar way (Chowdhury and Wardlaw, 1978). Studies in wheat have shown that while starch synthesis appears to be sensitive to heat, not all the enzymes of the starch synthetic pathway are sensitive to high temperature (Keeling *et al.*, 1993). Indeed, some enzymes show a characteristic increase in the rate of reaction with elevated temperatures. UDPglucose pyrophosphorylase, for example, has shown increased activity up to 50°C (Keeling *et al.*, 1993). In the case of soluble starch synthase, maximum enzyme activity depends on the temperature to which grains are exposed (Rijven, 1986; Keeling *et al.*, 1993) and the period of exposure (Rijven, 1986; Hawker and Jenner, 1993; Keeling *et al.*, 1993). Different responses to temperature by the various enzymes in the starch synthetic pathway may also indicate that the control strength of particular enzymes varies as temperatures vary. While control theory dictates that control of flux through a metabolic pathway may be associated with one enzyme in the pathway, control may also be shared by several enzymes (Heinrich and Rapoport, 1974).

Two distinct mechanisms of response to elevated temperatures which may lead to reduced grain weight due to reduced starch deposition have been proposed by Jenner (1994). In the high temperature range (above 30°C) he postulated a threshold response resulting in knockdown of soluble starch synthase activity, a reduction in starch synthesis and consequent loss of yield. At intermediate temperatures the reduction in starch synthesis is largely due to changes in the kinetic properties of starch synthetic enzymes. Where high temperature exposure is brief and grains continue development under cooler conditions until harvest ripeness, the potential for recovery of diminished enzyme activity is of particular importance. There is evidence that recovery of a high percentage of lost enzyme activity may occur under cool recovery conditions following periods of heat (Savin and Nicolas, 1996), however Hawker and Jenner (1993) found that the degree of recovery varied with wheat genotype and the enzyme of interest.

The objectives of this study were to assess the limitations to starch synthesis in barley grains grown under conditions of increasing ambient temperatures for 2 days, and approximately 3 days of sustained high temperature (35°C). These conditions are similar to those encountered in some cereal growing areas in Australia (Stone and Nicolas, 1994). Changes in enzyme activity and in the concentration of metabolites involved in the pathway of starch synthesis have been determined during the moderate and high temperature conditions and during the cooler conditions following heat treatment.

5.2 **Materials and methods**

5.2.1 **Sampling**

Endosperm dry weight accumulation data were collected (Section 3.4.1) and the activities of a number of enzymes of starch synthesis were measured during grain development. The activities of starch synthetic enzymes and metabolites were measured in Experiments 1-5 (see Chapter 3, Table 3.2). Enzyme analysis was performed on endosperm samples collected 15, 18, 21, 24, 27, 30, 33, 36 and 39 daa; in the case of β-amylase analysis, additional samplings were made 42 and 45 daa (Table 5.2). Five endosperms were dissected (Section 3.3.1) from 3 ears, collected from individual pots, during the developmental time course and enzyme activities measured from freshly prepared crude extract preparations (Section 3.5.1).

5.2.2 **Enzyme assays**

Sucrose synthase, UDPglucose pyrophosphorylase (UDPGp), ADPglucose pyrophosphorylase (ADPGp), soluble starch synthase (SSS), branching enzyme (BE) and granule bound starch synthase (GBSS) were assayed for activity immediately following extraction. The methods used have been outlined in Chapter 3 (Sections 3.5.1 - 3.5.7). Starch hydrolysing enzymes α-amylase and β-amylase were also assayed from extract preparations using Megazyme™ procedures (Section 3.5.8 and 3.5.9).

5.2.3 **Analysis of sugar nucleotides**

Detection of sugar nucleotides was carried out using a 'Waters™ Associates' high performance liquid chromatography (HPLC) system as described by Jenner (1991a). Collection and preparation of samples has been detailed in Section 3.4.4.5. The sugar nucleotides, ADPglucose (ADPG) and UDPglucose (UDPG) were identified using standard preparations which had retention times of 21.5 and 23.5 minutes respectively,

and recoveries were determined by spiking one sample per batch with a known quantity of sugar nucleotide prior to extraction.

5.2.4 **Endosperm sugars**

Soluble sugars were extracted from 10 endosperms in boiling 80% ethanol according to the method outlined in Section 3.4.4.4. Measurement of endosperm sucrose and maltose was performed using water-elution HPLC, with sugars separated isocratically by a Waters™ 'Dextro-Pak' column. Sucrose and maltose were identified using standard preparations and had retention times of 9.0 and 8.2 minutes respectively. A number of other peaks were integrated in the course of reprocessing and analysis and these had retention times of 7.7 minutes (and containing glucose, (Millipore Corporation, 1993)) and 11.4 minutes (unknown sugar). Both have been plotted as arbitrary area units.

5.2.5 **Statistical analysis**

Analysis of variance (ANOVA) was carried out incorporating a block design when analysing pooled data from several experiments. Significance has been presented for both the main treatment effect (control vs high temperature) and the interaction between treatment and time (development). Error bars have been plotted using standard errors of means (SEM) derived from ANOVA. A two-way classification of unequal numbers was used to analyse data sets for ADPGp, SSS and β-amylase activity and tables of means were constructed from predicted values arising from ANOVA. The Tukey method was used for the comparison of means where the main treatment effect was established by ANOVA.

5.3 **Results**

5.3.1 **Effect of high temperature on accumulation of endosperm starch**

Under moderately high temperatures (up to 32°C), the rate of starch synthesis showed an apparent increase over the control rate, but a reduction in rate of accumulation of endosperm starch occurred from 24 daa (Chapter 4, Fig 4.6) so that at harvest ripeness, endosperms of heat treated grains contained 33% less starch than control grains. The overall reduction in starch accounted for approximately 90% of the reduction in final grain weight (Chapter 4, Table 4.3). The significance of the effects of high temperature alone, and its interaction with time through development, on the components of the pathway of starch synthesis appear in Table 5.1.

Table 5.1 Results of analysis of variance (ANOVA) for attributes measured with respect to starch synthesis in the control and heat treated developing endosperm. ANOVA for the main effect (temperature treatment) and the interaction between temperature and time (development), the number of samplings and residual degrees of freedom (res.df) are shown.. ***significantly different from control P<0.001,** P<0.01 and * P<0.05; ns, not significant.

					ANOVA	
	Parameter	**Expt**	**samplings**	**res. df**	**heat**	**heat*time**
	Starch	1-3	16	113	***	***
Enzyme activity	Sucr synthase	1	16	77	***	***
	UDPGp	1	16	79	***	**
	ADPGp	1,4,5	16	155	***	***
	SSS	1,4,5	16	75	***	***
	GBSS	1	16	77	**	***
	Branching enzyme	2	16	32	ns	*
Hydrolytic enzyme	α-amylase	2	16	32	ns	***
	β-amylase (free)	3	18	46	***	***
	(total)	3	18	46	***	***
Sugars	sucrose	2,3	16	74	***	ns
	maltose	2,3	16	74	**	***
	glucose	2	16	29	***	***
	unknown (RT 11.4min)	2	16	29	*	***
Sugar nucleotides	UDPG	5	10	22	ns	ns
	ADPG	5	10	22	ns	***

Most attributes which showed a significant response to treatment (high temperatures) also showed significant effects with the interaction between temperature and time. Sucrose levels while significantly different in response to the main effect (high temperature), showed no significant effect in relation to the interaction between temperature and time. Details of these interactions are illustrated in Table 5.2 which shows a pair-wise comparison of means for attributes measured during development.

Table 5.2 Pair-wise comparison of means, calculated using the Tukey method for enzyme activities, metabolites and endosperm sugars during development. **significantly different from control P<0.01 and * P<0.05, - not significant.

Parameter		**Days after anthesis**									
		18	**21**	**24**	**27**	**30**	**33**	**36**	**39**	**42**	**45**
	Starch	-	-	-	-	-	-	**	**		
Enzyme activity	Sucr synthase	**	**	*	-	*	*	-	-		
	UDPGp	-	-	**	**	*	-	-	-		
	ADPGp	-	**	**	-	**	-	-	-		
	SSS	**	*	-	-	-	-	-	-		
	GBSS	-	-	-	*	-	-	-	-		
	Branching enzyme	-	-	**	-	-	-	-	-		
Hydrolytic enzyme	α-amylase	**	**	-	**	**	**	**	-		
	β-amylase (free)	-	-	-	-	**	-	-	-	-	-
	(total)	-	-	-	-	**	-	-	-	-	**
Sugars	sucrose	-	-	-	-	-	-	-	-		
	maltose	-	-	-	-	-	-	**	**		
	glucose	-	-	-	-	-	-	**	-		
	unknown$^{(RT11.4min)}$	-	-	-	**	-	-	-	-		
Sugar nucleotides	UDPG	-	-	-	-			-			
	ADPG	-	-	-	-			-			

Data for specific endosperm attributes and enzyme activities have been plotted on the basis of thermal time (degree days (°Cd) after anthesis) employing a base temperature of 7.1°C (Goyne *et al.*, 1996), in addition to the chronological plot. This discriminates between direct effects on endosperm development due to increased temperature and changes in endosperm metabolism as a result of exposure to high temperatures.

5.3.2 **Effect of high temperature on endosperm sucrose**

The amount of sucrose in heat treated barley endosperms increased relative to control endosperms throughout development. Similar amounts of sucrose were observed in control and heat treated endosperms during the heating period, and the differential between treatments appeared to increase from 21 daa (Fig 5.1).

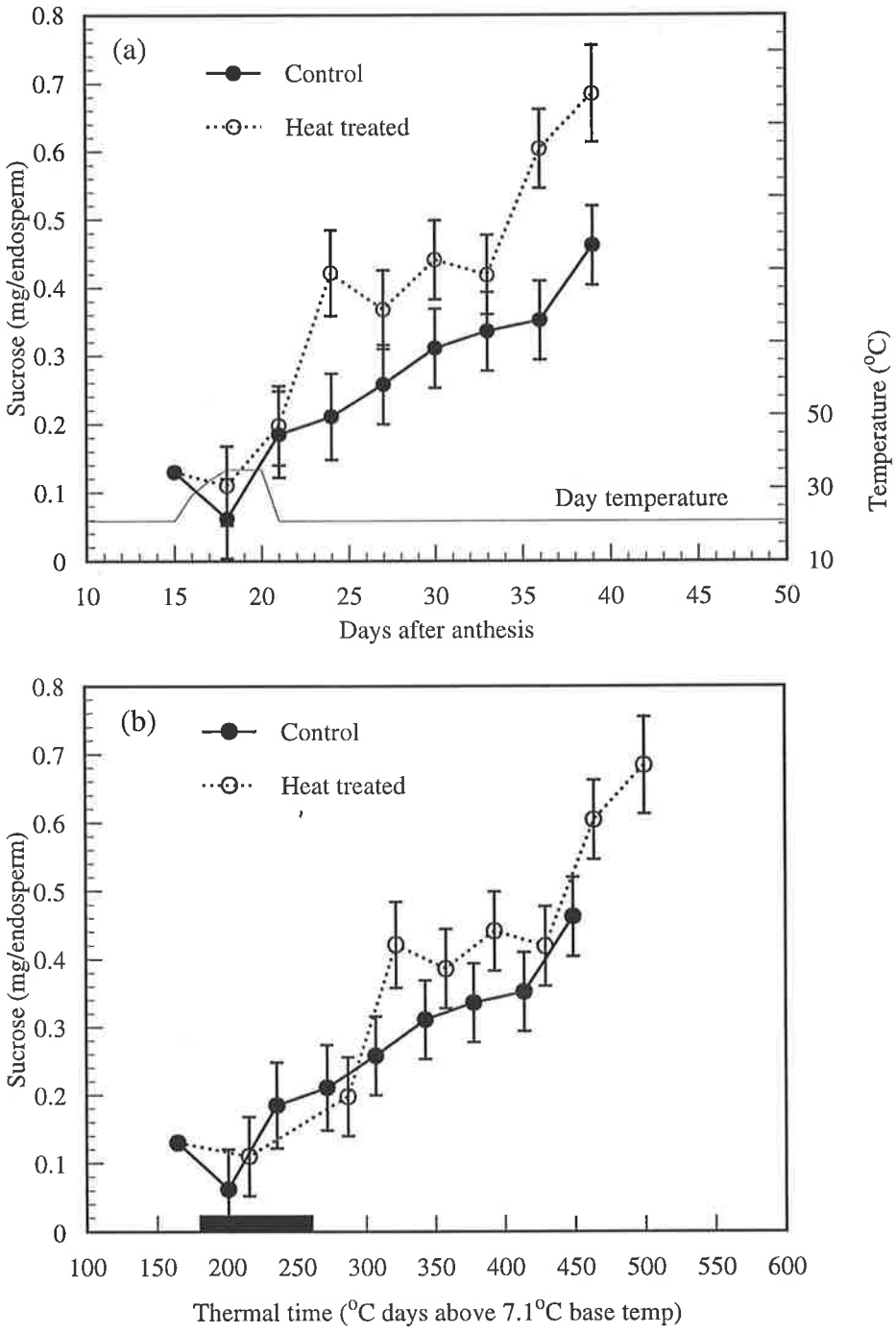


Fig 5.1 Changes in **sucrose** in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar shows high temperature period (b).

5.3.3 Effects of high temperature on the activity of enzymes in the pathway for starch synthesis

5.3.3.1 Sucrose synthase

Sucrose synthase activity was not static in the developing grain, but declined from 21 daa until maturity (Fig 5.2a). The immediate reduction in activity of this enzyme at the conclusion of the heating period was consistent with hastened development (Fig 5.2b), however, between 24 and 30 daa the level of activity in heat treated grain diminished and remained lower than control grain until 39 daa. Consideration of enzyme activity with respect to accumulated thermal time revealed an apparent delay in the recovery of activity of sucrose synthase following exposure to high temperature. Above about 400°C days, enzyme activity rates of control and heat treated grain were comparable (Fig 5.2b).

5.3.3.2 UDP-glucose pyrophosphorylase (UDPGp)

The initial decline in UDPGp activity under high temperature conditions cannot be accounted for as hastened development (Fig 5.3b). The greatest effects of high temperature, a reduction in activity of 70% of control, appeared ~~at mid-development~~ just after the high temperature treatment. The return to cooler conditions, between 24 and 33 daa, produced a recovery of activity to the level of enzyme activity observed under control conditions.

5.3.3.3 ADP-glucose pyrophosphorylase (ADPGp)

The detrimental effects of heating only became apparent in ADPGp activity after exposure to high ambient temperatures (35°C) (Fig 5.4). Enzyme activity was reduced by more than 50% at 21 daa as a consequence of heating, and activity remained low upon return to cooler conditions. This temperature effect could not be attributed to accelerated development (Fig 5.4b). Some recovery of ADPGp activity took place at cooler temperatures, between 24-33 daa, (not significant) and the enzyme activity of both control and heat treated grain coincided in thermal time from about 425°C days.

5.3.3.4 Soluble starch synthase (SSS)

The activity of SSS declined from 15 daa and throughout the period of development in this study. Elevated temperatures had an immediate effect ~~on~~ depressing enzyme activity which could not be explained entirely by hastened development (Fig 5.5b). Although sensitive to high temperatures, SSS showed a higher degree of recovery than ADPGp, with the

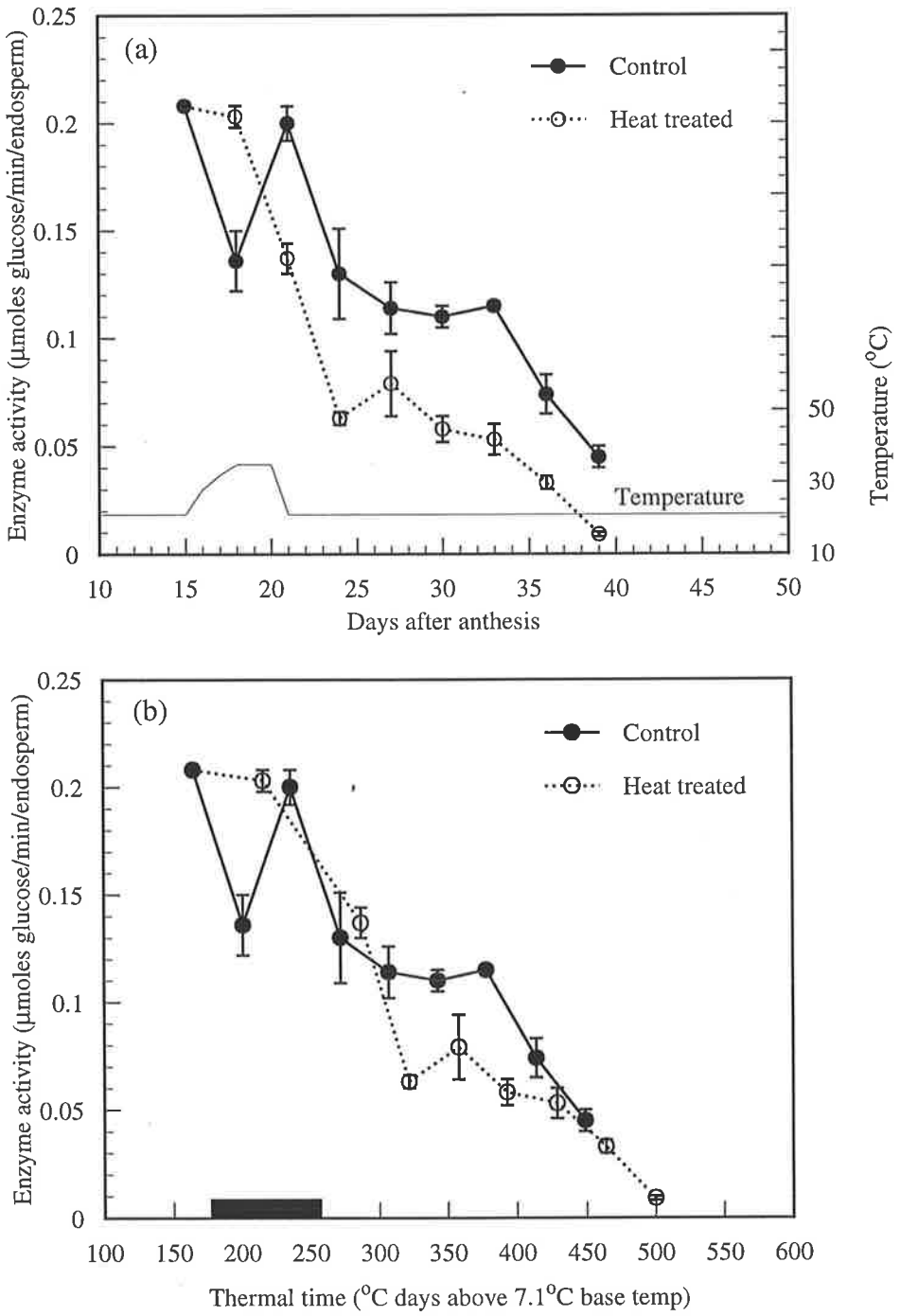


Fig 5.2 Changes in **sucrose synthase** activity in control and heat treated grain during development (a) and plotted in thermal time (b). Line indicates average daytime temperature (a) and bar indicates high temperature period (b).

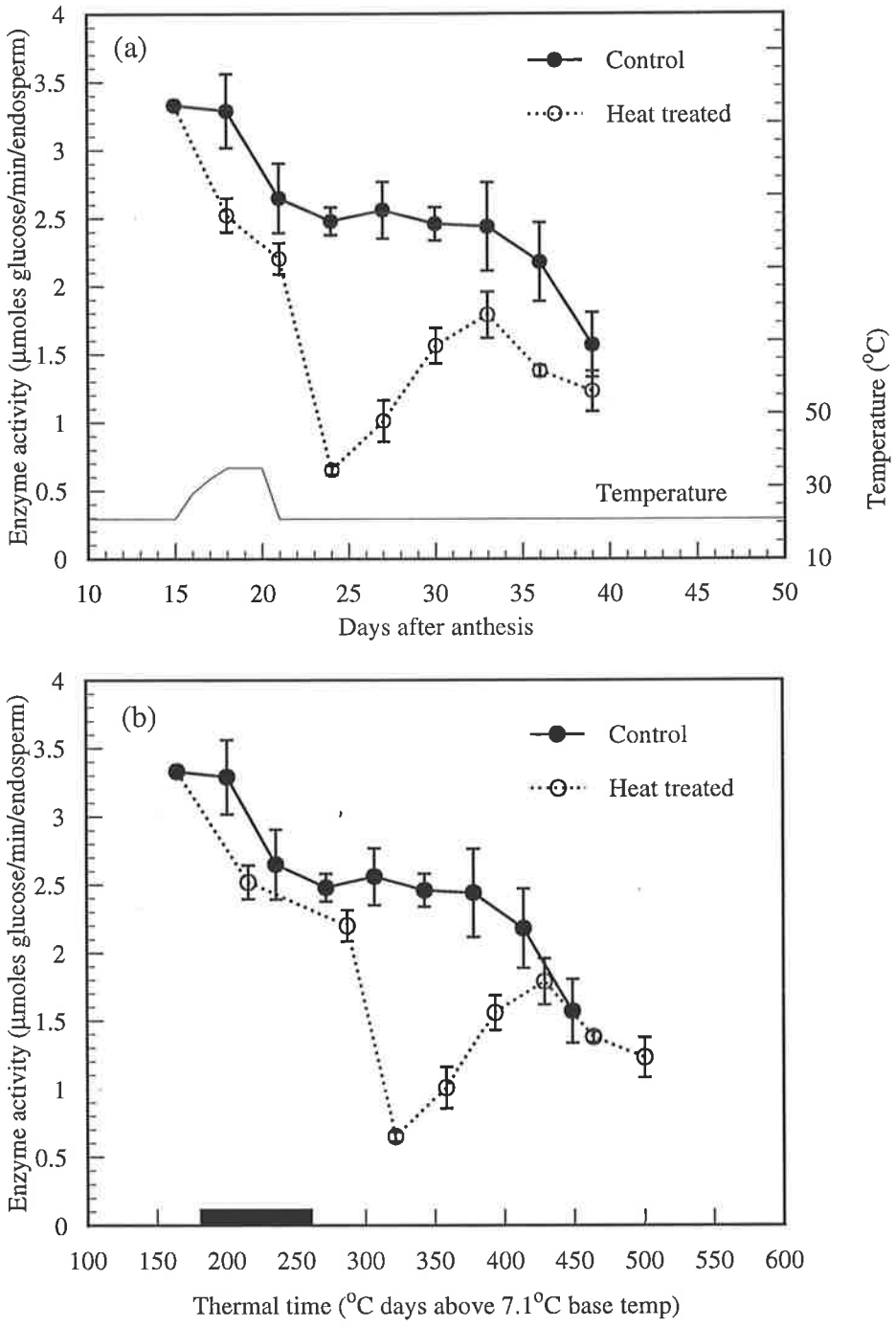


Fig 5.3 Changes in **UDP-glucose pyrophosphorylase** activity in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar shows high temperature period (b).

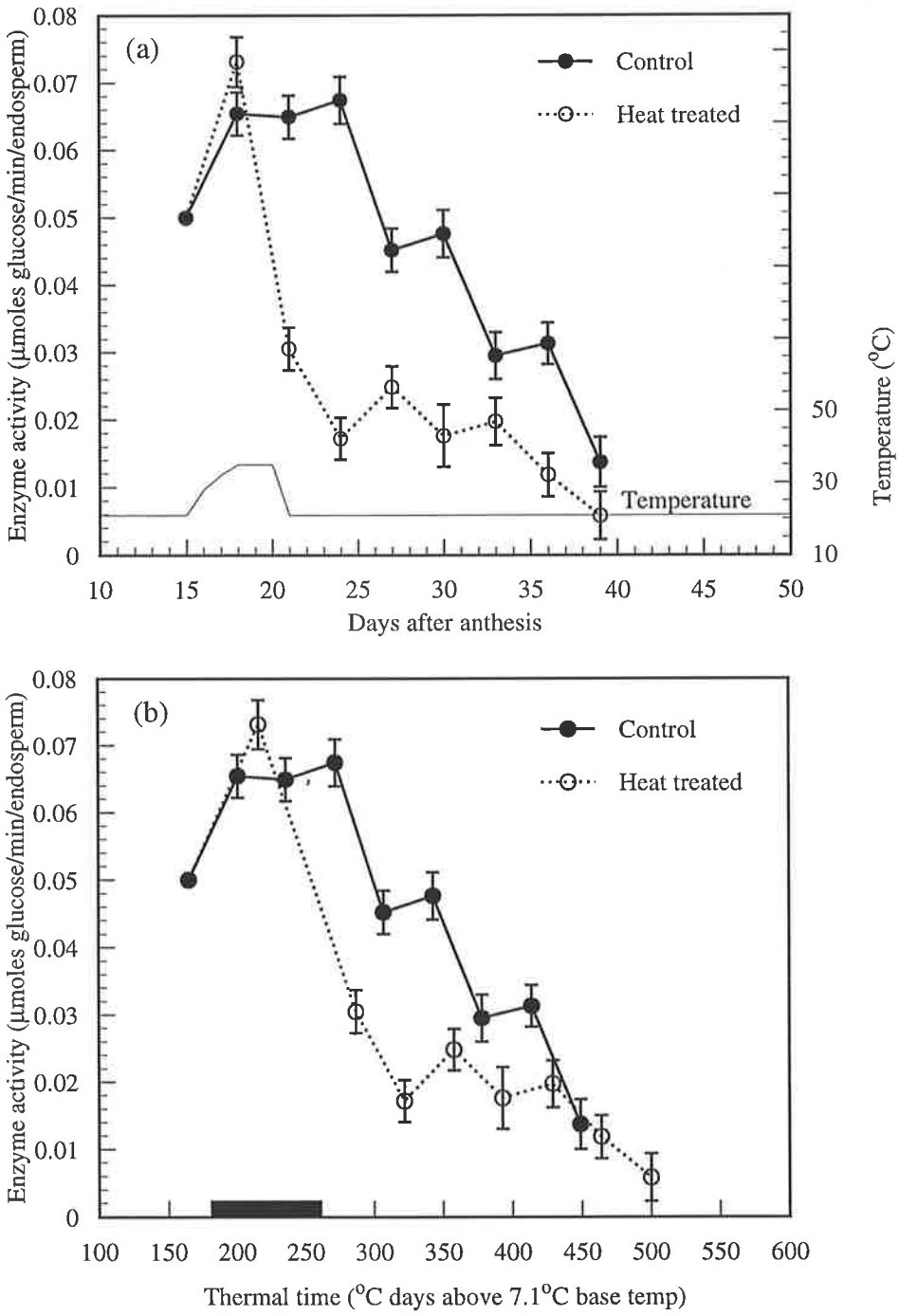


Fig 5.4 Changes in **ADP-glucose pyrophosphorylase** activity in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

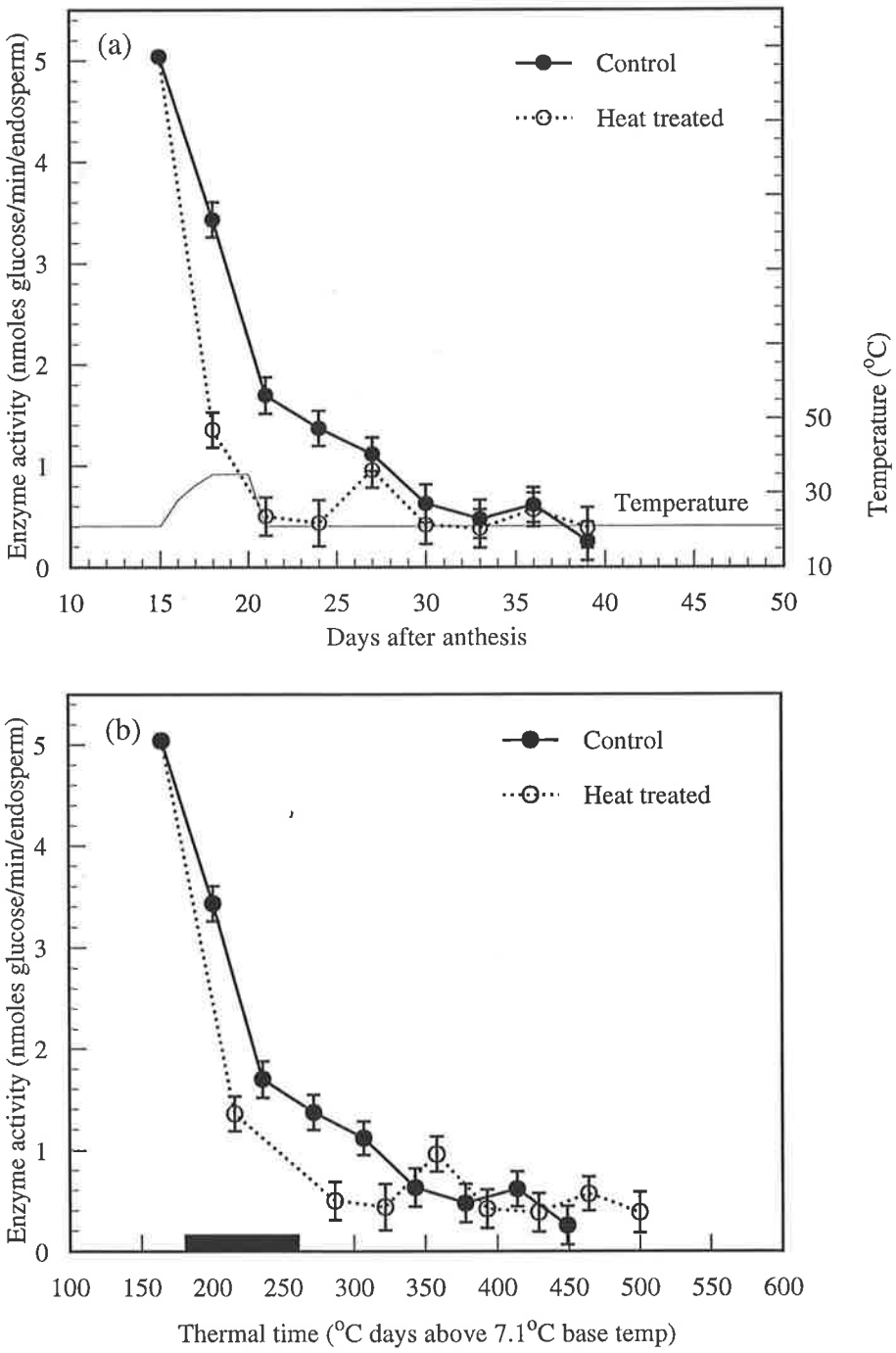


Fig 5.5 Changes in **soluble starch synthase** activity in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

enzyme activity of heat treated grain exceeding that in controls at 27 daa and, although overall enzyme activities remained low, they were comparable until 39 daa.

5.3.3.5 **Branching enzyme (BE)**

The activity of BE increased as grain development progressed (Fig 5.6). The initial increase in enzyme activity during temperatures up to 32°C appeared to indicate a temperature effect on development (Fig 5.6b), but this was not the case following high temperature exposure when enzyme activity failed to increase further. ~~Like SSS~~, BE appeared to show some recovery during the cooler conditions following the heat treatment.

5.3.3.6 **Granule bound starch synthase (GBSS)**

GBSS activity was not detrimentally affected during exposure to high temperatures (Fig 5.7). The severe effects of heat appeared to be delayed until after the heat treatment period, with little further increase in GBSS activity until 39 daa.

5.3.4 **Effect of high temperature on the metabolites of the starch synthetic pathway**

5.3.4.1 **UDP-glucose (UDPG)**

Levels of this sugar nucleotide in the developing endosperm varied in control grain between 15 and 35 daa, but response to high temperature treatment was not significantly different between temperature treatments (Table 5.1; Fig 5.8). While the levels of this metabolite to some extent depend on the activities of sucrose synthase and UDPGp in the starch synthetic pathway, there appeared no relation between the activity of these enzymes either during or following high temperature exposure and endosperm levels of UDPG.

5.3.4.2 **ADP-glucose (ADPG)**

Levels of endosperm ADPG appeared unaffected by the high temperature treatment (Fig 5.9). At 18 daa for example, when activity of SSS was reduced, ADPGp activity increased and starch accumulation was slightly elevated (Fig 4.6), there was no net change in this nucleotide sugar.

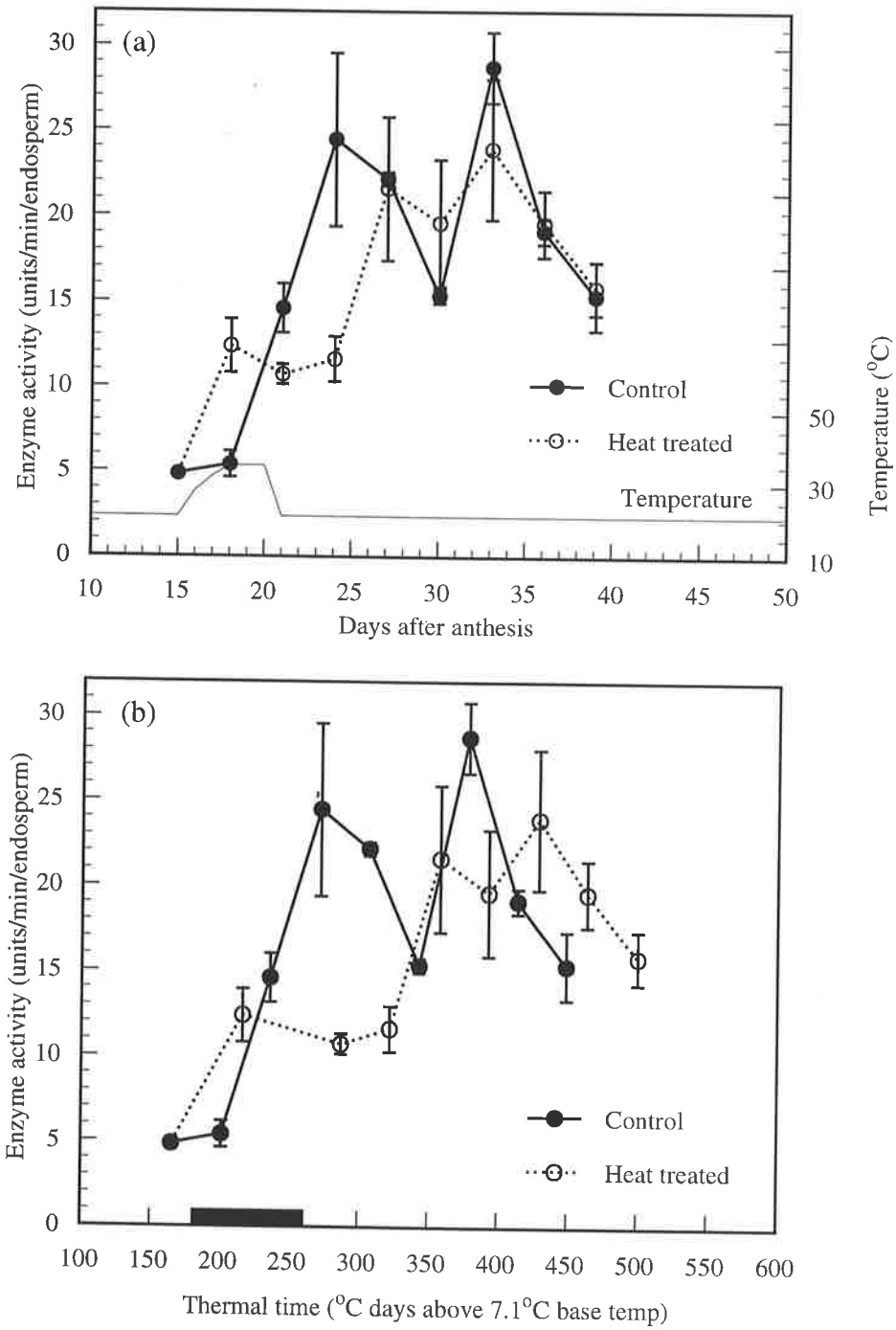


Fig 5.6 Changes in **branching enzyme** activity in control and heat treated grain during development (a) and plotted in thermal time (b). Bar indicates high temperature period.

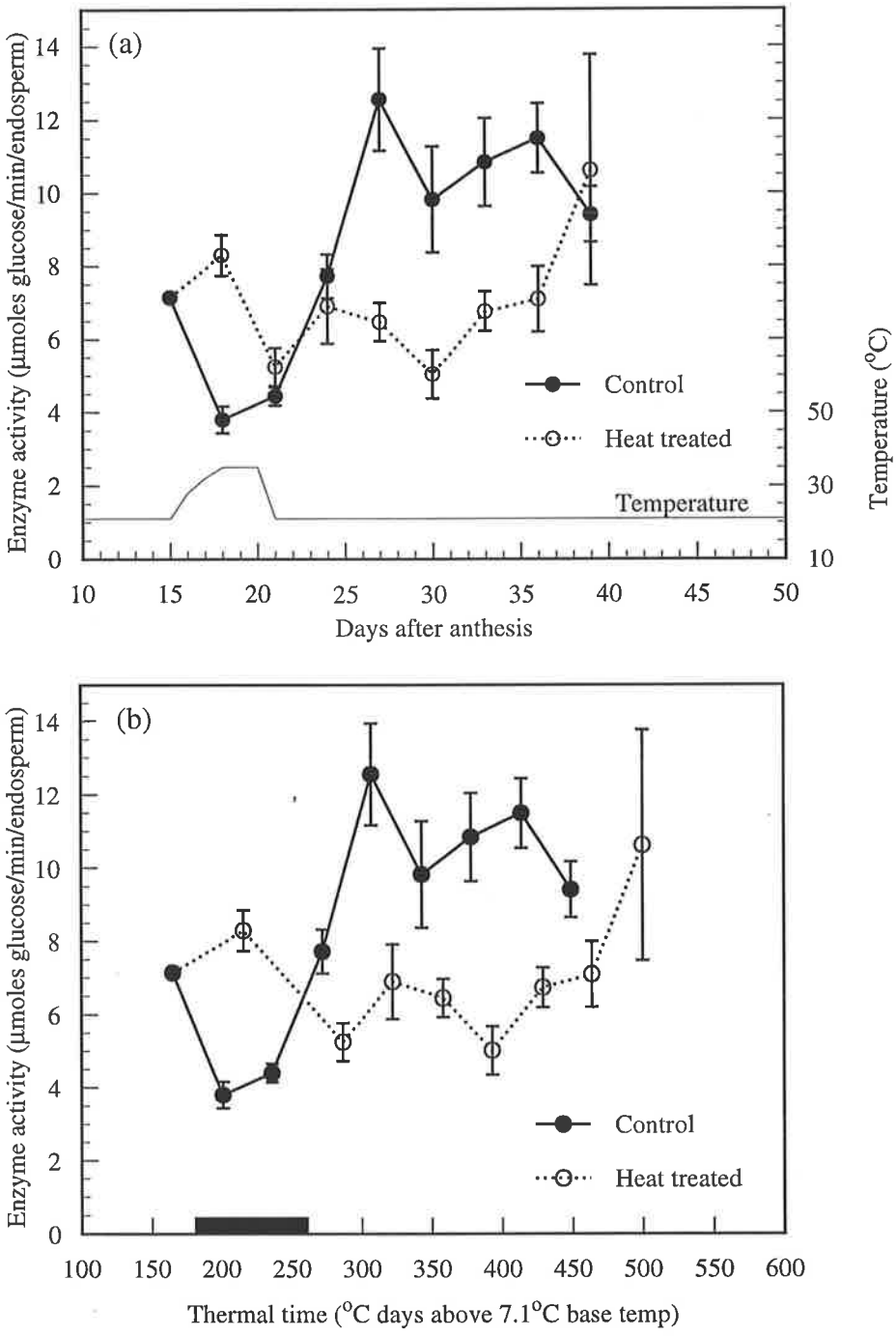


Fig 5.7 Changes in **granule bound starch synthase** activity in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

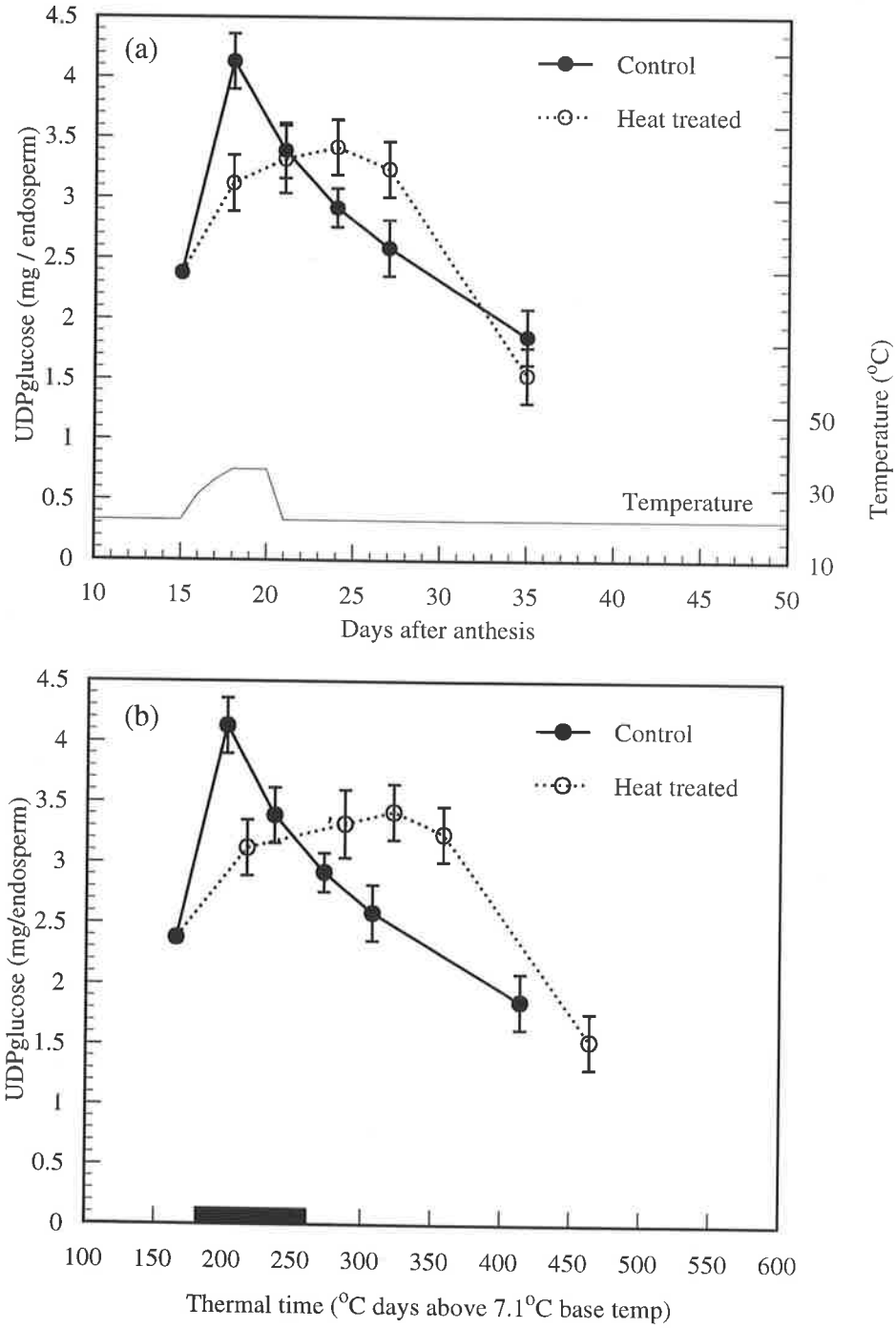


Fig 5.8 Changes in **UDPglucose** in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

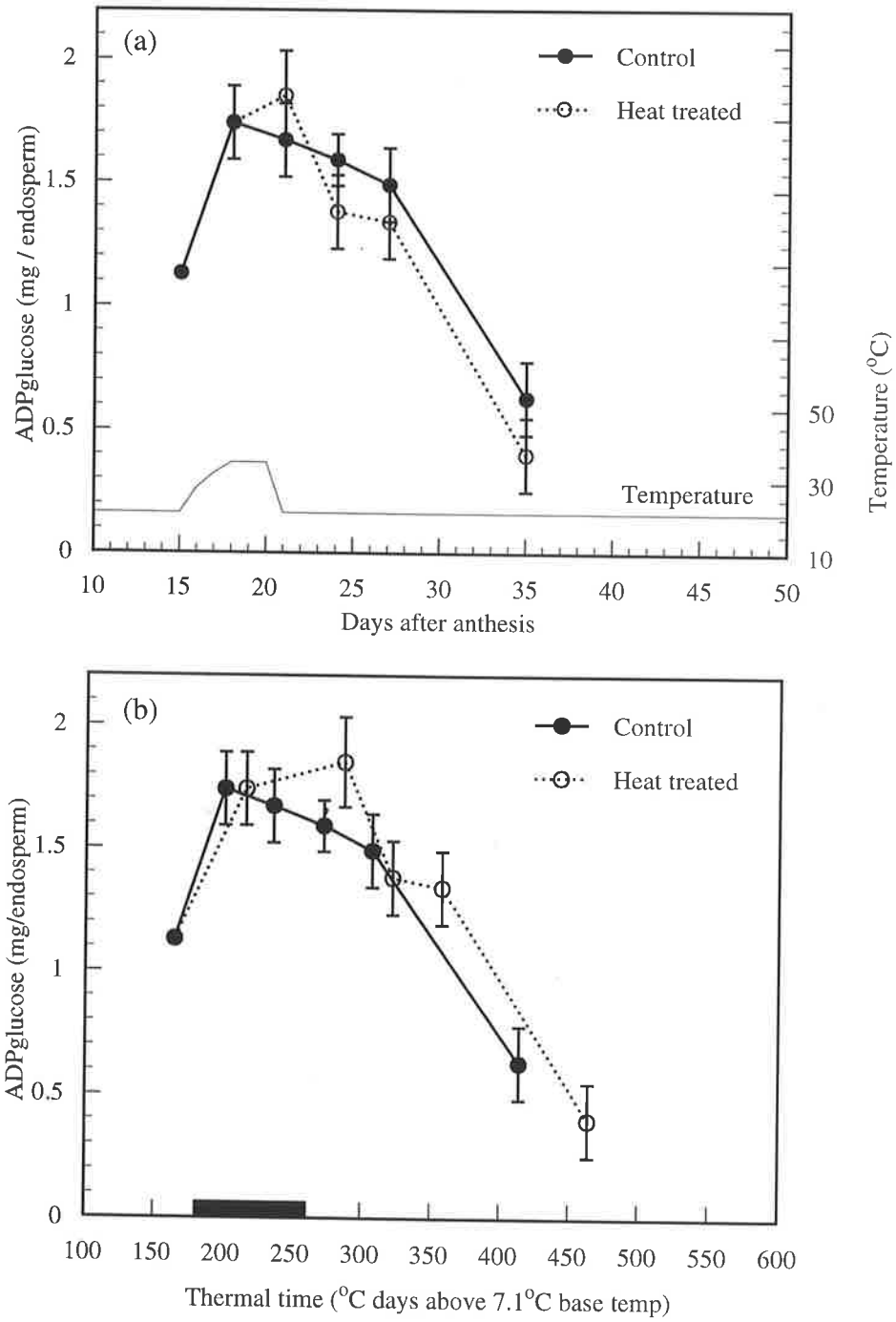


Fig 5.9 Changes in **ADPglucose** in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

5.3.5 Effect of high temperature on starch degrading enzymes

5.3.5.1 α-amylase

α-amylase activity was detected in developing endosperm tissue at low levels from 15 daa until maturity (Fig 5.10). The catalytic activity of α-amylase increased during the heating period, declining thereafter but showing some recovery of activity after 27 daa so that by 39 daa α-amylase activity was similar in grains of both treatments.

5.3.5.2 β-amylase

The change in accumulation of β-amylase in response to high temperatures could be ascribed to accelerated development, with no indication of any significant alterations to metabolism (Fig 5.11b). The rate of accumulation of endosperm β-amylase in heat treated grain increased 10 fold between 42 and 45 daa. Without comparative data for β-amylase levels in harvest-ripe control grain it is not possible to determine whether accumulation patterns differed between control and heat treated grain, although overall protein levels in harvest-ripe grain of both treatments were similar (Chapter 4, Table 4.3). During the period of observation most of the β-amylase was detected in the free form for both control and heat treated grain (data not shown) and at 45 daa free β-amylase still constituted 83 and 91%, respectively, of the total β-amylase detected.

5.3.6 Effect of high temperature on endosperm soluble sugars

Levels of endosperm maltose remained negligible in control grain throughout development (Fig 5.12). Endosperm maltose levels were low in heat treated grain until 33 daa after which a five-fold increase was recorded over the six days to 39 daa. Changes in relative quantities (arbitrary area units) indicated that the glucose level changed little during development in control grain (Fig. 5.13). A three-fold increase in the peak containing glucose was observed 36 daa in heat treated grain but by 39 daa levels were again comparable with those in control endosperm. The unknown sugar (RT 11.4 min) indicated a developmental pattern quite unrelated to maltose or glucose under high temperature conditions, despite its steady increase in the course of development under control conditions (Fig 5.14). Levels of this sugar peaked 27 daa following high temperatures, but diminished thereafter until 39 daa. No significant changes in levels of these sugars were observed during the heating period.

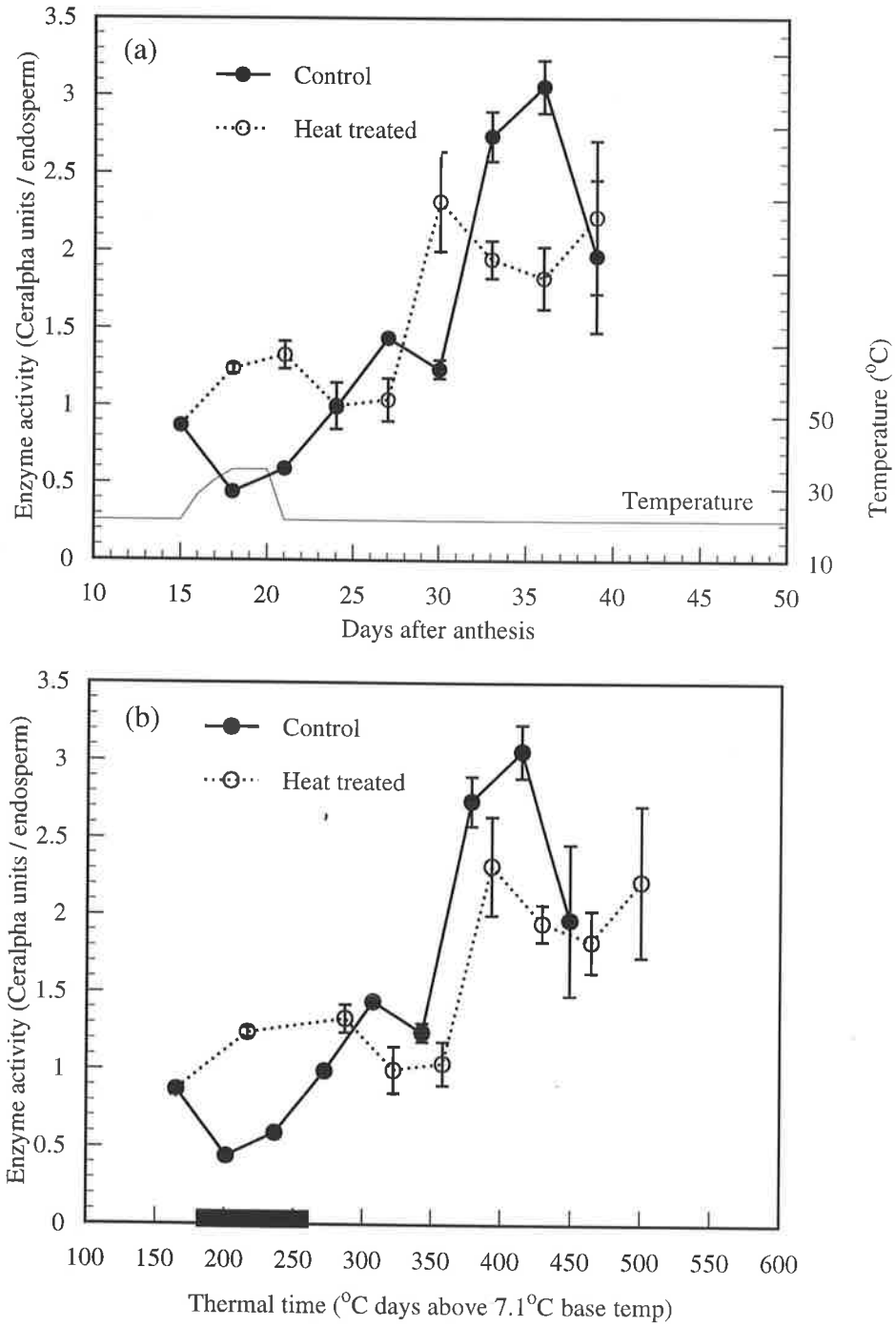


Fig 5.10 Changes in **alpha-amylase activity** in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

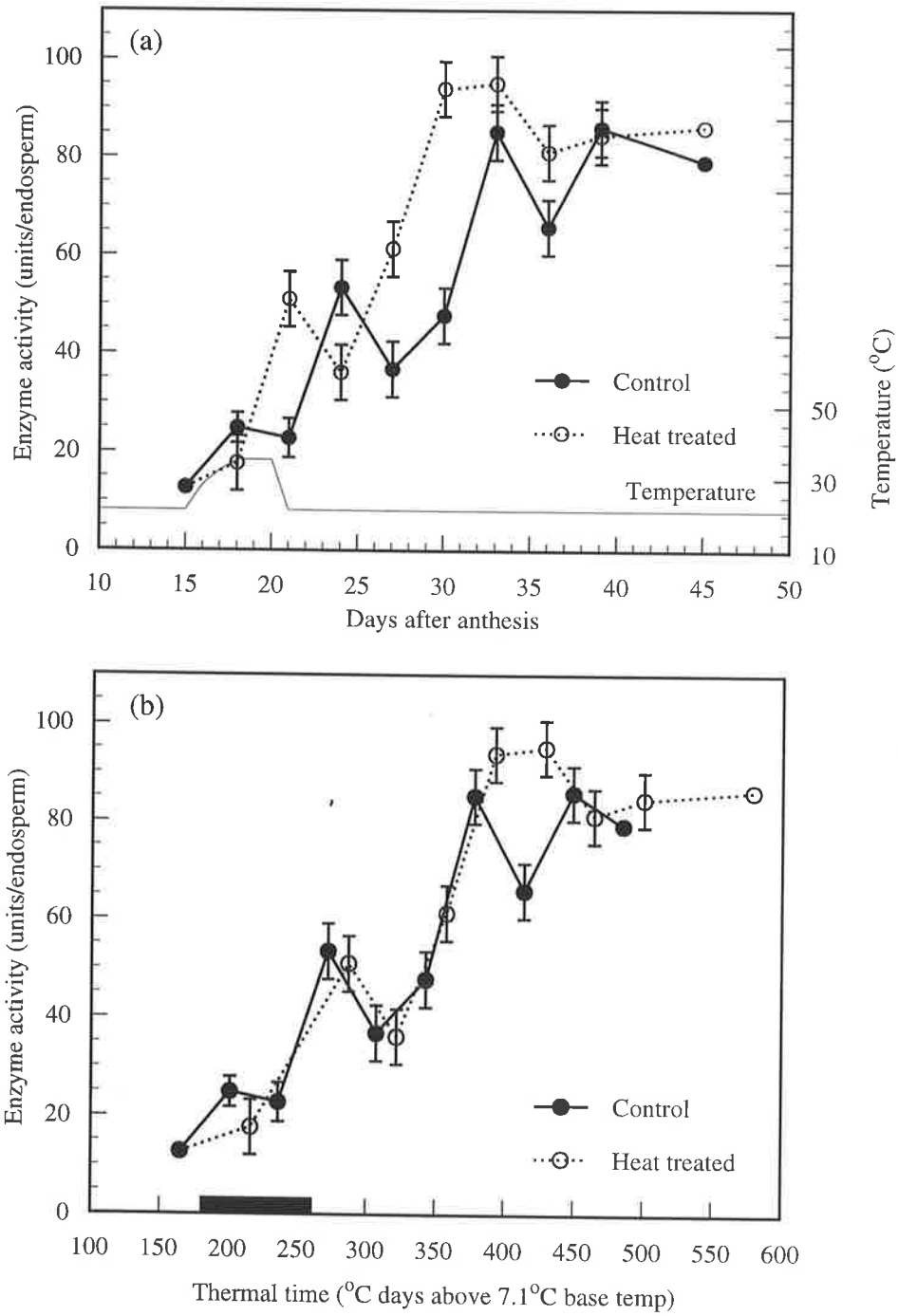


Fig 5.11 Changes in **total beta-amylase** activity in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

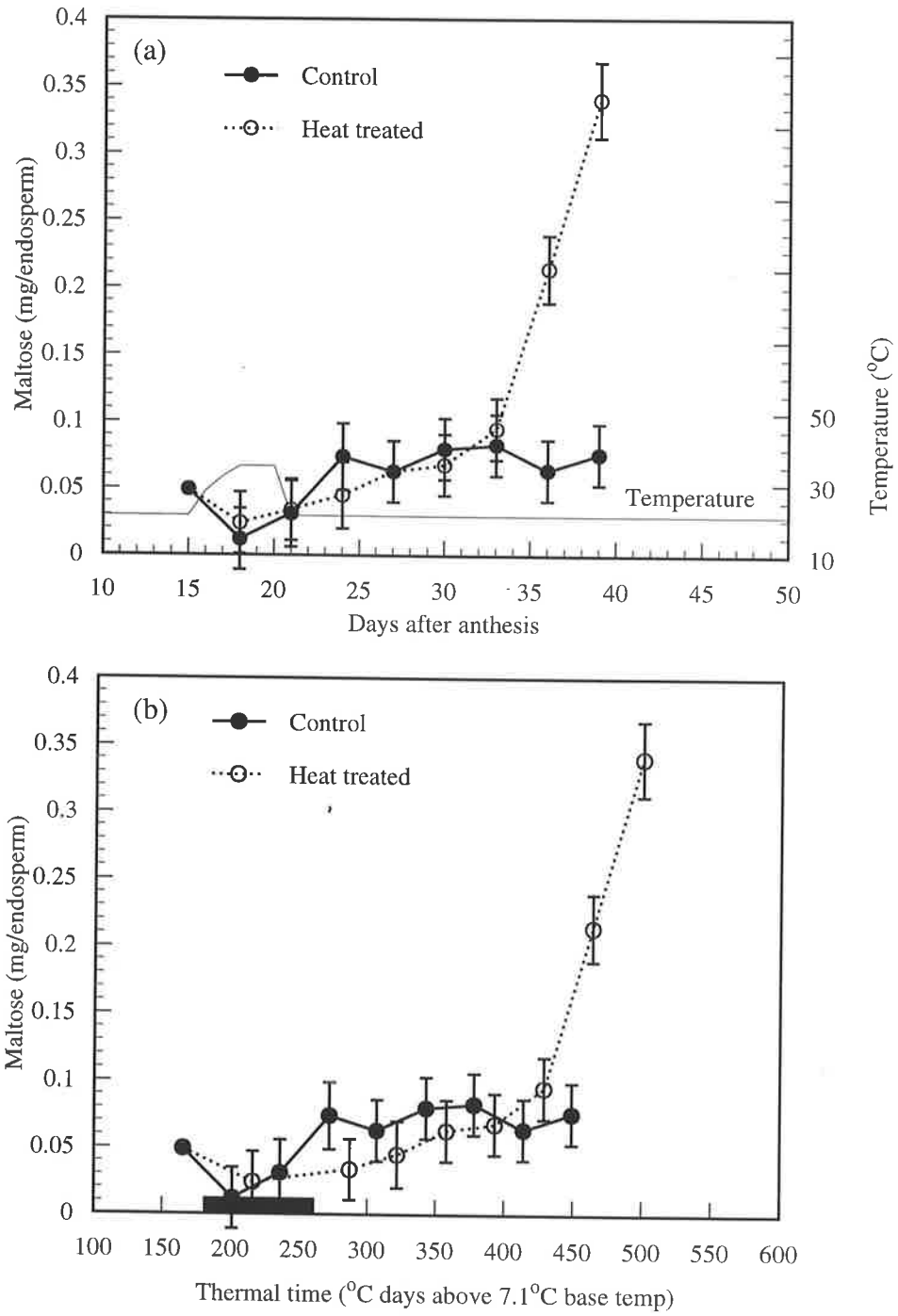


Fig 5.12 Changes in **maltose** in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

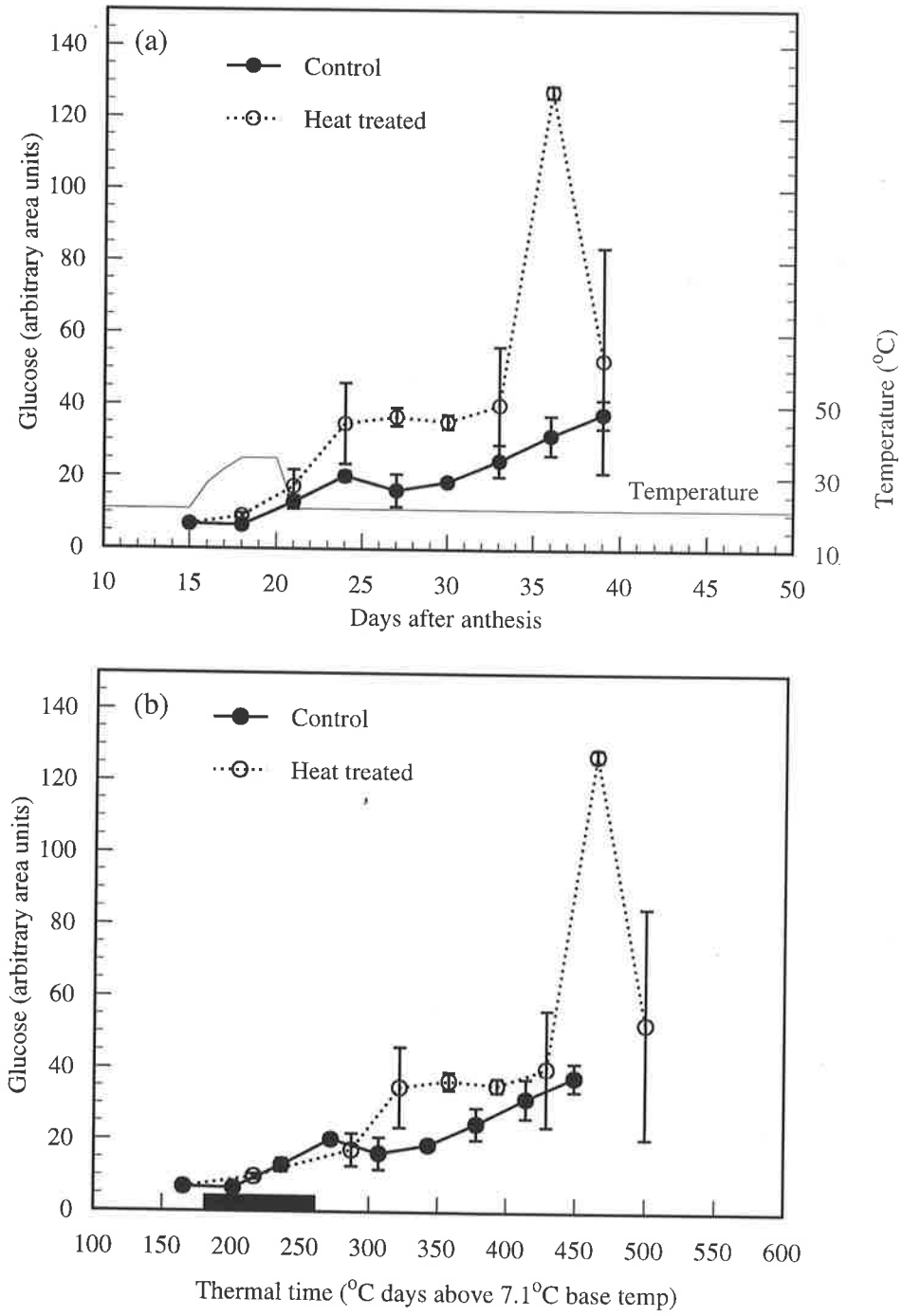


Fig 5.13 Changes in **glucose** in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

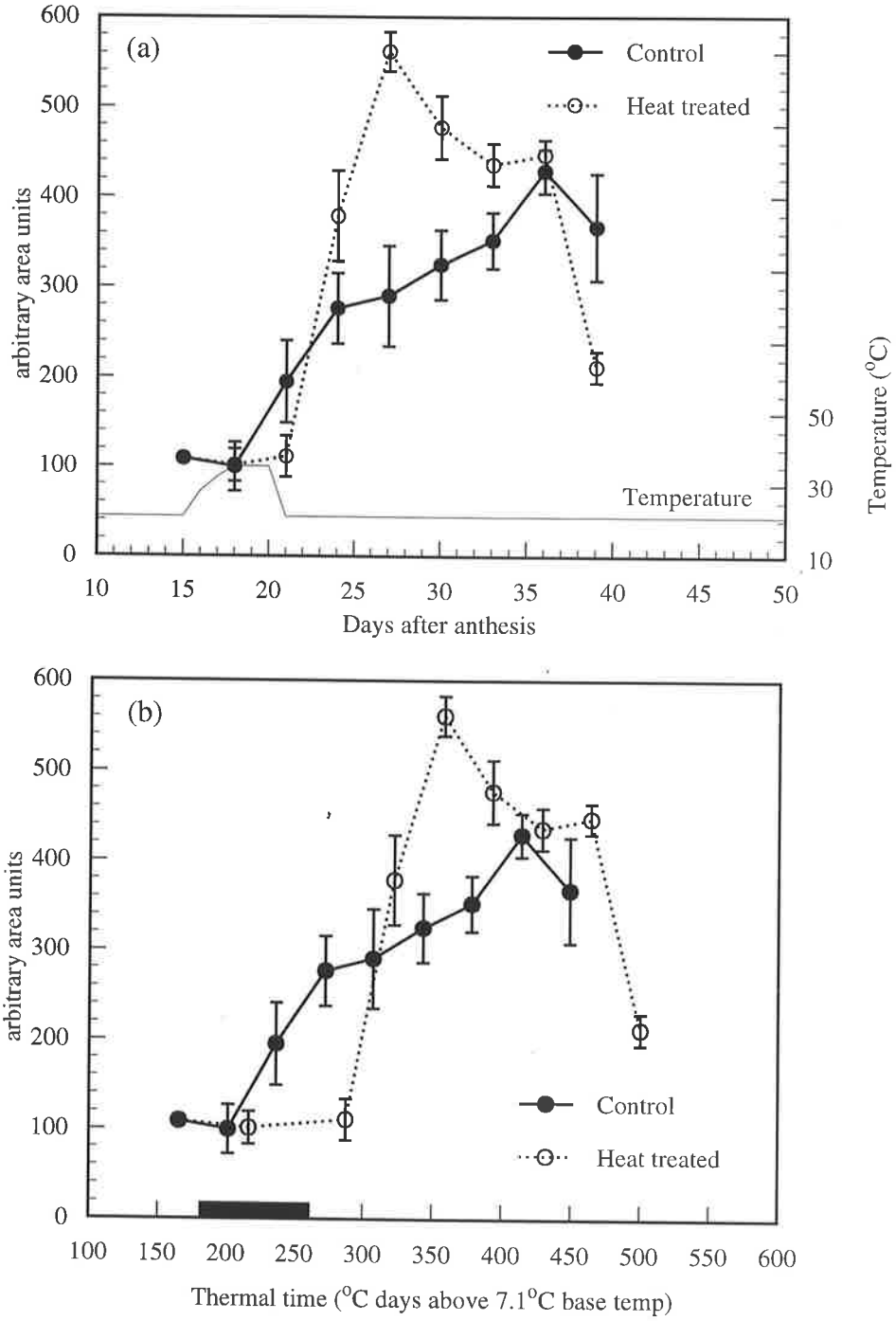


Fig 5.14 Changes in **(RT 11.4 minutes) sugar** in control and heat treated grain during development (a) and plotted in thermal time (b). Profile shows average daytime temperature (a) and bar indicates high temperature period (b).

5.3.7 Limitations to starch synthesis following exposure to high temperatures during development

ADPGp activity exceeded the rate of starch synthesis in both control and heat treated grain by a factor of 10 throughout development (data not shown). A comparison between the rate of starch synthesis (in nmoles of glucose equivalents per min per endosperm) and SSS (~~Fig 5.13~~ Table 5.3 : data not statistically analysed) provided some insight into possible limitations to starch synthesis following exposure to high temperatures. Under control conditions it appeared that the catalytic activity of SSS was lower than the rate of starch accumulation (Table 5.3). Heat treatment appeared to reduce the level of activity still further.

Table 5.3 Comparison between the average rate of starch synthesis (nmoles glucose equivalents per grain per min) and the catalytic activity of soluble and granule bound starch synthase for control and heat treated grain.

		Average rate of enzyme activity and starch synthesis (nmoles glucose equiv / grain /min)		
	Days after anthesis	**Starch**	**SSS**	**GBSS**
Control	15-21	5.06	3.39	5.12
	21-30	4.09	1.20	10.03
	30-39	1.97	0.49	10.37
Heat treated	15-21	4.57	2.30	6.9
	21-30	2.60	0.58	5.91
	30-39	0.38	0.44	7.37

5.4 Discussion

The results of this study confirmed other reports that elevated temperatures reduced the conversion of sucrose to starch in the cereal endosperm (Bhullar and Jenner, 1986; MacLeod and Duffus, 1988a). The reduction in starch synthesis appeared to result from the combined effects of diminished catalytic activity of a number of enzymes in the committed pathway of starch synthesis, and/or delayed recovery of enzyme activity in the cooler recovery conditions. Reductions of between 11 and 75% in the activity in the enzymes under investigation were recorded 24 daa following high temperature (35°C) exposure. The supply of photosynthate, sucrose, was not reduced in heat treated grain as a result of exposure to heat. In addition, ADPGp, BE and GBSS showed increased

activity during exposure to moderate temperatures, while SSS showed an immediate loss of activity, even at moderate temperatures. Sucrose synthase and UDPGp showed the greatest reduction in catalytic activity only after plants were returned to cooler conditions. Individual enzymes showed variation in the level of recovery under the cooler temperature conditions which followed the heating period.

5.4.1 **Effect of high temperature on the supply of sucrose**

The increase in endosperm sucrose observed following high temperature treatment may have arisen because increased quantities of this photosynthate were mobilised within the plant, or because of a slower rate of starch synthesis. While high temperatures in excess of 30°C have been associated with disruption of photosynthesis (Al-Khatib and Paulsen, 1984), there is some evidence that high temperatures alter the flow of nutrients to other plant parts (Kuroyanagi and Paulsen, 1985). At temperatures up to 30°C, sucrose uptake by isolated grains has been found to mimick apparent growth rate (starch accumulation) according to Wardlaw *et al.* (1995). However, the decrease in the rate of starch synthesis was observed during prolonged heating in the current study and reported in Chapter 4 (Section 4.3.4.1, Fig 4.6). Reduced starch synthesis was also observed by Savin and Nicolas (1996), who exposed plants to temperatures up to 40°C. Both results suggest that at elevated temperatures the consumption of sucrose in developing grain may also be reduced.

The reduction in accumulation of starch in the developing barley endosperm following exposure to a period of elevated temperatures did not appear to be due to a reduction in the supply of photosynthate, sucrose. This research finding is in line with that of Nicolas *et al.* (1984) and Jenner (1991a) in wheat. MacLeod and Duffus (1988a) reported an immediate elevation in endosperm sucrose in response to high temperature in barley. The effect of high temperature on sucrose mobility within the grain still requires further investigation.

5.4.2 **Effect of high temperatures on the enzymes of starch synthesis**

Under control conditions most enzymes of the starch synthetic pathway showed declining activity from about day 15 onwards, with BE and GBSS the only enzymes to exhibit increased activity with grain maturity. GBSS is associated with the surface of the starch granule (Denyer *et al.*, 1993) and catalytic activity would therefore be expected to increase with increased starch deposition. Exposure at fractured surfaces of some of the GBSS proteins incorporated into the structure of individual starch granules may have occurred during extract preparation (Section 3.5.1) and contributed to some of the variation in the activity of this enzyme (Fig 5.7).

Although under elevated temperature conditions starch synthesis showed a relatively small apparent increase, other contributing metabolic processes related to starch synthesis were more responsive. Only ADPGp showed an increase in the amount of catalytic activity during the initial stages of heating. However, it is likely that increasing ambient temperatures would have caused an immediate increase in the kinetic activity of most (if not all) the enzymes in the committed pathway of starch synthesis (Keeling *et al.*, 1994). That is, during the initial period at elevated temperatures, changes in molecular energy distribution would have led to an increase in the rate of all enzyme-catalysed reactions (Hochachka and Somera, 1984). According to Keeling *et al.* (1993), such effects may be observed up to 50°C and are reversible. It is likely that an increase in kinetic activity was responsible for the apparent increase in starch 18 daa, despite reductions in the catalytic activity of several enzymes in the starch synthetic pathway, even at moderate temperatures. The binding of enzymes such as GBSS and BE into the structure of starch granules has been associated with protection from reduction in activity and heat inactivation (Denyer *et al.*, 1994) and may explain the increases in the catalytic activity observed in these enzymes under moderately high temperatures.

At higher temperatures (35/25°C) there was evidence of loss of enzyme catalytic activity, termed 'knockdown' by Keeling *et al.* (1993) and which is slow to reverse. There appeared to be a time factor and possibly a threshold temperature effect to this change in enzyme catalytic activity. Sucrose synthase, UDPGp, ADPGp and BE all showed the greatest reduction in enzyme activity 24 daa, 3 days after the high temperature period had ended. A similar delayed response to heat has also been reported in sucrose synthase by MacLeod and Duffus (1988a), who found that the reduction in activity became

increasingly pronounced several days after heat exposure. In the present study, both UDPGp and ADPGp appeared more sensitive to elevated temperatures in barley than in wheat (Hawker and Jenner, 1993). Clarification of a carry-over response of enzyme activities in wheat still requires further investigation. In wheat, the small reduction in catalytic activity of sucrose synthase and UDPGp in response to 4 days of high temperature was explained by Hawker and Jenner (1993) on the basis of accelerated development. However, these workers failed to measure enzyme activities during an ensuing recovery period so any subsequent knockdown of enzyme catalytic activity would not have been detected. Indications from this study are that the immediate response to high temperature does not provide a complete account of enzyme response to heat. Enzyme activity during high temperature conditions may not fully provide an indication of genetic superiority in heat tolerance and under conditions where high temperatures are experienced for several days, recovery of lost enzyme activity may be at least equally important in determining heat tolerance and the overall success of grain filling under these conditions.

Lower GBSS activity is likely to be related to lower starch quantity in heat treated grain compared with control grains and it is not possible to be categorical as to whether this result was the cause or consequence of the treatment.

5.4.3 Limitations to starch synthesis following exposure to high temperature treatment

The results of this study are consistent with other reports that SSS is involved in the response of starch synthesis to high temperature (Jenner *et al.*, 1993; Keeling *et al.*, 1993) although this enzyme may not be wholly responsible for the reduction in conversion of sucrose to starch. For example, it has recently been reported that there are cytosolic as well as plastidic forms of ADPGp in barley endosperm (Thorbjornsen *et al.*, 1996). The role of cytosolic ADPGp in starch synthesis is as yet unknown so the significance of the effects of heat on ADPGp activity is difficult to interpret.

A decline in sucrose synthase activity has been associated with a steady increase in endosperm sucrose (MacLeod and Duffus, 1988a; compare Figs 5.1 and 5.2). Although this has been interpreted as providing an indication of the importance of this enzyme in determining the extent of grain filling (MacLeod and Duffus, 1988a), ADPGp also

showed a similar response in the present study (Fig 5.4). While neither is likely to have overriding importance in the effects of temperature on the synthesis of starch (Denyer *et al.*, 1994) levels of sucrose and the precursor for starch, ADPG, were not reduced as a result of elevated temperature.

5.4.4 Effects of high temperature on grain development and metabolic activity

Results from this study provide evidence that accelerated development cannot entirely explain the effects of heat on the activity of enzymes and the levels of metabolites in the committed pathway of starch synthesis. Minor changes in UDPGp and sucrose synthase activity were observed by Hawker and Jenner (1993) in response to high temperature and attributed simply to advanced development. In the present study significant reductions in the activity of these enzymes did not occur until some time following the heat treatment. The application of the degree day concept (Hunt *et al.*, 1991), showed that these changes could not be accounted for on the basis of accelerated development (see Figs 5.2b and 5.3b). Hawker and Jenner (1993) however did report inconsistencies between enzyme activity and increases in thermal time which were suggestive of a non-linear response with respect to several starch synthetic enzymes to the application of heat.

5.4.5 Factors contributing to the reduction in endosperm starch following exposure to high temperatures

Reductions in the amount of endosperm starch may be due to either decreased synthesis and/or increased starch breakdown during grain development. Evidence has already been presented for reduced starch synthesis in heat treated grains in Chapter 4 (Section 4.3.4.1). Changes in the activity of starch hydrolysing enzymes, α- and β-amylase, and increases in the levels of the products of degradation, maltose and glucose (and possibly RT 11.4 sugar) in this study suggest that some starch degradation may occur in developing heat treated grains. α-amylase activity was recorded at levels far below that of germinating grain (Graham, pers.comm.), and while its activity did increase during the high temperature treatment this was not directly associated with increases in the degradation product glucose (Fig 5.13a). Elevated levels of free β-amylase in maturing heat treated grains (Fig 5.11) did however coincide with a dramatic rise in maltose (Fig 5.12). The increase in this degradation product of starch hydrolysis did not occur during

heat exposure but occurred as a carry-over effect of high temperature. Whether the rise in maltose as a consequence of heating was due to the increased level of β-amylase activity, or to a change in the vulnerability of starch to enzymatic attack cannot be resolved with the information available.

There have been a number of reports of starch degrading enzymes (in particular, α-amylase) occurring during development in grains of barley (Hill and MacGregor, 1988; MacGregor and Dushnicky, 1989a) and in harvest ripe wheat (Mares *et al.*, 1994). α-amylase has been identified in intact barley grains during the early stages of grain development (Duffus and Rosie, 1973) and at later stages α-amylases similar to those in germinating grain have been found in the endosperm (Lindblom, 1985). Growth conditions have been implicated in the precocious formation of α-amylase at late-maturity in wheat (Mares, 1993) and by aleurone cells in barley (Cornford and Black, 1985), although not necessarily associated with high temperatures. Further, Kermode and Bewley (1989) observed the synthesis of developmental and germinative proteins in developing seeds of *Ricinus communis*, while Finkelstein and Crouch (1984) reported concurrent developmental and germination-specific processes in immature embryos. Notwithstanding this evidence, in the present study the greatest decline in the rate of starch synthesis (Table 4.4) in the heat treated grains during the period 18-21 daa was characterised by an increase in the activity of α-amylase but not β-amylase and although there was an apparent elevation in the level of glucose, other sugars, in particular maltose, showed no significant increase. Consequently, there appeared insufficient evidence for starch hydrolysis in the heated barley endosperm to account for the overall reduction in the rate of starch accumulation at this time. Rather, it appeared that a reduction in starch synthesis was the major cause of the decrease in starch in the heat treated endosperm.

5.5 **Conclusions**

Reduced starch deposition in barley endosperms as a result of exposure to high temperatures was due to diminished conversion of sucrose to starch. The supply of substrate, sucrose, was not found to be limiting in heat treated grains and was elevated following heating. Reduced catalytic activity was observed during the heating period in SSS, while other enzymes, including sucrose synthase and UDPGp, responded with the greatest reduction in activity during the cooler recovery period following the high

temperature treatment. The data suggest that SSS has an important role in the accumulation of starch during and following exposure to high temperature conditions. Definitive evidence of the nature of this role requires further investigation, particularly in the light of the discrepancy between the rate of activity of SSS and the rate of starch synthesis during the later stages of development.

Increased levels of starch degrading enzymes (α- and β-amylase), together with starch breakdown products (including maltose and glucose) were measured in heat treated endosperms, suggesting that starch degradation may occur in grains following high temperature exposure. However, insufficient quantities of enzymes and breakdown products were detected to support the possibility that starch degradation made the greatest contribution to the reduced starch levels in these grains. An investigation of the changes in structure in both control and heat treated grains was undertaken to document the effects of heat on the structure of the developing grain and on its final composition (Chapter 6).

Chapter 6

Effect of high temperature during grain filling on the structure of developing and malted barley grain.

6.1 Introduction

The major tissues of the barley grain (Fig 2.1) play important roles in the malting and brewing processes. The outer layers comprising the husk (palea and lemma) adhere to the grain and protect the embryo during grain handling. During the malting process the husk serves to maintain critical water relations of the grain (Palmer, 1989) and protect the developing acrospire. During brewing the husk provides an efficient filter bed for the mash (Briggs, Hough and Stevens, 1971). The inner and outer pericarp and the testa are important in protecting grain during storage and later in restricting the access of substances such as CO_2, water and dissolved solutes during germination (Collins, 1918; Briggs, 1987; MacGregor, 1991). Together these tissues represent about 15% of the grain weight.

The embryo is a complex organ comprising scutellum and root-shoot axis. The developing embryo is initially isolated from the endosperm, and is a powerful sink for nutrients as it grows and differentiates (Smart and O'Brien, 1983). The embryo drives the process of germination ensuring the secretion of enzymes which hydrolyse the contents of the endosperm to provide energy and substrates for seedling growth. The embryo is pivotal to the malting process.

The endosperm is the largest tissue of the barley grain, comprising about 70% by weight. It is a store of carbohydrate, as starch and non-starch polysaccharides, and protein, which normally provides energy and substrates for germination and early seedling growth. The outer-most layer of endosperm cells, the aleurone layer is particularly rich in protein (Palmer, 1989). Generally 2-3 cells thick, a single layer of aleurone cells is present over the scutellum (Smart and O'Brien, 1979) and covering the embryonic axis (Pogson *et al.*, 1989) and this has been termed the germ aleurone. Endosperm texture may be loose and friable (mealy) or dense and vitreous (steely) depending on grain composition (Allison *et al.*, 1979; Palmer, 1989) and variety (Schildbach *et al.*, 1990).

The crushed cell layer (CCL) comprises principally remnant endosperm cell wall material and at grain maturity represents a barrier between the embryo and its nutrient supply. Water uptake and retention in the region of the CCL (Collins, 1918) and its position between the embryo and the endosperm (MacGregor, 1991) imply an, as yet undefined, role in germination and malting. Following malting the endosperm components provide an energy source for yeast during fermentation and contributes to final beer flavour and composition.

Malting quality barley should be high in starch and low in β-glucan, that is, with little endosperm cell wall material, while satisfying a stringent protein requirement (MBIBITC, 1995). These important quality parameters are generally measured biochemically. There have been many investigations of the structure of the developing barley grain (Cochrane, 1985, 1994; Cochrane and Duffus, 1979, 1980, 1983; MacGregor *et al.*, 1989; MacGregor and Dushnicky, 1986, 1989a,b) and of the mature barley endosperm in relation to malting (Schildbach *et al.*, 1990; MacGregor, 1991; Palmer, 1980, 1989). Despite this, the relationship between grain development and final quality remains unclear (Palmer, 1980; Schildbach *et al.*, 1990). In particular, the formation of the CCL in relation to the metabolism and catabolism of starch and β-glucan is poorly understood (MacGregor and Dushnicky 1986, 1989b). Further, the effects of high temperatures during grain filling on grain structure are not clear. Periods of high temperature during grain development have been found to reduce the activity of a number of starch synthetic enzymes (Chapter 5) and to result in changes to endosperm composition (Chapter 4). While periods of high temperature are common in cereal growing regions of Australia (Stone and Nicolas, 1994), these conditions may not only alter the accumulation of endosperm products, but also result in changes to the physical structure of the grain.

In this study the structure of developing grain which had been subjected to a period of 5 days of high temperature during grain filling was investigated. Control and heat treated grain were compared at several stages of development up to and including harvest-ripeness, and following malting to relate grain structure to malting quality.

6.2 **Methods**

6.2.1 **Sampling**

Methods for sampling of Schooner grain for microscopy and details of microscopic

techniques have been presented in Chapter 3 (Materials and Methods). Details of the number of grains observed microscopically are presented in Table 6.1. Schooner grain was collected during Experiment 1 to be used in a preliminary study of grain structure during development. Harvest-ripe grains from this experiment were sampled for observation of endosperm structure using the Scanning Electron Microscope (SEM). Grain and malt collected from Experiment 3 were observed in the light microscope and using the SEM. Embryos were sampled to determine changes in dry weight during development. The sampling schedule for Experiment 4 was as follows; prior to heat treatment at 16 days after anthesis (daa), followed by 18, 21, 24, 30, 33, 40 and 45 daa. Exposure to increasing high temperatures, up to 35°C (14h day), was between 16 and 20 daa. Control conditions were maintained at 21/16°C, 14h day.

6.2.2 **Light microscopy**

Grains were bisected longitudinally into 2.5% glutaraldehyde and fixed for 1-2 days, dehydrated through an alcohol series and embedded in GMA. Each half grain was flat embedded to facilitate sectioning in the vertical longitudinal plane. 2.5μm sections were collected on slides and stained for observation. The columnar cells of the scutellar epithelium were used to ensure true longitudinal orientation. This was required for measurement of the thickness and number of cells of the CCL.

Histochemical stains used to identify grain structure also facilitated identification of cell components. Staining protocols, based on methods detailed in O'Brien and McCully (1981) appear in Chapter 3 (Materials and methods). Toluidine blue O was used for survey work. The PAS-Toluidine blue O stain (PAS/TBO) combination was used for general grain structure, cell wall material (cell wall polysaccharides), proteinaceous material, and cytoplasm. Starch deposits/granules were PAS positive. This stain resulted in starch and cell wall polysaccharides (and some phenolics) appearing red or magenta. Cell walls, containing mixed linkage β-glucan, fluoresced under UV light when stained with the optical brightener Calcofluor, and with the fluorescent stain Congo red. Autofluorescence was observed in mature grain tissues, as a result of the inherent fluorescence of lignins and some smaller molecular weight phenolic compounds. However, the limitation of these various stains and staining combinations lies in their variable specificity for components of plant tissues.

Table 6.1: Number of grains observed at each sampling time using the light microscope (LM), scanning electron microscope (SEM) and confocal laser scanning microscope (CLSM). Figures shown are the number of control **(heat treated)** grains observed.

	Experiment 1		**Experiment 3**		**Experiment 4**
Sampling time (daa)	LM	SEM	LM	SEM	LM/CLSM
16	2				6
18	1 *(4)*				6 *(6)*
21	2 *(2)*				7 *(7)*
24					6 *(4)*
30					5 *(6)*
33	5 *(3)*				5 *(5)*
40	4 *(3)*				4 *(3)*
45	1 *(1)*				5 *(4)*
harvest-ripe grain	1 *(1)*	3 *(3)*	4 *(7)*	3 *(3)*	3 *(3)*
malted grain		3 *(3)*	6 *(8)*	3 *(3)*	

6.2.3 **Scanning Electron Microscopy (SEM)**

Harvest-ripe and malted grain were sampled for SEM by halving grains and fixing to stubs with Superglue™ (Selleys Chemical Company, Australia). Specimens were coated with carbon and gold/palladium and observed in a Cambridge Stereoscan S250 SEM. Images were recorded using Ilford FP4 film.

6.2.4 **Confocal Laser Scanning Microscopy (CLSM)**

Sections of developing and harvest-ripe grains prepared for conventional light and fluorescence microscopy, were stained with Congo red and observed using a Bio-Rad MRC-1000 confocal laser scanning microscope connected to a krypton-argon laser (described in Section 3.8.3). Digital images were collected using the CLSM at excitation λ, 488 nm (blue), and emission filter 605 nm (red), using a 60x oil immersion lens (Zeiss).

6.2.5 **Embryo dry weight**

Embryos were sampled throughout development (Experiment 3), at 15, 18, 21, 24, 27, 30, 33, 36, 40 and 45 daa. Embryos were dissected from each of 10 grains per ear, dried at 80°C for 24h, and the dry weight recorded. The data have been plotted in chronological time and also expressed on the basis of thermal time (Russelle *et al.*, 1983). Thermal time was calculated for post anthesis development only, assuming a base temperature of 7.1°C (Goyne *et al.*, 1996). Details of calculations appear in Chapter 3, section 3.4.3.

6.2.6 **Micromalting**

Malted grain was sampled following micromalting in the Waite Barley and Malt Quality Evaluation Laboratory using the micromalting schedule outlined in Chapter 3 (Materials and Methods).

6.2.7 **Statistical analysis**

Data analysis was carried out using the GENSTAT statistical package (Genstat 5 Committee, 1987). Analysis of embryo dry weight was based on samples collected in a completely randomised design with the two factors of treatment (control vs heat treated) and time (sampling days after anthesis). Data collected on CCL development were analysed as a two way classification of unequal numbers of samples and the table of means was constructed from predicted values arising from the analysis of variance. The

Tukey method (Zar, 1984) was used for the comparison of means where the main treatment effect was established by analysis of variance.

6.3 **Results**

6.3.1 **Structural changes during development in control and heat treated grain**

6.3.1.1 **Structure of control grain prior to heat treatment (16 daa)**

At the time of heat treatment (16 daa) the developing grain had a small embryo and a clearly differentiated endosperm with a cell wall network present (Plate 6.1a). Endosperm cells contained developing A-type starch granules and small protein deposits (not shown), with evidence of smaller B-type granule initiation in the central (older) endosperm cells (Plate 6.1b).

Cell division continued after 16 daa and had ceased by 45 daa. During this time endosperm cell enlargement and associated storage product accumulation took place (Section 2.2). The endosperm accumulated approximately 70% of its final dry weight (Section 4.3.4) and the embryo developed to maturity.

6.3.1.2 **Embryo**

The scutellar epithelial layer of the 16 daa embryo appeared as a clearly differentiated, starch free layer of cells (Plate 6.1b). Scutellar epithelial cells abutting the CCL began to elongate at 18 daa in both control and heat treated grain and attained the columnar appearance characteristic of harvest-ripe grains between 21 and 24 daa (Plate 6.1c).

From 18 daa onwards the scutellar epithelial layer could be distinguished from the scutellar parenchyma, not only by the regular cell shape, but also by the accumulation and then loss of small starch granules during development. The granules were first observed in control grain at the ventral end of the scutellum, progressing over time towards the central and dorsal regions, and by 18 daa small starch granules were visible throughout the length of the epithelial layer adjacent to the CCL (Plate 6.1c). These small starch granules disappeared between 40 and 45 daa. Starch granule formation in the scutellar epithelium in heat treated grain did not differ from that in controls. However, there was no apparent loss of starch granules later in development from either the scutellar epithelium or the scutellar parenchyma cells in the heat treated grains and

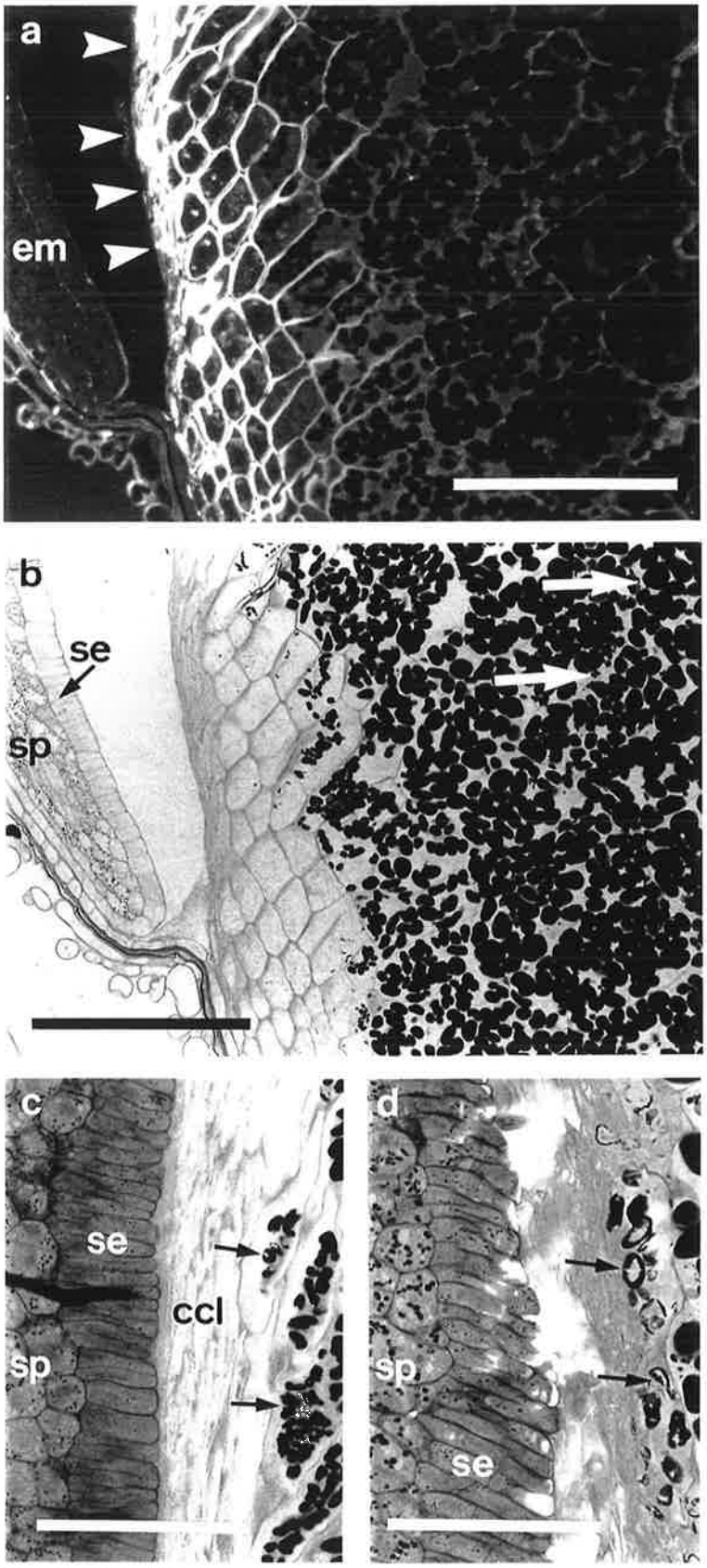
Plate 6.1

(a) Fluorescence micrograph of a longitudinal section stained with Calcofluor of a developing control grain 16 daa at the embryo-endosperm junction on the dorsal side of the grain showing the edge of the scutellum of the immature embryo (em) and a portion of the cell network present throughout the endosperm. Note that cell wall fluorescence appeared brightest in the region of the developing CCL (arrow-heads).
Bar represents 200μm.

(b) Adjacent section to 1(a) stained with PAS/TBO showing starch granule distribution in the endosperm cells. Small, developing B-type starch granules were present in the older cells towards the centre of the grain (arrows) with well developed A-type granules. Scutellar parenchyma (sp) cells had small starch deposits while the scutellar epithelial layer (se) remained starch free.
Bar represents 200μm.

(c) Light micrograph of a longitudinal section stained with PAS/TBO through a developing control grain 18 daa showing the scutellar parenchyma cells (sp) and the elongated cells of the epithelial layer of the scutellum (se) abutting the developing CCL (ccl). Note the presence of degraded starch granules adjacent to the CCL (arrows).
Bar represents 100μm.

(d) Light micrograph of a longitudinal section stained with PAS/TBO through a heat treated grain 40 daa showing the scutellar parenchyma cells (sp) and the scutellar epithelial layer (se). Starch granules were present in cells of both layers. Degraded starch granules were observed in cells adjacent to the CCL (arrows).
Bar represents 100μm.


a
em
b
se
sp
c
se
ccl
sp
d
sp
se

starch was observed throughout this tissue from 40-45daa (Plate 6.1d, 6.2c).

In contrast to the epithelial layer, the irregularly shaped scutellar parenchyma cells developed larger starch granules (Plate 6.1c). Towards the end of development these granules had developed into complex, often lobed, structures (not shown). Between 40 and 45 daa in control grain they declined in abundance progressively from the epithelial layer towards the meristematic region of the embryo. This loss of starch occurred sequentially from scutellar epithelial cells, via the scutellar parenchyma to the procambial region of the embryo. No starch was present in scutellar tissue of control grains 40 daa (Plate 6.2a) but was evident in heated grains 45 daa (Plate 6.2c).

Increase in embryo dry weight took place between 16 and 45 daa in both control and heat treated grain (Fig 6.1), but the dry weight of heat treated embryos appeared higher at 18 daa and from 24 daa than control embryos (Fig 6.1a). When plotted in thermal time, the effects of heat treatment on embryo dry weight did not become apparent until more than 240 degree days (^{o}Cd), when the plots diverged (Fig 6.1b). The effects of heating became progressively more pronounced after this time and the course of embryo dry weight accumulation appeared to differ in heat treated grain, compared with controls.

At 40 daa the control embryo had a well developed axis (Plate 6.2a). Increased extension of the acrospire was observed in heat treated (Plate 6.2b) as compared with control grains and the coleoptile had penetrated through the germ aleurone layer in all heat treated grain sampled after 40 daa (Plate 6.2c). Development did not proceed beyond initial growth of the acrospire and there was no evidence of rootlet emergence.

6.3.1.3 **Endosperm**

6.3.1.3.1 **Starch granules**

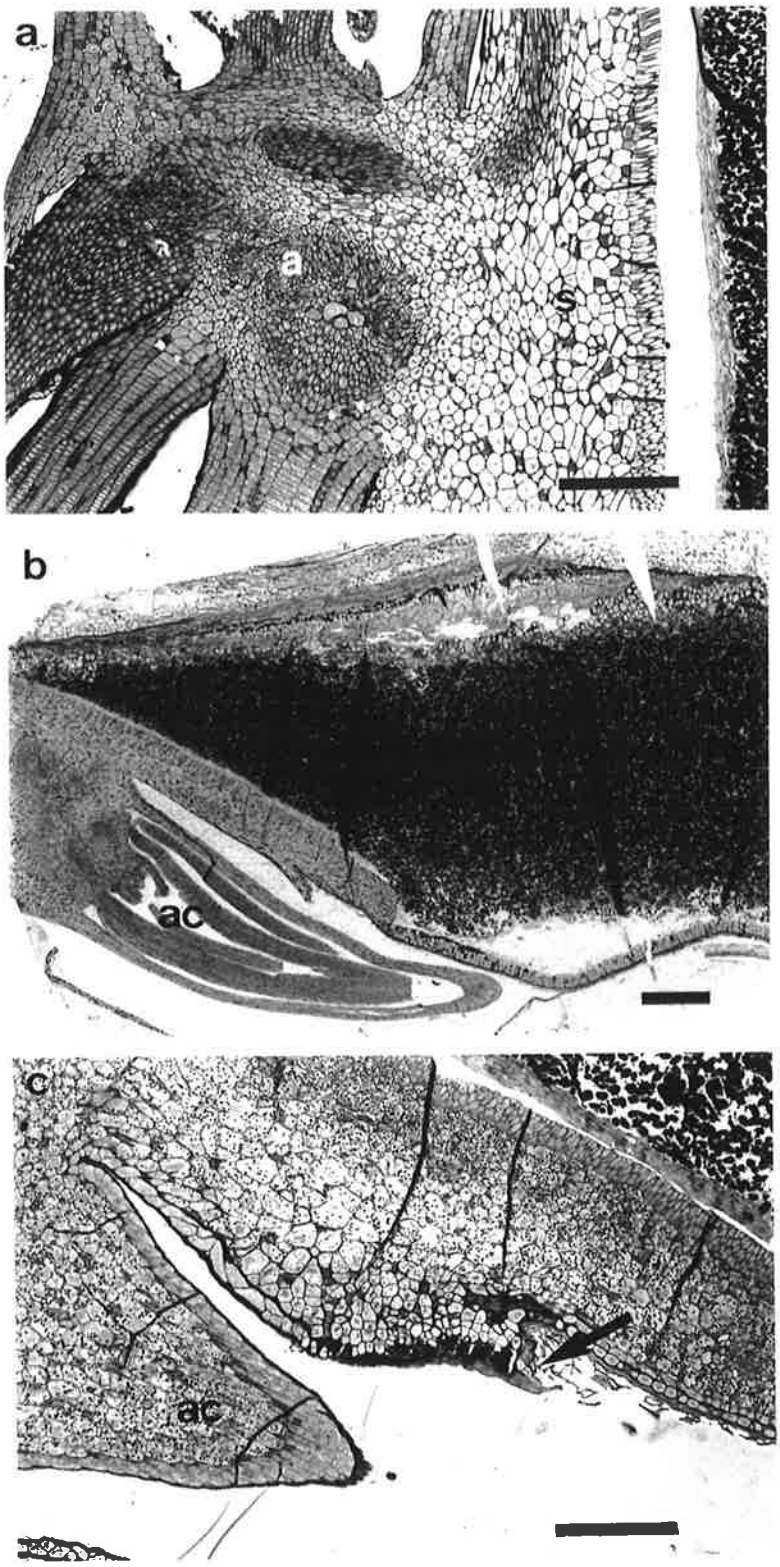
At 16 daa the aleurone layer was undergoing cell division at the periphery of the endosperm. Initiation of A-type starch granules occurred in the sub-aleurone cells and these growing cells were progressively displaced towards the centre of the grain. A-type starch granules continued growing up to 40-45 daa and were of uniform size within cells of similar age. In sub-aleurone layers small A-type granules were present in heat treated and control grain 40 daa (Plate 6.3c,d) and at harvest-ripeness. B-type starch granule

Plate 6.2

(a) Light micrograph of longitudinal section through the well developed control embryo 40 daa showing the axis (a) and scutellum (s). No starch was present in the embryo.
Bar represents 100μm.

(b) Light micrograph of longitudinal section stained with PAS/TBO through a heat treated grain 30 daa showing elongation of the acrospire (ac) along the dorsal face of the grain.
Bar represents 200μm.

(c) Light micrograph of a longitudinal section through a heat treated grain 45 daa stained with PAS/TBO. The expanding acrospire (ac) has broken through the aleurone layer (arrow).
Bar represents 100μm


a
a
s
b
ac
c
ac

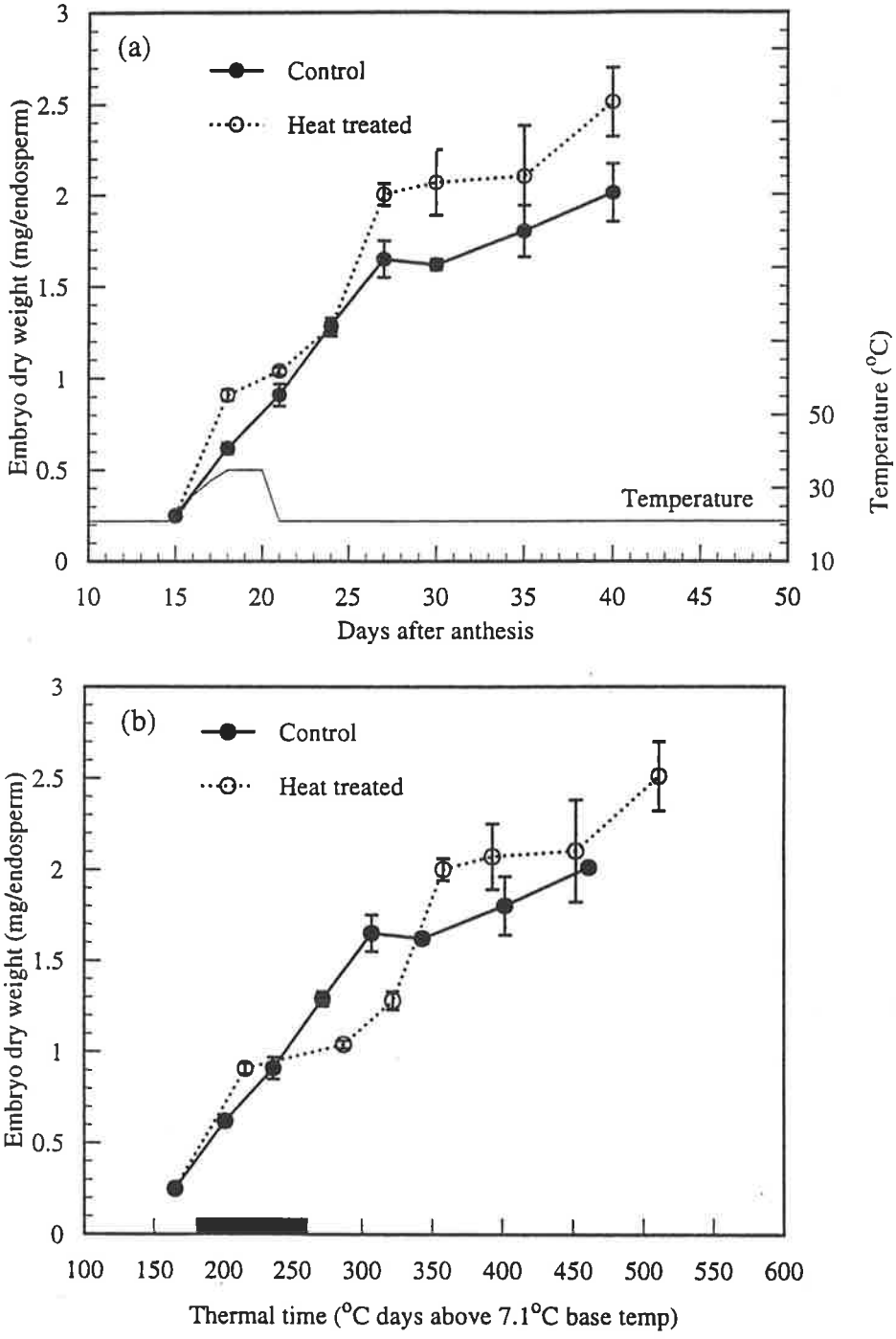


Fig 6.1 **Embryo dry weight** during development of control and heat treated grain (a), also plotted in thermal time (b), showing standard errors. Bar indicates high temperature period in (b).

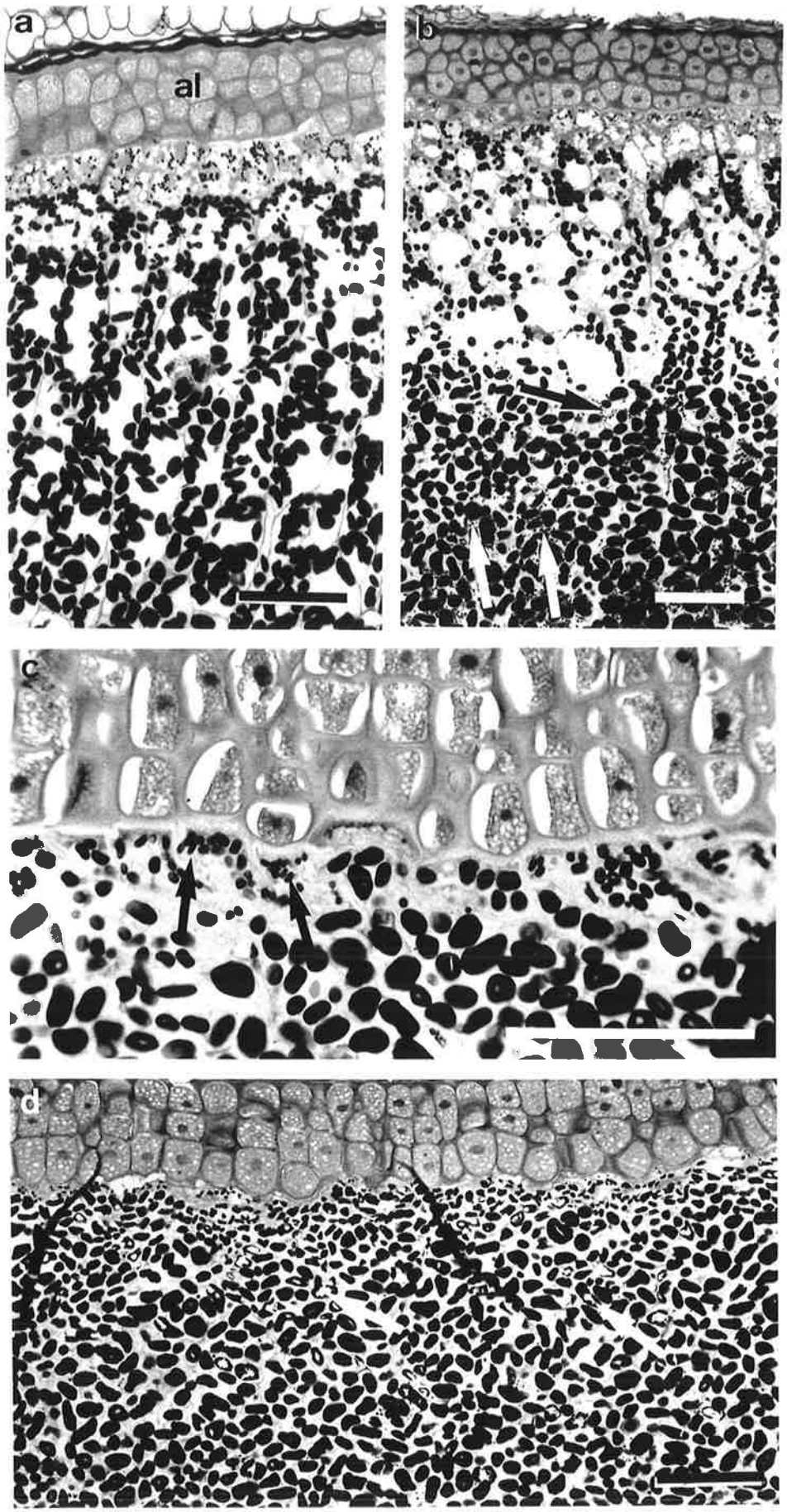
Plate 6.3

(a) Light micrograph of a longitudinal section through the dorsal endosperm of a control grain 24 daa stained with PAS/TBO. This profile shows aleurone (al) and new starchy endosperm cells containing developing A-type starch granules, with larger A-type granules in cells displaced towards the centre of the grain.
Bar represents 100μm.

(b) Light micrograph of a longitudinal section through the dorsal endosperm of a heat treated grain 22 daa stained with PAS/TBO and showing comparable region of the grain to (a). The subaleurone cells were vacuolate and contained fewer and smaller starch granules than (a). Older cells contained numerous small B-type starch granules (arrows).
Bar represents 100μm.

(c) Light micrograph of a longitudinal section through the dorsal aleurone of a control grain 40 daa stained with PAS/TBO. Small A-type granules are visible in sub-aleurone cells (arrows).
Bar represents 100μm.

(d) Light micrograph of a longitudinal section through the dorsal endosperm of a heat treated grain 40 daa stained with PAS/TBO. Degradation of endosperm starch was apparent (arrows).
Bar represents 100 μm.


a
al
b
c
d

initiation commenced prior to 16 daa in older cells located towards the centre of the grain (Plate 6.1b).

Following heat treatment the most recently formed endosperm cells had fewer starch granules when compared with control grain (compare Plates 6.3a,b), with developing A-type granules appearing smaller in size and distributed around the margins of the cells. In heat treated grain, starch remained concentrated in the central region of the endosperm at 45 daa. Some degraded starch granules were observed in cells adjacent to the CCL throughout development, in both control and heat treated harvest-ripe grain (Plate 6.1c and 6.1d, 6.4d respectively). In heat treated grain, however, localised starch degradation was also observed in cells in the region along the dorsal side of the grain below the aleurone, but remote from the embryo (Plate 6.3d) .

SEM observations showed that the central endosperm cells of harvest-ripe grain were packed with A- and B-type starch granules embedded in a protein matrix (Plate 6.4a). The control endosperm had a steely (close packed starch granules) appearance. Endosperm texture appeared relatively homogeneous, with components densely packed within the cells. In heat treated grain few B-type starch granules were observed in cells adjacent to the CCL and many A-type starch granules in this region were pitted or showed surface erosion (Plate 6.4d). A-type starch granules of heat treated grains appeared smaller than those in control grain (compare Plate 6.4a,b). The endosperm of harvest-ripe heat treated grains appeared more heterogeneous and generally had a more mealy (loose packing) appearance than control endosperm tissue. B-type starch granules were rarely visible within the central endosperm cells and some large protein rich deposits were present (Plate 6.4b).

6.3.1.3.2 **Cell walls**

A network of cell walls fluoresced with Calcofluor throughout the endosperm by 16 daa. The intensity of fluorescence varied between different regions until about 24 daa, when it became relatively uniform throughout the grain. Initially those cell walls adjacent to the developing CCL appeared bright and clear, while those nearer the centre of the grain appeared finer and fluoresced less brightly (Plate 6.1a). Dorsi-ventral differences were also apparent, with a gradation in cell wall fluorescence from the dorsal side to much reduced fluorescence in the vicinity of the ventral furrow. Harvest-ripe control grain

Plate 6.4

(a) SEM image of the central endosperm of harvest-ripe control grain showing A-type (s) and B-type (arrows) starch granules embedded in a protein matrix. Endosperm cell walls were also visible (arrowheads).
Bar represents 40μm.

(b) SEM image of the central endosperm of a harvest-ripe heat treated grain. Thick protein deposits (arrow), but few B-type starch granules, were visible in central endosperm cells.
Bar represents 40 μm.

(c) SEM image showing the junction between the embryo and endosperm in a harvest-ripe control grain. Limited erosion of starch granules was seen in cells adjacent to the CCL (arrow).
Bar represents 40 μm.

(d) SEM image similar to (c) in a harvest-ripe heat treated grain. Starch granules in adjacent endosperm cells showed evidence of surface erosion (arrowhead).
Bar represents 40 μm.

a
S
S
b
c
d

contained a network of brightly fluorescent cell walls throughout the endosperm (Plate 6.5a).

During the period of exposure to high temperatures, there were no changes in the endosperm, with the fine network of cell walls still visible throughout the endosperm. Rather, changes in distribution and intensity of cell wall fluorescence were observed in heat treated grain from 24 daa., after plants had been returned to control temperatures. There was reduced staining in the central endosperm from 30 daa and adjacent to the dorsal aleurone from 33 daa (Plate 6.5b).

6.3.1.3.3 **Crushed cell layer**

After 18 daa there was no significant increase in the number of cell layers contributing to the CCL (Table 6.2, Plate 6.6a), but its thickness continued to increase in control grain until 30-33 daa. No significant changes occurred in the thickness or the number of cells contributing to the CCL during the high temperature period (ie 18-21 daa) so that this layer was significantly thinner than in control grain by 30 and 33 daa (Table 6.2; Plates 6.6a,b). By 40 daa CCL fluorescence in heat treated grain was also very patchy (Plate 6.6d).

Table 6.2 Crushed cell layer development measured in changes to CCL thickness and the number of contributing cell layers between 18 and 33 daa. Mean values predicted from regression models (ANOVA). Significance of variation from control values shown as: *P<0.05; **P<0.01.

Crushed cell layer development

	Mean number of cells		**Thickness (μm)**	
Days after anthesis	Control	Heat treated	Control	Heat treated
18	8.3	7.8	49	43
21	8.4	8.2	56	56
24	9.0	7.5	60	51
30	9.8	8.5	71	46**
33	9.4	7.3	70	49*

Plate 6.5

(a) Fluorescence micrograph of a longitudinal section through a harvest-ripe control grain stained with Calcofluor showing the network of endosperm (e) cell walls and the CCL (ccl). Arrows indicate regions where loss of fluorescence has occurred in the CCL.
Bar represents 500μm.

(b) Fluorescence micrograph of a longitudinal section through a harvest-ripe heat treated grain stained with Calcofluor. Brightly fluorescent cell wall material was visible in the CCL region and along both the dorsal and ventral surfaces of the endosperm with only a faint cell wall network discernible in the centre of the grain.
Bar represents 500 μm.

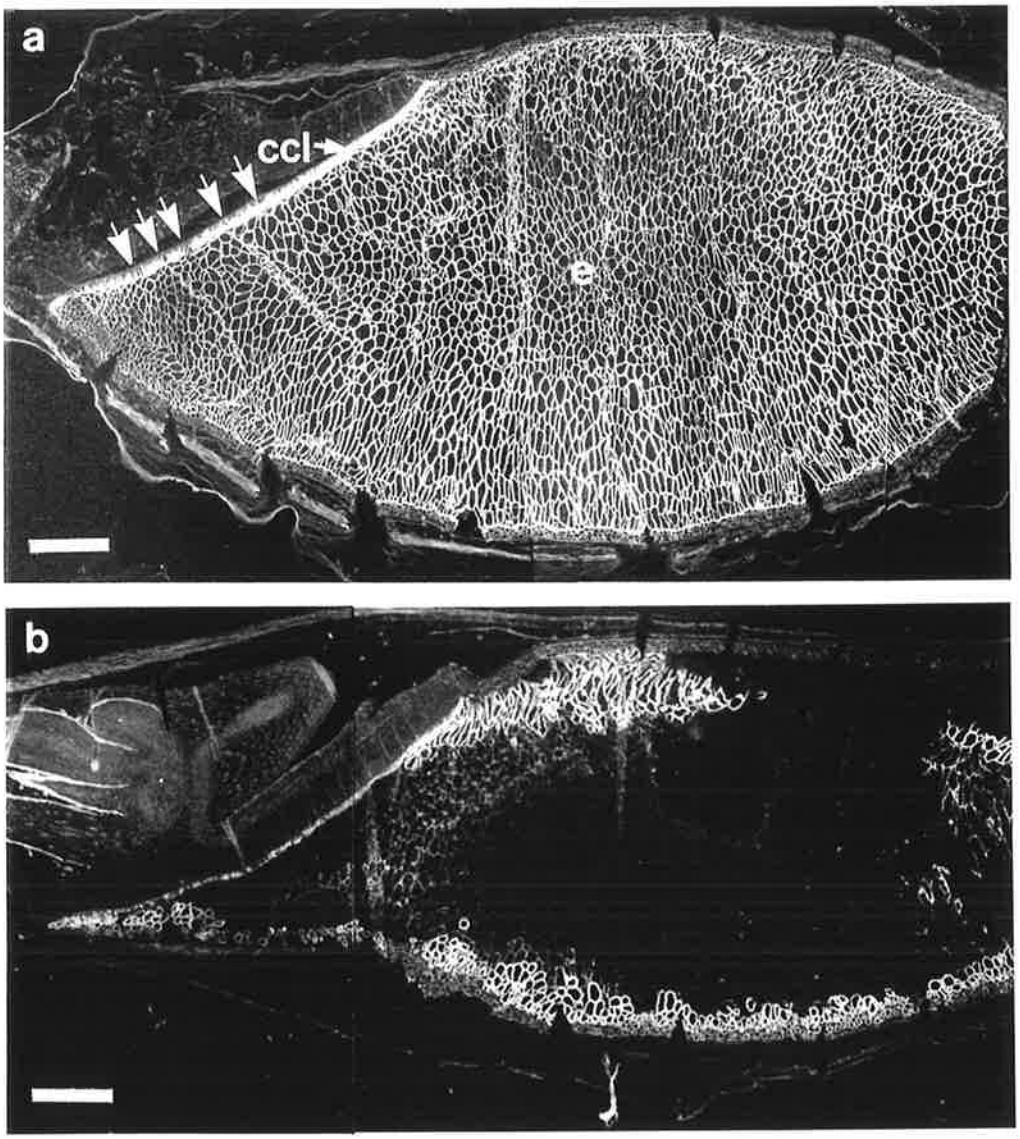

a
ccl
b

Plate 6.6

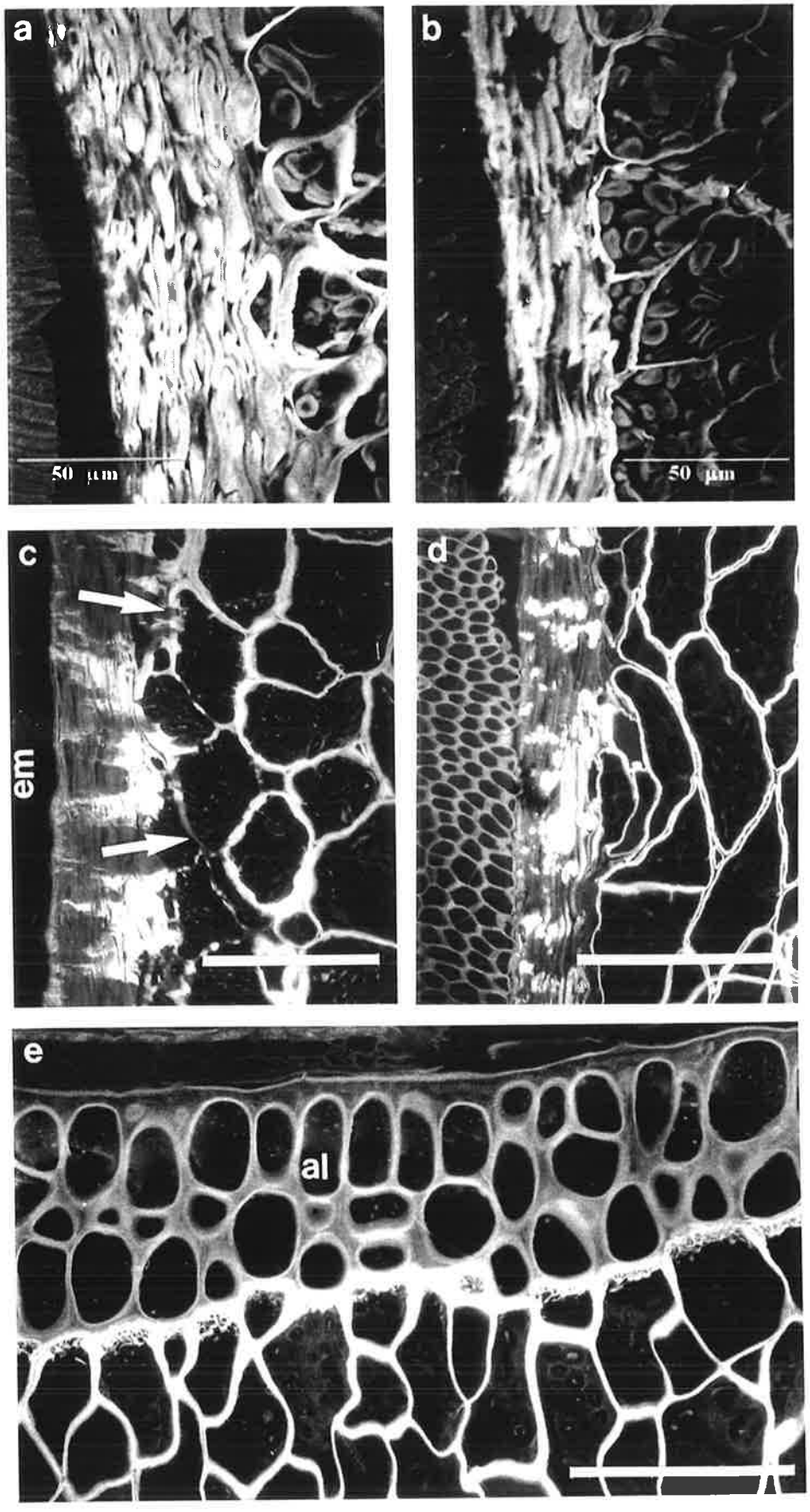
(a) Confocal image of a longitudinal section of developing control grain 33 daa stained with Congo red, showing the compressed cells contributing to the CCL.
Bar represents 50 μm.

(b) Confocal image, as in (a) of a heat treated grain 33 daa stained with Congo red. Some patchiness in the fluorescence of the CCL was observed in heat treated grain, although contributing cell layers were clearly distinguished.
Bar represents 50 μm.

(c) Fluorescence micrograph of a longitudinal section through a control grain 40 daa stained with Calcofluor. Loss of wall fluorescence appeared as channels extending from the adjacent embryo (em) through the CCL and into adjacent starchy endosperm cells (arrows).
Bar represents 100 μm.

(d) Fluorescence micrograph of a similar region to (c) in a heat treated grain 40 daa. Loss of Calcofluor fluorescence in the CCL is patchy, and adjacent endosperm cell walls continue to fluoresce and appear intact.
Bar represents 100 μm.

(e) Fluorescence micrograph of a longitudinal section of a control grain 33 daa stained with Calcofluor. Thick walled mature aleurone (al) cells showed reduced fluorescence while subaleurone and starchy endosperm cell walls remained highly fluorescent.
Bar represents 100 μm.


a
50 µm
b
50 µm
c
em
d
e
al

Loss of CCL fluorescence was observed in control grain 40 daa as channels from the scutellar epithelium towards the starchy endosperm (Plate 6.6c), and these were also visible in harvest-ripe grain (Plate 6.5a, arrows). Where the loss of CCL fluorescence was complete, loss of endosperm cell wall fluorescence was localised in adjacent cells (arrow in Plate 6.6c). The loss of CCL fluorescence progressed from the ventral to the dorsal side of the grain.

6.3.1.3.4 **Aleurone layer**

The aleurone layer comprised 2-4 layers of cells which were irregular in shape (Plate 6.3a). The region where the endosperm and embryo abut, separated by the CCL, formed the transition between the meristematic aleurone layers surrounding the starchy endosperm and the single germ aleurone layer. The endosperm aleurone cells developed thickened walls and became more cuboid in shape 24 daa in control grain. From 33 daa aleurone cell walls began to fluoresce differently to other endosperm cells, showing strong autofluorescence and diminished Calcofluor staining (Plate 6.6e). The boundary between the aleurone cells and the endosperm cells fluoresced more brightly with Calcofluor than the thickened axial cell walls.

In heat treated grains autofluorescence of the aleurone cells developed more quickly than in control grains, consistent with more rapid development, although the developmental pattern appeared similar. Reduced Calcofluor fluorescence was observed by 30 daa (data not shown).

6.3.2 **Structural changes following malting of control and heat treated grain**

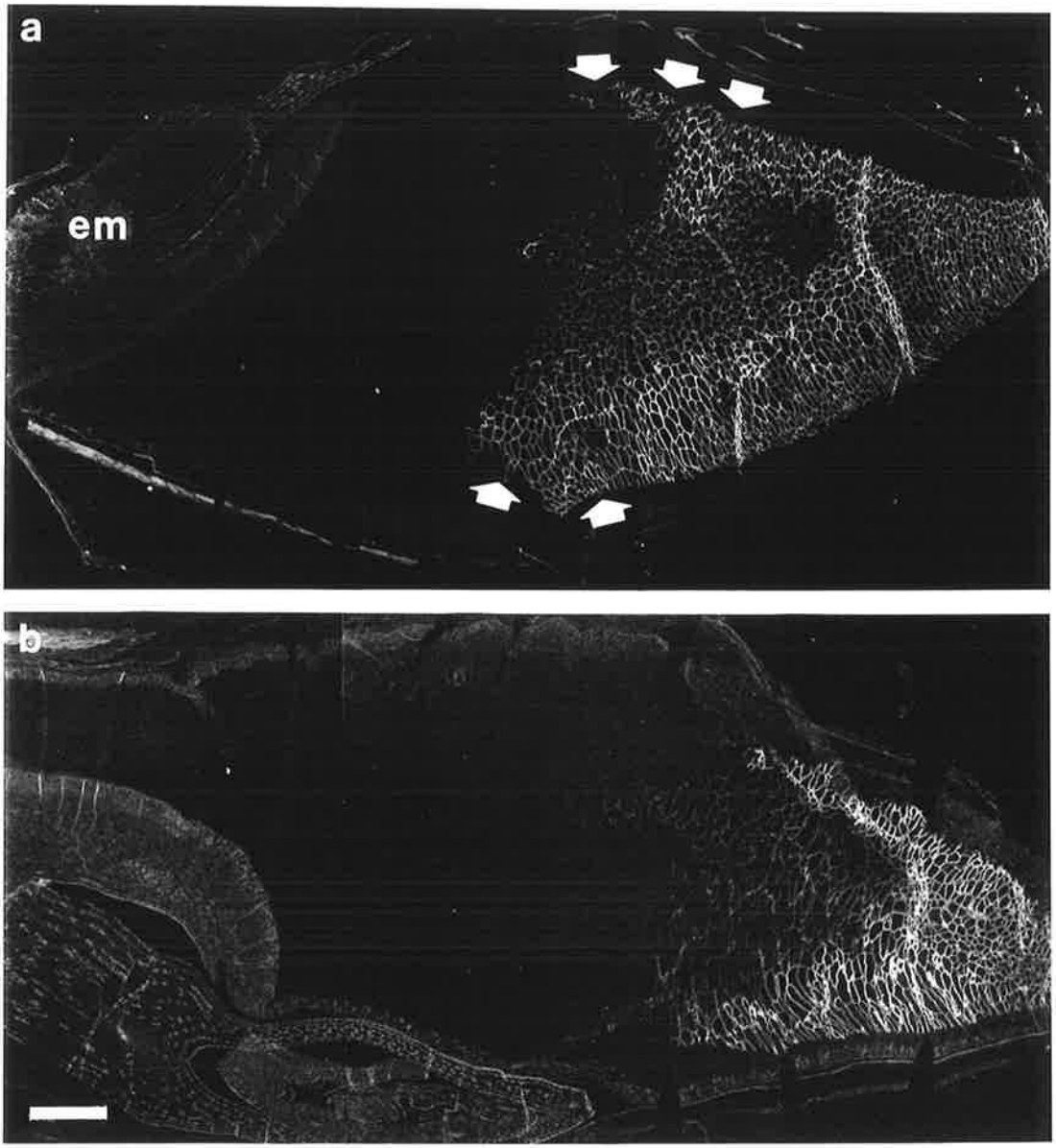
Both heat treated and control grain germinated successfully during malting and showed no dormancy or water sensitivity (failure to grow in an excess of water).

The degree of modification (enzymic breakdown of the endosperm) of control grain varied but always followed a pattern of loss of cell wall fluorescence parallel to the scutellum and penetrating towards the distal region of the grain (Plate 6.7a). This pattern was not symmetrical, with greater loss of cell wall fluorescence on the periphery of the grains (Plate 6.7a, arrows). Extensive loss of protein (not shown) and some loss of starch in the form of loss of B-type starch granules and pitting in A-type granules in the scutellar region (not shown) also characterised malted control grain. The central endosperm increased in mealiness and although B-type starch granule numbers

Plate 6.7

(a) Fluorescence micrograph of a longitudinal section through a malted control grain stained with Calcofluor. The pattern of endosperm modification was essentially parallel to the scutellum, with some loss of fluorescence adjacent to the surrounding aleurone cells (arrows). Embryo (em).
Bar represents 500 μm.

(b) Fluorescence micrograph of a longitudinal section through a malted heat treated grain stained with Calcofluor. Little endosperm cell wall material remained following malting and only in the distal region of the grain.
Bar represents 500 μm.


a
em
b

decreased, there was no evidence of loss of starch from A-type starch granules, as evidenced by pitting, in this region during malting (Plate 6.8a).

Heat treated grains were over-modified during malting[1], with very little cell wall fluorescence present in malted grain (Plate 6.7b). Some cell wall material remained in the distal region of the grain. Loss of protein and B-type starch granules was observed in the scutellar and central regions of the grain and contributed to an open, loose-packed appearance. Pitting of A-type granules was observed in the central region (Plate 6.8b).

6.4 **Discussion**

The developing barley grain responded to heat in a number of important ways. Among these were reduced starch accumulation and a reduction of Calcofluor fluorescent cell wall material in the endosperm and the CCL. The cultural conditions which induced changes in the metabolism of the heat treated grain from 30 daa, were associated with alterations to the appearance of the contents of cells within the starchy endosperm and with continued and excessive growth of the acrospire. Following malting there was evidence in the heated grains of increased starch pitting and granule erosion.

6.4.1 **Starch distribution**

Heat treatment appeared to reduce starch granule development and resulted in alterations to starch granule distribution and growth. Under elevated temperatures the initiation of starch granules and the synthesis of endosperm starch did not appear to keep pace with cell division and cell enlargement and the peripheral endosperm cells remained relatively empty of starch granules. This was consistent with the reduction in starch synthesis due to high temperature exposure which has been reported elsewhere in this study (see Chapters 4 and 5) and by other workers (MacLeod and Duffus, 1988a; Jenner, 1994; Savin and Nicolas, 1996). The supply of sucrose for starch synthesis has not been found to be limiting under high temperature conditions (Nicolas *et al*, 1984; Jenner and Rathjen, 1975) and may even be increased (MacLeod and Duffus, 1988a; Section 5.3.2). It is the conversion of sucrose to starch which is affected by high temperature exposure (MacLeod and Duffus, 1988a) due to reduced activity of one or several of the starch synthetic enzymes (Jenner, 1994; Section 5.3.3).

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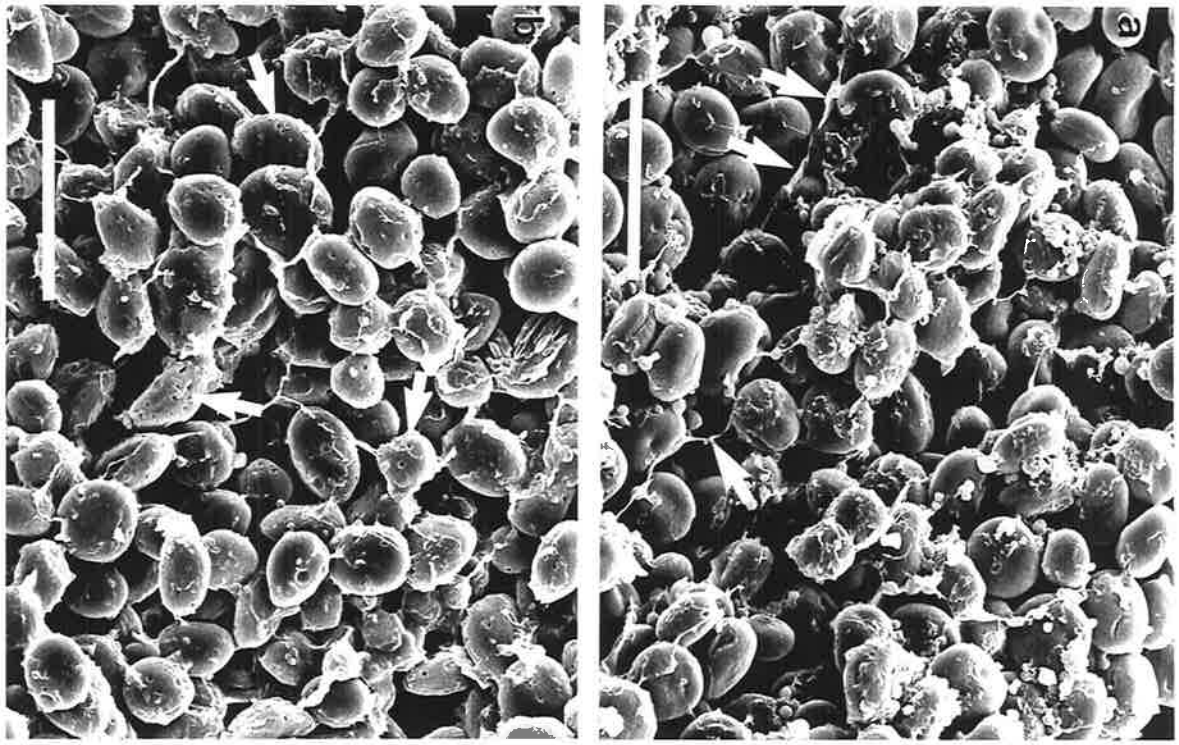
[1] The author acknowledges discussions with Dr. Sandy MacGregor (Canadian Grain Commission) regarding the appearance of well modified field grown malt. In the experience of the author, Australian malting barleys rarely, if ever, exhibited no Calcofluor fluorescence at the distil end of the grain, in contrast to similar malts produced from Canadian varieties.

Plate 6.8

(a) SEM image of the central endosperm region of a malted control grain with open and friable appearance due to loss of protein and most of the B-type starch granules. Very little cell wall material remained (arrows) but there was no evidence of degradation of A-type starch granules.
Bar represents 40 μm.

(b) SEM image of the central endosperm region of a malted heat treated grain. Considerable loss of B-type starch, protein and cell wall material was apparent. Extensive pitting (arrows) was observed in A-type starch granules as a result of malting.
Bar represents 40 μm.


b
a

A-type starch granules within starchy endosperm cells appeared smaller in heat treated harvest-ripe grains compared with control grains (compare Plates 6.4a,b). Reduction in the size of A- and B-type starch granules in response to increased temperatures has been reported by a number of workers (Shi *et al.*, 1994; Tester *et al.*, 1991;1995) and associated with reduced starch accumulation. Although no observations of starch granule numbers were made in this study, other workers have found that high temperatures may also reduce the number of starch granules (MacLeod and Duffus, 1988b; Tester *et al.*, 1991; Savin *et al.*, 1997a). Although bi-modal overall (Bathgate and Palmer, 1973), the distinction between A- and B-type starch granules depends on granule diameter (MacLeod and Duffus, 1988b: Savin *et al.*, 1997a), as the size distribution within barley grains is broad and continuous (Goering *et al.*, 1973). It is possible that small, partially degraded A-type starch granules (Plate 6.3d) in the peripheral cells of mature heat treated grain may have contributed to the bi-modal distribution of B-granules reported by Savin *et al.* (1997b) following high temperature exposure. While a natural decrease in amyloplast numbers during development has been found (Briarty *et al.*, 1979), Tester *et al.* (1995) also observed a reduction in amyloplast numbers with high temperature exposure. These observations may explain the reduction in A-type starch granules reported by MacLeod and Duffus (1988b).

An apparent reduction in overall grain volume available for starch accumulation following high temperature exposure has been reported by Bhullar and Jenner (1983) and MacLeod and Duffus (1988b). From structural evidence in this study it appears that on an individual cell basis no physical restriction to the accumulation of starch was evident following high temperature treatment. Even though maximum cell number and individual cell size may have been smaller as a result of high temperature exposure (Nicolas *et al.*, 1984), the later formed cells on the periphery of the endosperm were not filled with starch (Plate 6.3b). It seems likely therefore, that the overall reduction in starch granule development in heat treated grain resulted from the combined effects of a reduction in starch synthesis and alterations to starch granule initiation and growth.

6.4.2 **Cell wall and CCL development**

MacGregor and Dushnicky (1986) postulated a physical compression component in the crushing of cells to the formation of the CCL. Using conventional microscopy, they found that an increase in CCL thickness coincided with embryo enlargement. Although consistent with the present findings for control grain, confocal microscopy clearly showed that from 18

daa the heat treated grain did not have any additional cell layers crushed into the CCL. If the formation of the CCL was associated with embyro growth then more cell layers would be expected to be incorporated in the CCL in heat treated grain between 18 and 33 daa, when the overall rate of embryo growth was greater than in control grains. However, this was not the case, indicating that the formation of the CCL may be controlled independently of the embryo, and be sensitive to the influence of the environment.

The thickness of the CCL remained unchanged in heat treated grain between 18 and 33 daa, while it increased significantly in control grains. From the appearance of cell layers in the CCL in Plate 6.3a, it seems likely that β-glucan deposition continued in the CCL of control grains from 18 daa. This is supported by evidence that synthesis of this cell wall component takes place later in grain development (Coles, 1979; Aman *et al.*, 1989; Section 4.3.3.3). However, the possibility of increased β-glucan degradation in heat treated grain during this period cannot be ruled out. The patchy appearance of the CCL and the reduced Calcofluor fluorescence within the starch endosperm are consistent with partial hydrolysis of β-glucan. While the most reliable molecular weight estimations for these cell wall polysaccharides range from 150,000 to 300,000, they are heterogeneous with respect to size, solubility and molecular structure (MacGregor and Fincher, 1993). Where the molecular weight is less than 10,000, β-glucan may not be detected by Calcofluor fluorescence (Foldager and Jorgensen, 1984).

6.4.3 **Embryo development**

Increased growth of the embryo took place in heat treated grain. It is likely that the apparent increase in embryo dry weight observed during the heating period (Fig 6.1) was simply a temperature-driven growth response and could have been a direct result of temperature on the rate of growth and hastened embryo development (Fig 6.1b). This is consistent with the reduction in duration of grain filling, or shortened period of grain development, that has been documented as a response to a period of high temperature in both barley (Nicolas *et al.*, 1984; Savin and Nicolas, 1996; Savin *et al.*, 1997a) and wheat (Jenner, 1994; Stone and Nicolas, 1995a).

Changes in embryo growth from 27 daa until harvest ripeness, however, indicated a significant divergence from the control development pattern (Fig 6.1b). Heat treated embryos grew rapidly from 21 daa and were significantly larger than the control embryos by 40 daa.

The growth of the heat treated embryo may have been associated with an increase in available nutrients resulting from hydrolysis of endosperm cell contents below the dorsal aleurone, degradation of cell wall material within the CCL, and/or higher levels of sucrose (Section 5.3.2). Uptake of nutrients, in particular sucrose, by growing embryos has been indicated up to 50 daa (Cochrane, 1985) and active uptake of sucrose by the embryo has been established *in vivo* (Cameron-Mills and Duffus, 1979). Net inflow of sucrose by diffusion into the embryo could be expected to increase when external sucrose concentrations are high (i.e. following heat treatment). Although a pathway of nutrient movement into the embryo during development has not been clearly established, the development pattern of the scutellum provides a route for the movement of nutrients between the embryo and endosperm as they mature. Vascularisation of the scutellum occurs from 10 daa with two bundles developing from the base of the stem meristem and along the face of the scutellum, extending with the dorsal tip (Merry, 1941). Growth of the heat treated acrospire may have been promoted by the availability of nutrients as a result of the heat treatment, in combination with the presence of a transport route between the endosperm and the embryo.

6.4.4 **Endosperm degradation**

Microscopic observations revealed that some loss of endosperm structural components occurred in control grain during development. The presence of degraded starch granules in cells adjacent to the CCL was observed throughout development in this study, in both control and heat treated grain (Plate 6.1d, 6.4d). This has been reported elsewhere as indicating that starch hydrolysis in immature barley endosperms plays a role in normal grain development, in particular the formation of the CCL from 10 daa (MacGregor and Dushnicky, 1989b), although the source of α-amylase responsible has not been clearly established. In the present study there was also evidence in control grain of reduced Calcofluor fluorescence of cell wall material in the CCL (Plate 6.5a (arrows)). The areas of β-glucan degradation (loss of Calcofluor fluorescence) were first detected in the ventral end of the CCL and corresponded to the appearance of the CCL during the early stages of germination, between 12 and 24 hours after imbibition (MacGregor *et al.*, 1994). This evidence suggests that control grains showed signs associated with precocious germination before they attained harvest ripeness. It is possible that the 'switch' between embryonic development and germination may not occur as abruptly as previously suggested on the basis of the spectrum of proteins synthesised in the endosperm (Dasgupta and Bewley, 1982; Kermode and Bewley, 1989). Seed development and germination are generally considered distinct physiological stages in the plant life cycle,

with development characterised by the accumulation of endosperm storage reserves and germination by reactivation of existing metabolic systems, supplementation of new components and the mobilisation of endosperm reserves. However there is evidence that developmental events and germination/growth events can occur simultaneously. For example, precociously germinated, immature rape seeds continue to accumulate storage protein in newly formed tissues (Finkelstein and Crouch, 1984) Further investigation is required to establish the significance of concurrent developmental and germination processes within developing grain. Two potential regulatory factors have been identified, ABA and restricted water uptake (Bewley and Black, 1983), and there are indications that receptors may be membrane-associated with the controlling 'switch' between development and germination involving a series of temporal cues (Kermode, 1990). A greater understanding of the mechanisms involved may be particularly important in relation to such issues as water sensitivity, the timing of malting after harvest and tolerance of pre-harvest sprouting (Mares, 1993).

Microscopic observations of the developing grain provided evidence of premature breakdown within the starchy endosperm as a result of exposure to high temperatures. Reduced Calcofluor fluorescence of cell wall material was apparent in mature heat treated grain (Plate 6.5b) and loss of starch below the aleurone on the dorsal surface (Plate 6.3c), consistent with the pitting and internal digestion of large starch granules by α-amylase (Palmer, 1989). These changes may have arisen from precocious production of β-glucanase and α-amylase, and/or through the increased vulnerability of cell walls or starch granules to enzymic attack.

Specific cultural conditions of light (high intensity sodium lights) and temperature (27°C) during grain development have been reported to result in the production of hydrolytic enzymes by distal halves of mature barley grains prior to harvest ripeness (Nicholls, 1983; MacGregor *et al.*, 1983). Production of α-amylase and subsequent breakdown of starch was associated with these changes in regions of the grain below the dorsal aleurone (MacGregor et al. 1983). This was observed in the present study, which also used high-intensity sodium lights and high temperatures.

Growth conditions have the potential to alter the metabolism of specific tissues within the developing grain. Increased aleurone sensitivity to gibberellic acid (GA), the hormone secreted by the germinating embryo but present in small quantities throughout the grain, has

been observed under various cultural conditions by a number of workers (Armstrong *et al.*, 1982; Cornford and Black, 1985; Skadsen, 1993). Sole *et al.* (1987) also detected natural variation in aleurone viability within individual grains.

It is possible that the composition of starch formed under high temperature conditions may have resulted in increased susceptibility of starch granules to enzymic attack. For example, observations by Greenwood and Thompson (1959) suggest that amylopectin may be attacked faster than amylose in the germinating grain. Increased levels of amylose and starch lipid have been associated with high temperature exposure (Shi *et al.*, 1994; Tester *et al.*, 1995), although such alterations to starch granule composition would be expected to reduce rather than promote enzymic degradation (Palmer, 1989; Swanston *et al.*, 1995).

6.4.5 **Effect of high temperature treatment on malting**

The results of this study indicate that grain exposed to high temperatures during grain filling may significantly overmodify under commercial malting conditions. Loss of fermentable sugars, in the form of degraded starch, would represent a considerable financial penalty. However, while modification proceeded rapidly in heat treated grains in this study, some exposure to elevated temperatures during grain filling may have been beneficial for malting where it altered grain endosperm texture. The increased mealiness observed in the heat treated endosperm has been associated with increased susceptibility to enzymic attack (Axcell *et al.*, 1983) due to increased 'water free space' and alterations to the hydration pattern of steeped grain. Uniform hydration is desirable during malting as it allows the greatest flexibility and control of the process (by water and temperature) (Axcell *et al.*, 1983) and results in more even enzyme modification of the endosperm (Brookes *et al.*, 1976; Briggs, 1987b).

6.5 **Conclusions**

High temperatures applied during grain filling had a profound effect on the structure of the mature grain. Structural observations showed that final grain composition resulted from the combined effects of synthesis and degradation of endosperm storage products. Biochemical analysis of grain measured the net effect of these processes between sampling days (Chapter 4). These structural observations indicated that in addition to their overall concentration, altered distribution of endosperm storage components has the potential to affect malting performance. Some evidence has been presented for a continuum between grain

development and germination, and this requires further investigation. The final structure and texture of the barley endosperm is important in determining malting performance. In particular, the relationship between water penetration and distribution ultimately determines the mobility and accessibility of enzymes to their substrates during germination (Axcell *et al.*, 1983: Schildbach and Rath, 1994). At present there is a lack of evidence linking the growth behaviour of the different tissues of the embryo with the biochemical changes occurring within the grain as a whole during early germination and seedling growth (Palmer, 1980; Briggs, 1992). If knowledge in this area were improved through coordinated structural observations and biochemical analyses, this would enable better control of embryo development and germination and provide a significant impact on malting technology.

Chapter 7

Changes in grain and malt quality following exposure to a period of heat during grain filling.

7.1 Introduction

Malting involves the germination of barley grain under controlled conditions. The suitability of barley varieties for malting is determined by a large number of characteristics of grain and malt. Commercial specifications for barley for malting tend to be comprehensive and usually reflect the major aim of malting, which is to maximise the levels of fermentable sugars, amino acids and vitamins available to yeast during fermentation of beer, while minimising those characteristics which may cause processing problems.

The improvement in quality of new malting barley varieties in Australia by barley breeders is influenced by the requirements set out by the Malting and Brewing Industry Barley Technical Committee (MBIBTC). The MBIBTC classification system has been developed in association with the malting and brewing industry. It is based on five quality parameters, namely malt extract, diastatic power (DP), viscosity and modification (Kolbach Index, KI) (MBIBTC, 1995). These important parameters depend on grain having the correct balance of components, including protein, starch and β-glucan. For example, although low protein together with high starch concentration is desirable for malting, very low protein (less than 8%) may cause difficulties in the brewing process (MBIBTC, 1995) with reduced levels of desirable foam proteins (Gromus, 1988). Because of the complex interrelationship between the various grain components researchers continue to try and determine the most important factors in both grain and malt contributing to final malting quality (MacLeod *et al.*, 1993; Allan *et al.*, 1995).

In addition, the stability of these malting quality parameters is important. As has been shown in Chapter 4, environmental effects, in particular high temperature exposure, have important effects on grain composition (see Section 4.3.3). In order to effectively select for stable malting quality characteristics in the extremely variable environments

found in many of the Australian cereal growing regions breeders require a clearer understanding of the stability of these quality characteristics.

The effects of high temperature have been investigated on a number of malting quality parameters, including malt extract (Eagles *et al.*, 1995; Dunn *et al.*, 1996; Savin *et al.*, 1997a,b), DP (Eagles *et al.*, 1995; Savin *et al.*, 1997a,b), β-glucan (Savin *et al.*, 1997a,b); and wort viscosity (Perez-Vendrell *et al.*, 1996). Such studies have shown that high temperatures during the grain filling period lead to both beneficial and detrimental effects on individual components of malting quality. For example, high temperatures have been associated with reduced starch accumulation (MacLeod and Duffus, 1988a; Savin and Nicolas, 1996) and elevated protein levels in barley (Savin and Nicolas, 1996). While this combination of attributes leads to reduced malt extract (Savin *et al.*, 1997b), high protein has been associated with increased DP in malt (Arends *et al.*, 1995), a potentially desirable malting quality outcome (Henry, 1990).

In order to identify genotypes with desirable heat-stable characters, it is important to understand how the components which contribute to malting quality are altered under these conditions. Harvest-ripe grain from Experiments 1-4 has been used for malting quality analysis in order to investigate the malting quality of grain which has been subjected to 5 days of elevated temperatures during mid grain filling. The changes in the accumulation of endosperm storage components and final grain composition have already been discussed in Chapter 4 (Sections 4.3.3 and 4.3.4).

7.2 **Experimental materials and methods**

7.2.1 **Analysis of harvest-ripe grain**

Starch, nitrogen (protein) and β-glucan concentrations were determined in samples of harvest-ripe whole grain from Experiments 1-4 (Chapter 4) using methods detailed in Sections 3.4.2.1, 3.4.2.2 and 3.4.2.3 respectively. Whole grains were ground to a fine powder using a mortar and pestle. Calculation of flour moisture (Section 3.3.4) allowed attributes to be expressed as percentage on a dry weight basis.

7.2.2 **Micromalting of grain**

Harvest-ripe control and heat treated grain was collected at the completion of Experiments 1-4 and micromalted in a Phoenix™ Automatic Micromalting System in

the Waite Barley and Malt Quality Evaluation Laboratory (WBMQEL), according to the procedure in Section 3.6.

7.2.3 **Malt analyses**

Malting loss (outlined in Section 3.6.1) was measured. Hot Water Extract (Section 3.7.1), DP and its major components, α- and β-amylase (Sections 3.7.2 and 3.7.3), and malt starch (Section 3.4.2.1), β-glucan (Section 3.7.4) and protein (Section 3.7.5) were determined for each sample. Viscosity (Section 3.7.7) and malt β-glucanase (Section 3.7.8) were also measured. β-glucan degradation was calculated as the difference between grain and malt β-glucan, expressed as a percentage of grain β-glucan (Stuart *et al.*, 1988).

7.2.4 **Statistical analysis**

Analysis of variance was carried out for all grain and malt quality attributes to compare treatments (control and heat treated) based on a block design with 4 replicates (experiments) and the significance of mean differences was established from f-probabilities.

7.3 **Results**

7.3.1 **Effects of high temperature on composition of control and heat treated grain**

In this study, the average grain dry weight of heat treated grain was significantly lower ($P<0.01$) than that of control grain (Table 7.1) as shown in Chapter 4 (Section 4.3.1). All harvest-ripe grain from Experiments1-4 was required to produce a 15 gm sample of grain for micromalting. Consequently, grains less than 2.25 mm in diameter, not normally regarded as meeting malting quality standards (MBIBTC, 1995), were included in the sample for malting quality analysis. The smaller grain came predominantly from heat treated plants.

Final grain weight was 20% lower in heat treated grain than in control grains. The endosperm storage component of final grain dry weight was reduced more than 6% in these grains despite an overall increase in grain protein of almost 4% (Table 7.1). Grain components, starch and protein, appeared to be closely correlated, so that the increase in protein (7%) which occurred following high temperature treatment was

balanced by an equivalent decrease in starch concentration (6%). Grain β-glucan was reduced by 23% in heat treated grain.

Table 7.1 Comparison of grain attributes of control and heat treated grain before and after micromalting. Significance of variation from control values shown as: **P<0.01, *P<0.05.

Treatment	Mean grain weight (mg)		% Starch		% Protein		% β-glucan	
	grain	malt	grain	malt	grain	malt	grain	malt
Control	47.4	42.2	58.4	59.6	14.0	13.7	4.45	1.87
Heat treated	37.9**	31.7*	49.1*	52.2*	17.8**	18.2*	3.43*	1.04*

7.3.2 **Effects of high temperature on malting quality of control and heat treated grain**

Malting loss in converting barley to malt (Hough *et al.*, 1971) was 11.0% for control grain but was higher in heat treated grain (16%). β-glucan concentration was reduced to a greater extent than starch (23% compared with 16%) following exposure to heat, while protein increased by 27%.

The greater reduction in dry weight in heat treated grain following malting also affected the concentration of endosperm constituents. Starch concentration, for example, increased following the malting of heat treated grain. Although an increase in starch was also observed in control grain, this component always remained higher than in heat treated grain (Table 7.1). Grain protein concentration, which increased in heat treated grain, remained high following malting.

Table 7.2 Comparison of malt quality characters for control and heat treated grain following micromalting. Significance of variation from control values shown as: ***P<0.001, **P<0.01; ns, not significant.

Treatment	Hot Water Extract	DP	α-amylase	β-amylase	Viscosity	Malt β-glucanase
Control	80.08	671	160	510	2.08	409
Heat treated	74.16**	714^{ns}	136^{ns}	578^{ns}	1.77^{ns}	275***

Malt extract was significantly lower in heat treated grain than in control grain. The 13% increase in β-amylase provided the greatest contribution to the overall increase in DP (not statistically significant) following high temperature exposure while α-amylase activity was reduced. β-glucan degradation was increased significantly in heat treated grain (70% compared with 58% in control grain), despite reduced levels of malt β-glucanase detected in heat treated compared with control malt. The lower viscosity level in heat treated grain was associated with reduced β-glucan in grain and malt.

7.4 **Discussion**

A number of quality attributes of barley grain were modified significantly in plants exposed to high temperatures during mid grain filling. A reduction in starch and increase in protein content were accompanied by a decrease in β-glucan. While lower starch is likely to have contributed to the lower malt extract produced from heat treated grain, lower β-glucan has been associated with improved malting performance (Bourne and Wheeler, 1984; Smith *et al.*, 1987; Molino-Cano *et al.*, 1993).

Expression of endosperm constituents on the basis of concentration is commonly used for quality attributes within bulk grain samples (MBIBTC, 1995; WBMQEL, 1995). While it gives a measure of relative composition, it does not reveal variability of grain size nor differences in the contribution of non-endosperm tissues (including husk and embryo) to final grain dry weight. Significantly lower final grain weight has been recorded following high temperature treatment in this study (Chapter 4; Section 4.3.1) and screenings (grains < 2.25mm) in excess of 13% have been recorded in similar studies where they reflected reductions in dry grain weight of between 10 and 35% following high temperature exposure (Savin *et al.*, 1997a,b). Increased embryo growth

following high temperatures has also been reported in Chapter 6 (Section 6.3.1.1) with significant divergence from the control development pattern observed from 27 daa (Fig 6.1).

In this study the main storage components of the endosperm, namely starch, protein and β-glucan together comprised a greater proportion of control grains (77%) than heat treated grains (70%). Malting loss which has been attributed mainly to the growth and metabolism of the embryo (Hough *et al.*, 1971), is generally expected to be around 10-11% of total grain dry weight (Smith and Gill, 1986). While control grain recorded a malting loss similar to this predicted value, the higher malting loss recorded in heat treated grain could not be attributed entirely to the loss of endosperm storage components (Table 7.1). Despite not screening grain samples, malt extract values of between 74 and 80% were comparable with those in other studies under controlled environment conditions (Macnicol *et al.*, 1993) and with malt extracts obtained following micromalting of field grown barley (WBMQEL, 1995). Thus, while it is possible that small grains may give good yields for malt extract, in a competitive market overseas buyers place great emphasis on grain size specifications, along with consistency of grain protein levels (Anon., 1995).

Grain protein concentrations in this experiment were generally high and outside the range specified by the MBIBTC (MBIBTC, 1995) and normally accepted for malting quality barley (Henry, 1990). A reduction in starch was accompanied by an increase in protein concentration so that the inverse relation between grain starch and protein percentage (Henry, 1990; Jenner *et al.*, 1990) was sustained in grain subjected to high temperature treatment. The resultant lower malt extract in this study was in line with the results of a number of other workers (Morgan and Riggs, 1981; Coles *et al.*, 1991; Allan *et al.*, 1995; Eagles *et al.*, 1995), who reported negative correlations with protein concentration. A low protein content, together with increased starch level, has been associated with high malt extract (Smith *et al.*, 1987).

β-glucan has complex effects on malting quality since its degradation during malting affects the diffusion of catabolic enzymes through the barley endosperm, and thus modification during malting. It shows both genetic and environmental variation (Gill *et al.*, 1982; Smith *et al.*, 1987; Stuart *et al.*, 1988; Perez-Vendrell *et al.*, 1996).

Interactions between β-glucan and other major grain components, such as protein (Palmer, 1971; Forrest and Wainwright, 1977), have important consequences for final malting quality (Henry, 1990). Consequently, there are limitations to the value of this grain attribute in predicting malting quality (MacLeod *et al.*, 1993). While total β-glucan levels in grain are not significantly correlated with malt extract, there is evidence for a relationship between malt β-glucan and malt extract (Bourne and Wheeler, 1984; Stuart *et al.*, 1988), which is reflected in the correlation between malt extract and β-glucan loss (Stuart *et al.*, 1988).

High temperature treatment made β-glucan more accessible to degradation during malting than β-glucan in control grains, a finding also reported by Savin *et al.* (1997a). Structural observations reported in Chapter 6 revealed that heat treated grain had a more open endosperm structure (see Fig 6.4c) and showed greater loss of cell wall fluorescence following malting than control grain (compare Figs 6.7a,b). This increased loss of cell wall material in malt from heat treated grain occurred despite a lower activity of malt β-glucanase than in control grain. It seems likely therefore that the alterations to overall grain texture (see Section 6. 3.1.3) and/or to endosperm cell wall structure played a role in the improved efficiency of β-glucan degradation.

High temperatures contributed to low levels of viscosity which are preferred for malting and brewing to avoid filtration problems during processing (MBIBTC, 1995). Although viscosity measurements are not necessarily correlated to β-glucan content (Ullrich *et al.*, 1986), in this instance lower viscosity corresponded to the lower β-glucan levels in heat treated malt compared with control malt. Exposure of grain to high temperatures during grain growth may therefore reduce filtration problems associated with the use of the resultant malt in a brewhouse.

Elevated DP in malt from heat treated grain was associated with increased protein concentration. Although a similar observation was made by Eagles *et al.* (1995), this is not always the case (Savin *et al.*, 1997a). Although in this study there was little difference in final grain nitrogen levels (Chapter 4, Section 4.3.3), heat treated grain appeared to synthesise β-amylase more rapidly during development than control grain (Chapter 5, Fig 5.11a). The complexity of the relationship between protein and DP has been attributed to the varying contributions made to DP by the starch degrading enzymes

which determine its activity (Arends *et al.*, 1995), although β-amylase has been found to make the greatest contribution to DP (Swanston, 1980). The elevated β-amylase activity recorded in malt following high temperature treatment was accompanied by reduced α-amylase activity (Table 7.2). This illustrates the weakness in any correlation between α- and β-amylase (Arends *et al.*, 1995), in part highlighting the different stages of development at which the enzymes are produced. It is possible that the effects of temperature on intermolecular disulphide bonds formed during the deposition of β-amylase (Ciaffi *et al.*, 1996) may have contributed to the increased activity recorded in heat treated malt by altering its activation from the bound state during germination (Evans *et al.*, 1993).

7.5 **Conclusions**

High temperatures during grain filling were associated with alterations to the composition of harvest ripe grain which led to both good and detrimental changes in the malting quality of that grain. Although DP, β-amylase, β-glucan and viscosity were improved following high temperatures (i.e. DP and β-amylase were increased, β-glucan and viscosity reduced), heat treatment was associated with inferior malt extract, a reduced quantity of starch and an increased protein concentration. The overall reduction in the amount of 'maltable' grain due to the smaller final grain size as a result of high temperature exposure, represents one of the most significant effects of this treatment.

Chapter 8

Comparison of the effects of a period of heat during grain filling on the accumulation of endosperm storage products and the grain and malt quality characteristics of three Australian malting barleys

8.1 Introduction

The malting potential of barley depends on the biochemical attributes of the grain endosperm, which in turn are influenced by the interaction between genotype and the environment (Eagles *et al.*, 1995; Logue *et al.*, 1994; MacLeod and Wallwork, 1991; Molino-Cano *et al.*, 1991, 1993; Perez-Vendrell *et al.*, 1996; Savin *et al.*, 1997a; Stuart *et al.*, 1988). Malting quality may vary on a regional basis due to differences in growing conditions, and from one season to the next (Table 8.1). This variation has a significant impact on the supply of high quality barley for malt to both local and overseas customers. For example, the 1994/95 season in South Australia was characterised by poor grain size and higher than average protein. This coincided with a harsh spring finish (high temperatures and low rainfall), so that maltsters were faced with trying to meet customers' specifications utilising barley of lower than normal quality (Bussell, pers.comm.).

Table 8.1 Area sown to barley and receivals in malting grade barley in South Australia (Australian Barley Board Report, 1994/95).

Season	Area sown (10^6 hectares)	Malting grade (10^3 tonnes)	Total (10^3 tonnes)
1990/91	2.51	225	1 360
1991/92	2.70	497	1 761
1992/93	2.96	473	1 762
1993/94	3.42	687	2 113
1994/95	2.50	239	609

In recent years, specifications for malting quality have become increasingly stringent with increasing quality consciousness in the market-place (Gill, pers.comm.). At the same time, the quality of malting barleys in Australia has generally fallen behind key international competitors (Anon., 1995). Stability of malting quality traits over successive, often variable seasons is of paramount importance in order to sustain a successful malting barley export industry with consistent production of high quality grain. An understanding of the underlying mechanisms of environmental effects on malting quality is therefore important in order to optimise yield and quality of current varieties and to assist breeders in improving the malting quality of new barley varieties.

The Australian cereal production areas encompass a wide climatic range (Nix, 1975). Based on the region for which barley varieties have been developed for cultivation, individual malting barley varieties receive an annual malting quality rating by the Malting and Brewing Industry Barley Technical Committee (MBIBTC, 1995). It is possible for some varieties to be successfully cultivated in climatic regions at the extremes of their adaptation (Eagles *et al.*, 1995) and attain malting grade, while others do not achieve malting grade even in the region for which they have been developed. Several studies have been made on the effects of environmental growth conditions on malting quality in Australian malting barleys grown under field conditions (Stuart *et al.*, 1988; Logue *et al.*, 1994; Eagles *et al.*, 1995; Dunn *et al.*, 1996). As a result, a number of malting quality variables have been identified which are influenced by environmental conditions such as temperature, rainfall and soil type (Logue *et al.*, 1994). These variables include protein, enzyme levels (specifically diastatic power), β-glucan and malt β-glucanase potential. In addition, correlation studies have highlighted the impact of agronomic and management factors (such as sowing date and plant nutrient levels) on malting quality. A number of developmental responses to environmental conditions have also been found to be dependent on variety (Long, pers.comm.).

Schooner is currently considered to be the most widely adapted, consistently performing malting quality variety in South Australia (South Australian Field Crops Evaluation Program, 1995). In this study a reduction in starch accumulation has been found in Schooner grains in response to exposure to high ambient temperatures (Chapter 4, Section 4.3.3) together with an increase in grain protein concentration (Chapter 7, Table 7.1). Similar changes in these grain components were reported by Savin and Nicolas

(1996). Changes to β-glucan synthesis (Chapter 4, Section 4.3.3.3) have also been reported in this study. These grain components all play important roles in determining malt quality (Palmer, 1990; MacGregor and Fincher, 1993). While there is evidence that some varieties of barley are more tolerant to high temperatures than others (Savin and Nicolas, 1996), the stability of quality and sustained yield are nevertheless important to all successful malting barley varieties.

The objective of this study was therefore to compare the development of grain and malting quality attributes of Schooner with two other malting barley cultivars in relation to the response to a period of high temperature during mid grain filling. The Victorian barley variety, Arapiles, and WI-2875*22, a potential new South Australian malting variety, were chosen for this study. Developed for a cool, relatively moist environment, Arapiles has responded differently to Schooner under a range of environmental conditions (Long, pers.comm.). WI-2875 shares significant genetic similarity to Schooner (Section 3.1) but shows improved malting quality for several attributes, notably higher DP and lower malt β-glucan (Waite Barley and Malt Quality Evaluation Laboratory (WBMQEL), 1994). In addition, both Arapiles and WI-2875 have consistently outperformed Schooner with respect to protein (lower) and DP (higher) over the past 2 seasons while maintaining comparable malt extract potential (Table 8.2; WBMQEL, 1994, 1995).

Table 8.2 Summary of results from 1995 Secondary Trial Report (WBMQEL, 1995) and South Australian Field Crops Evaluation Program (SAFCEP, 1995). Mean of 8 sites. Results expressed as a percentage of Schooner.

	Yield kg/ha	**% Extract**	**% Grain protein**	**DP**	**% Malt protein**	**% Malt β-glucan**
Schooner	100	100	100	100	100	100
Arapiles	98	99	96	105	99	171
WI-2875*22	107	100	97	123	99	59

8.2 Experimental materials and methods

8.2.1 Experimental design

Schooner, Arapiles and WI-2875*22 plants were grown in a constant environment room (CER) from germination to maturity. Seed was planted directly into pots (20 per pot) and CER conditions maintained at 18/13°C, 10h day, for the first 5 weeks of plant growth. Control conditions were then set at 21/16°C, 14h day, for the remainder of the development period until harvest ripeness. Fertiliser was applied fortnightly (see Chapter 3; Materials and Methods) until anthesis and regular watering throughout the experiment ensured that plants were not subjected to water deficit. Four of the 7 pots comprising the experimental unit for each variety were removed to an adjacent, identical CER for high temperature treatment between 16-20 daa (details appear in Chapter 3, Section 3.2.3). The temperature profile for this room during the period 16-21 daa has been reproduced in Fig 8.1.

Details of ear tagging, sampling schedules, endosperm dissection, and measurement of fresh weight, dry weight and grain moisture appear in Chapter 3, Materials and Methods. Only main stem ears were used, each tagged at anthesis (Day 0). Triplicate samples were collected 15 daa and then 18, 21, 24, 27, 30, 35 and 42 daa. Sampling took place during the first 3-4 hours of daylight on each prescribed day. Grain was harvested at the termination of the experiment for quality analysis (micromalting and malt quality analysis) by the WBMQEL. Micromalting took place 6 weeks after harvest.

8.2.2 Grain filling characteristics

Accumulation of endosperm fresh and dry weights were determined over each 3 day period and the maximum rate recorded as the greatest increase in fresh or dry matter obtained over successive samplings.

The duration of grain filling, the time between anthesis and physiological maturity, was derived from plots of endosperm dry weight and endosperm moisture expressed on the basis of thermal time (see Section 4.2.2).

8.2.3 Analysis of endosperm storage components

Analyses of endosperm starch, nitrogen and β-glucan were carried out using methods described in Chapter 3, on the dried endosperms used to determine dry weights during

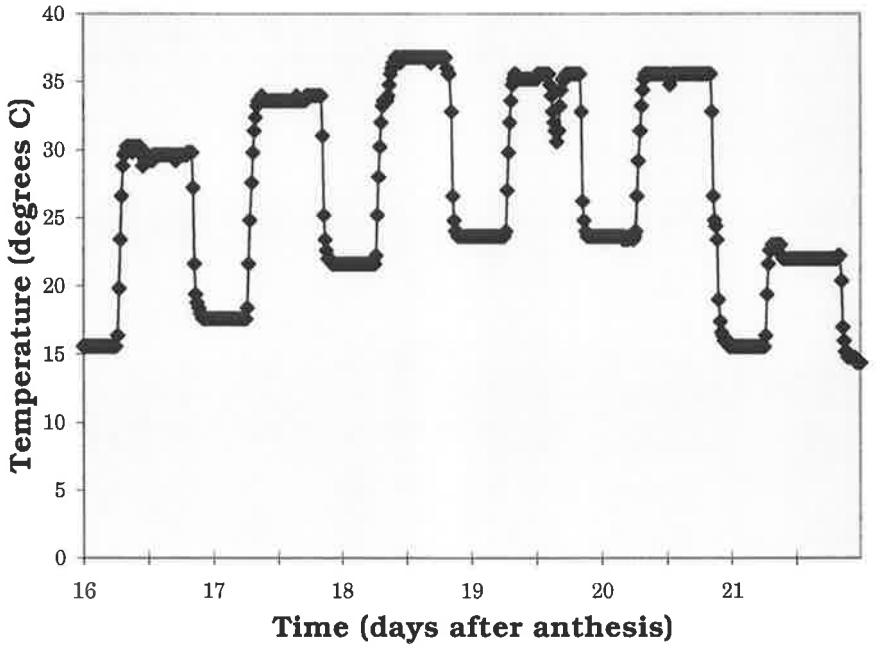


Fig 8.1 Temperature profile for constant environment room during heat treatment period, 16-21 daa.

Note: thermocouple output was approximately 2°C higher than that measured on a shielded thermometer at the level of the ear.

grain filling. This material was ground to a fine powder using a mortar and pestle. Moisture measurements made on samples of heat treated and control flour were used to express endosperm component data on a dry weight basis.

8.2.4 **Malting quality analysis**

Micromalting and subsequent malting quality analyses were carried out in the WBMQEL using the micromalting schedule and methods described in Chapter 3. Malt was analysed for malt extract, nitrogen (also presented as malt protein), total starch, β-glucan, DP, α- and β-amylase, and viscosity.

8.2.5 **Statistical analysis**

Analysis of variance was applied to experimental results to determine the differences between control and heat treated grain, based on the interaction between time (after anthesis) and treatment. Data were analysed separately for each variety and also collectively. Error bars (standard errors of means) have been plotted for individual data points (mean of 3 replicates). The Tukey method was used to provide pairwise comparisons of individual means (Zar, 1984).

8.3 **Results**

8.3.1 **Effect of high temperature on final grain weight between varieties**

Differences were observed between varieties in the number of flowers produced per ear (data not shown) and therefore in the potential number of grains per ear. Fertility was uniformly high within each variety, with rarely more than 1-2 empty glumes per ear. Schooner plants produced 29.3 grains per main stem ear, on average. Arapiles and WI-2875*22 produced lower average numbers of 24.6 and 26.0 grains per ear, respectively.

Under control conditions, final grain weights for Schooner and WI-2875*22 were similar, at 51.1 and 51.5 mg respectively (mean of 50 grains) (Table 8.3). Arapiles grains were smaller (46 mg). Following exposure to high temperature, all 3 varieties suffered significant reductions in final grain weight (Table 8.3). On a whole grain basis Arapiles appeared most sensitive to exposure to high temperature and suffered the greatest yield loss. The apparent discrepancy between the sensitivity rankings of the whole grain and the endosperm to high temperature exposure was due to differences between varieties in the effects of heat on non-endosperm grain components, estimated

by difference (Table 8.3). In Arapiles, both non-endosperm and endosperm dry matter were reduced at high temperature, whereas in the other two varieties reduction in endosperm dry matter was partly offset by an increase in non-endosperm dry matter. The proportion of the grain's non endosperm dry matter in Schooner and WI-2875*22 increased in heat treated grain but there was little effect on dry matter distribution in Arapiles grain.

All subsequent grain filling data have been derived and presented on a per endosperm basis to show the specific response of this grain storage organ to the effects of high temperature exposure during grain development.

Table 8.3 Final whole grain and endosperm dry weights in barley varieties, Schooner, Arapiles and WI-2875*22. Figures in brackets show percent reduction in dry weight due to high temperature treatment.

Variety		Whole grain dry wt (mg)	Endosperm dry wt (mg)	Grain-endosperm difference (mg)	Grain-endosperm as % whole grain
Schooner	C	51.52	47.15	4.37	8.5
	H	44.82 (13.0)	38.66 (18.0)	6.16 (-41.0)	13.7
Arapiles	C	45.99	40.15	5.84	12.7
	H	35.09 (23.7)	31.88 (20.6)	3.21 (45.0)	9.1
WI-2875*22	C	51.15	48.55	2.6	5.1
	H	43.52 (14.9)	37.63 (22.5)	5.89 (-126.5)	13.5

8.3.2 **Effect of high temperature exposure on the accumulation of endosperm dry weight and moisture between varieties**

Statistical analysis of individual barley varieties revealed differences in response to exposure to high temperatures during grain filling (Table 8.4). Endosperm moisture was not affected significantly by high temperature treatment for any of the varieties individually, nor when data were analysed collectively. Individual varieties responded differently however, with respect to the accumulation of dry weight, starch and β-glucan, but when analysed collectively, dry weight, starch and β-glucan accumulation was significantly different in heat treated plants from that in control plants. Nitrogen accumulation remained unaffected by increased ambient temperatures.

Table 8.4 Results of analysis of variance comparing grain filling patterns between control and heat treated plants for each of 3 barley varieties and the mean of all three varieties. ***significantly different from control P<0.001; **P<0.01; P<0.05; ns, not significant.

	Attribute (mg per endosperm)					
Variety	**Fresh wt**	**Dry wt**	**Moisture**	**Starch**	**Nitrogen**	**β-glucan**
Schooner	**	***	ns	**	ns	*
Arapiles	**	*	ns	ns	ns	ns
				(0.053)		(0.15)
WI-2875*22	ns	ns	ns	ns	ns	ns
		(0.06)		(0.085)		(0.16)
Mean	**	***	ns	***	ns	***

The pattern of accumulation of endosperm dry matter (Fig 8.2a) under control conditions appeared similar for Schooner and WI-2875*22, with a steady increase until approximately 30 daa, slowing until 42 daa, and resulting in similar dry weight values for both varieties. The accumulation of dry matter by Arapiles endosperms was slower than those of the other varieties until 24 daa. The accumulation of dry matter was very rapid from 24 daa in Arapiles grain, with 53.4% of total endosperm dry weight accumulated in just 6 days. As a consequence, Arapiles recorded a considerably higher maximum rate (3.87 mg per endosperm per day) than either Schooner or WI-2875*22 under control conditions (2.59 and 3.03 mg per endosperm per day, respectively). The rapid dry matter accumulation of Arapiles did not compensate for the initial delay in grain filling, so that by 42 daa these endosperms were smaller than those of both Schooner and WI-2875*22.

Little difference was observed in grain filling rates between control and heat treated grains of the barley variety, WI-2875*22 during and following the high temperature period (Table 8.5). Following an initial rise under moderate temperatures, the period at high temperature (35°C) caused the grain filling rates to fall in Schooner. Arapiles also responded with an increased rate of dry matter deposition under moderate temperatures, but the maximum rate of dry matter accumulation (2.79 mg per endosperm per day) was recorded when the plants were being exposed to high temperatures (35°C). As a consequence of this altered pattern of endosperm dry matter accumulation, developing

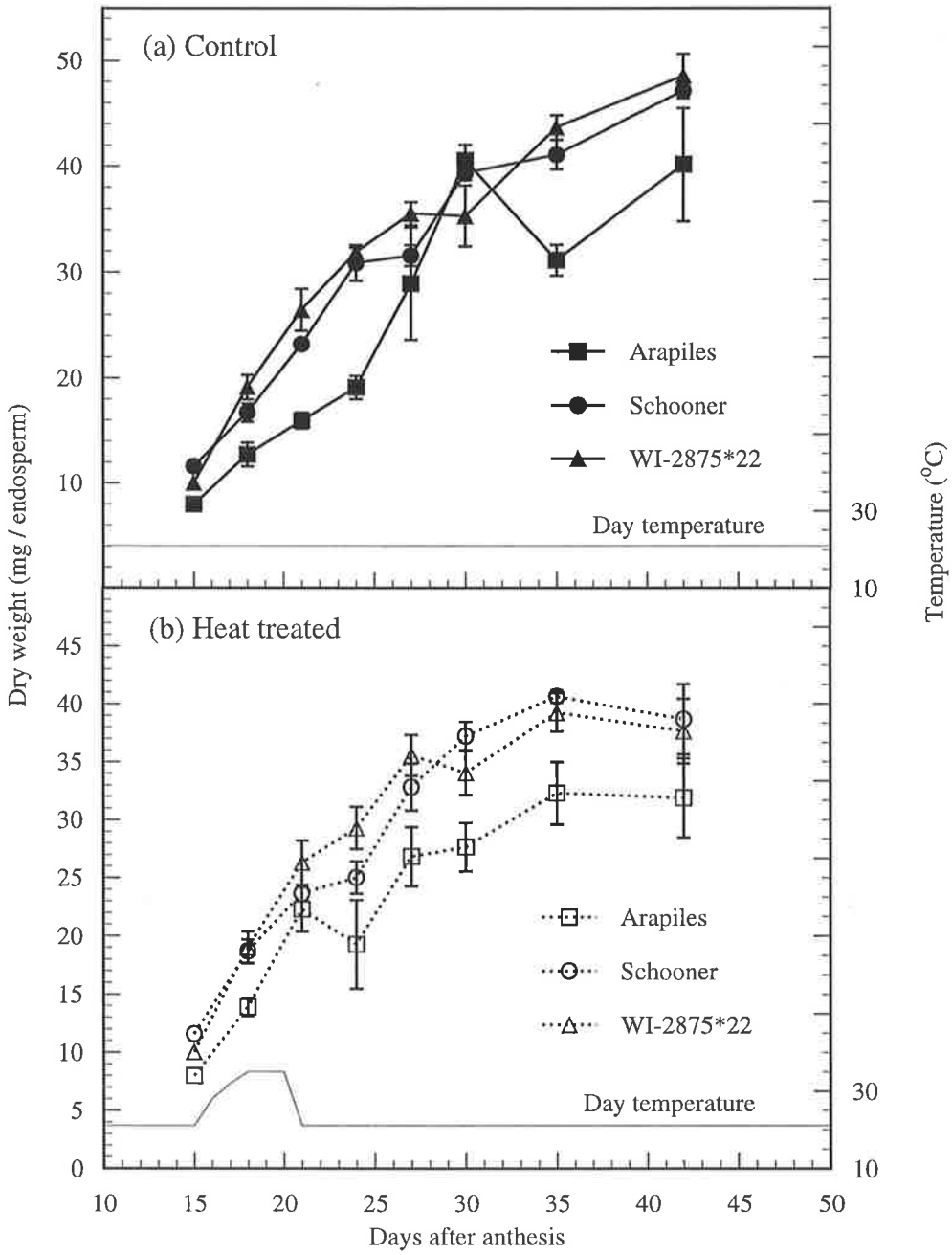


Fig 8.2 Accumulation of endosperm dry weight under control and high temperature conditions during development for barley varieties, Schooner, Arapiles and WI-2875*22.

endosperms of Arapiles barley were not significantly different (in terms of dry weight) from either Schooner or WI-2875*22 at 21 daa (Fig 8.2b). After a return to cooler conditions on 21 daa there was an abrupt decrease in the rate of accumulation of dry matter by Arapiles (Table 8.5), and these endosperms were smaller than those of the other varieties from 24 daa (Fig 8.2b). The maximum rate of dry weight accumulation in Schooner and WI-2875*22, although similar between treatments (2.6 and 3.0 mg per endosperm per day), occurred earlier in heat treated than in control grain.

derived data : not statistically analysed.

8.3.3 **Starch accumulation**

Under control conditions the pattern of accumulation of starch closely reflected the accumulation of endosperm dry weight (compare Figs 8.2 and 8.3). Schooner and WI-2875*22 both accumulated starch in a similar pattern throughout development. Under control conditions WI-2875*22 endosperm contained a slightly higher amount of starch (7%) than Schooner. As with dry weight accumulation, synthesis of starch occurred slowly in Arapiles until 24 daa; 57% of the total endosperm starch was then synthesised in the following 6 days and the rate of starch accumulation peaked at 2.98 mg per endosperm per day between 24 and 27 daa, exceeding the maximum rates for both of the other varieties. The average rates (21-30 daa) in Table 8.6 also conform to this pattern.

Under moderate temperatures the rate of starch synthesis increased for each variety between 15 and 18 daa (Table 8.6). Three days of high temperature (35°C) however proved deleterious to both Schooner and WI-2875*22 but provided further stimulation for starch synthesis in Arapiles. This variety recorded its maximum rate of starch accumulation (1.76 mg per endosperm per day) during this high temperature period. The rate of starch synthesis decreased in Arapiles (Fig 8.3) from 24 daa, and from 27 until 42 daa Arapiles grain contained less starch per endosperm than either Schooner or WI-2875*22.

8.3.4 **Nitrogen accumulation**

Despite a brief delay in Arapiles between 15 and 18 daa, control grain of all varieties accumulated nitrogen at a relatively constant rate, until 27 daa (Fig 8.4a). Similar quantities of endosperm nitrogen were present 42 daa in all three varieties.

Table 8.5 Rate of **endosperm grain filling** under control and heat treated conditions for Schooner, Arapiles and WI-2875*22 during the heating period (16-20 daa) and the cooler recovery period (21-30 daa), expressed as mg dry weight per endosperm per day.

Days after	**Schooner**		**Arapiles**		**WI-2875*22**	
anthesis	control	heated	control	heated	control	heated
15-18	1.70	2.36	1.58	1.97	3.03	2.99
18-21	2.17	1.65	1.08	2.79	2.44	2.42
21-30 (ave)	1.79	1.51	2.73	0.60	0.98	0.86

Table 8.6 Rate of accumulation of **starch** under control and heat treated conditions for Schooner, Arapiles and WI-2875*22 during the heating period (16-20 daa) and the cooler recovery period (21-30 daa), expressed as mg starch per endosperm per day.

Days after	**Schooner**		**Arapiles**		**WI-2875*22**	
anthesis	control	heated	control	heated	control	heated
15-18	1.13	1.58	1.11	1.42	2.26	2.42
18-21	1.58	0.98	0.28	1.76	1.71	1.66
21-30 (ave)	1.13	0.94	1.78	0.16	0.49	0.43

Table 8.7 Rate of accumulation of **nitrogen** under control and heat treated conditions for Schooner, Arapiles and WI-2875*22 during the heating period (16-20 daa) and the cooler recovery period (21-30 daa), expressed as mg nitrogen per endosperm per day.

Days after	**Schooner**		**Arapiles**		**WI-2875*22**	
anthesis	control	heated	control	heated	control	heated
15-18	0.06	0.06	0.02	0.05	0.06	0.06
18-21	0.02	0.06	0.06	0.04	0.05	0.07
21-30 (ave)	0.03	0.03	0.06	0.05	0.01	0.01

Table 8.8 Rate of accumulation of **β-glucan** under control and heat treated conditions for Schooner, Arapiles and WI-2875*22 during the heating period (16-20 daa) and the cooler recovery period (21-30 daa), expressed as mg β-glucan per endosperm per day.

Days after	**Schooner**		**Arapiles**		**WI-2875*22**	
anthesis	control	heated	control	heated	control	heated
15-18	0.06	0.09	0.04	0.14	0.09	0.09
18-21	0.09	0.12	0.08	0.11	0.11	0.14
21-30 (ave)	0.12	0.08	0.14	0.07	0.09	0.07

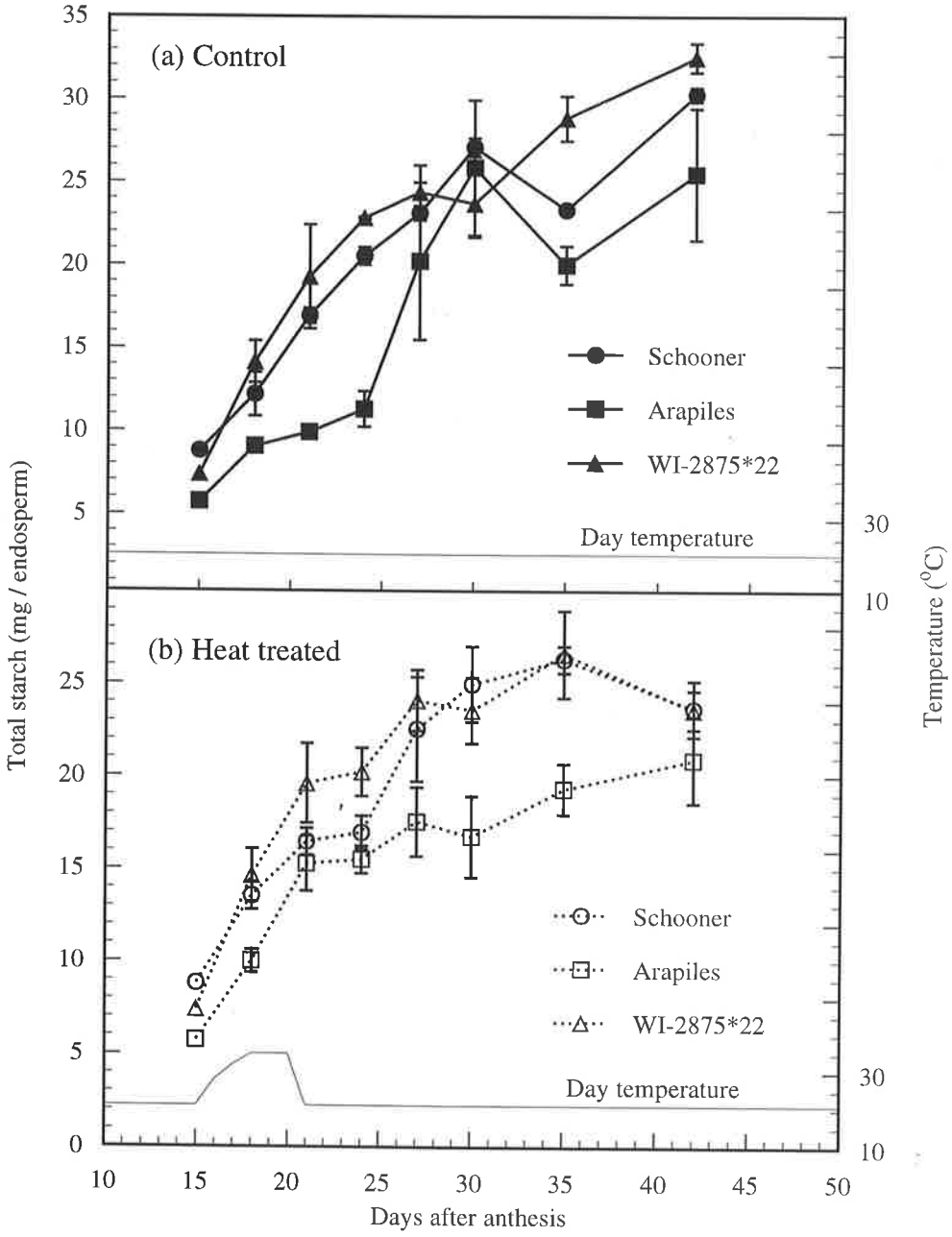


Fig 8.3 Accumulation of endosperm starch under control and high temperature conditions during development for barley varieties, Schooner, Arapiles and WI-2875*22.

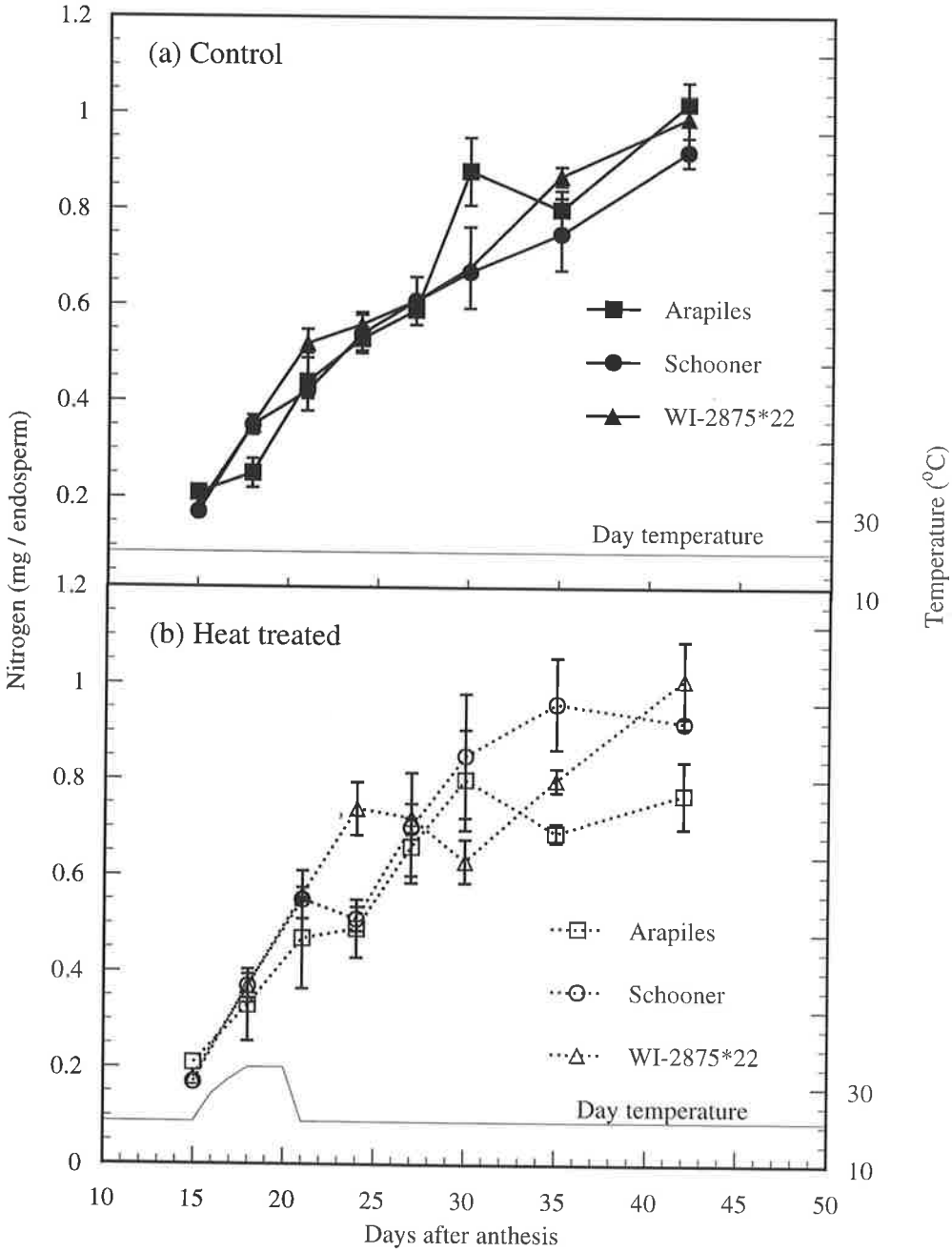


Fig 8.4 Accumulation of endosperm nitrogen under control and high temperature conditions during development for barley varieties, Schooner, Arapiles and WI-2875*22.

Moderately high temperatures, between 15 and 18 daa did not alter the rate of accumulation of nitrogen in Schooner or WI-2875*22 and rates remained unaffected during sustained high temperatures between 18 and 21 daa (Table 8.7). Arapiles, however, showed an immediate stimulation of nitrogen accumulation during exposure to moderately high temperatures. In contrast to Schooner and WI-2875*22, high temperatures in Arapiles resulted in a depression of nitrogen accumulation between 18 and 24 daa and despite increased nitrogen deposition between 24 and 30 daa, these grains contained apparently less nitrogen than the other two varieties from 35 daa (Fig 8.4b).

8.3.5 β-glucan accumulation

The rate of deposition of β-glucan increased for all varieties between 15 and 24 daa and continued until 42 daa under control conditions (Fig 8.5a). Arapiles recorded the highest rate of accumulation of β-glucan (0.23 mg per endosperm per day) between 24 and 27 daa and accumulated 45% of this component in just 6 days from 24-30 daa.

Elevated temperatures stimulated the deposition of β-glucan in all three varieties, although to a greater extent in Arapiles (Table 8.8). High temperatures did not appear to alter the pattern of β-glucan deposition until 35 daa, and although final endosperm levels were lower in the heated than in the control grains in all varieties, Arapiles was the only variety to show an apparent loss of β-glucan between 35 and 42 daa (Fig 8.5b) in the heated grains.

8.3.6 Effects of high temperature exposure on the final amount of accumulated endosperm storage products

The reduction in the synthesis of starch (Table 8.9) made the greatest contribution to reduced endosperm dry weight between treatments for all varieties. For WI-2875*22, the 27.5% reduction in starch synthesised following exposure to high temperature accounted for 82% of the corresponding reduction in endosperm dry matter accumulation and in Schooner this was 78%. Arapiles appeared less sensitive to high temperature than the other varieties. Although heat treated Arapiles endosperm contained 18% less starch per endosperm than control grain at 42 daa, there was an increase of 2% in starch concentration (Fig 8.6). This may be due in part to the reduction in endosperm nitrogen and β-glucan which was also recorded in Arapiles.

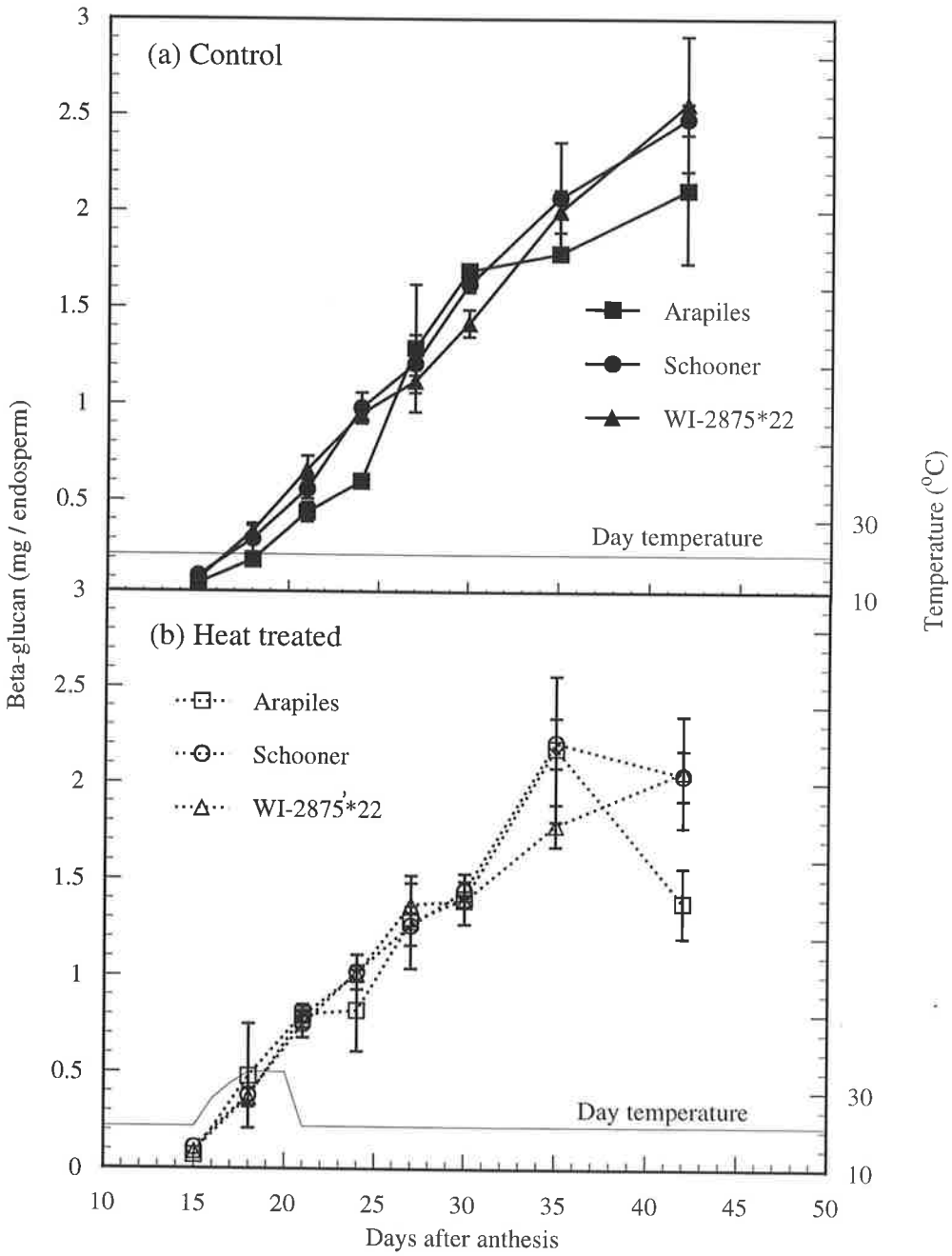


Fig 8.5 Accumulation of endosperm beta-glucan under control and high temperature conditions during development for barley varieties, Schooner, Arapiles and WI-2875*22.

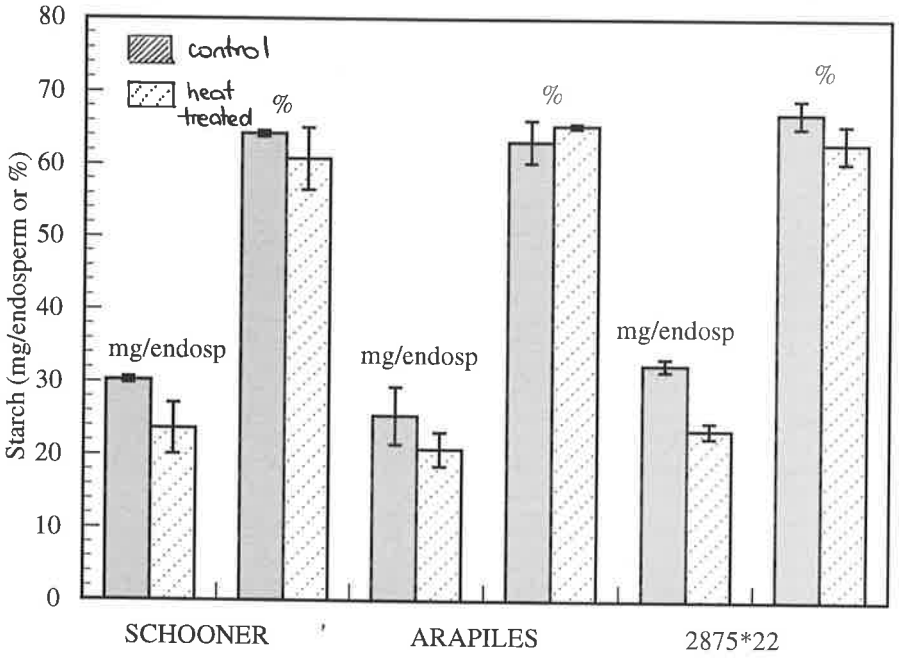


Fig 8.6 Differences in endosperm starch content between control and heat treated Schooner, Arapiles and WI-2875*22 endosperms 42 days after anthesis, expressed as mg/endosperm and % dry weight.

Table 8.9 Reduction in grain endosperm components (mg per endosperm) at 42 daa due to high temperature exposure during mid grain filling (expressed as percentage endosperm dry weight).

	% Reduction due to high temperature treatment		
Variety	**Starch**	**Nitrogen**	**β-glucan**
Schooner	21.9	-1.0	17.7
Arapiles	18.1	13.5	31.1
WI-2875*22	27.5	-2.0	19.5

The effect of high temperature on nitrogen accumulation (Table 8.9) varied, with Arapiles the only variety to record an overall decrease in endosperm nitrogen (from 0.89 to 0.77 mg per endosperm). When expressed on a percentage (endosperm) dry weight basis, all three varieties recorded an apparent increase in endosperm nitrogen (Fig 8.7). In Arapiles, the 9% increase in nitrogen within the heat treated endosperm occurred despite a 13% reduction in total dry weight of nitrogen. This was due to the reduced contribution to grain dry weight made by starch under high temperature conditions and the smaller grain size which resulted from this treatment.

High temperatures reduced the quantity of β-glucan in all 3 varieties (Fig 8.8). In Arapiles the amount of β-glucan, expressed as mg per endosperm, was reduced by 31%, far exceeding the 18 and 20% recorded by Schooner and WI-2875*22, respectively (Table 8.9).

8.3.7 **Effect of high temperature exposure on the duration of grain filling**

High temperature treatment resulted in the addition of 50 degree days (°Cd) relative to the control plants during the experimental period. Expressing endosperm development in thermal time (degree days above a base temperature of 7.1°C (Goyne *et al.*, 1996)) discriminated between changes in endosperm development due to high temperature treatment, and the effects of temperature on endosperm metabolism. Fig 8.9 shows the grain filling data for each variety plotted in this way. The arrows indicate relative dry matter of approximately 0.55gm/gm fresh weight, providing an indication of maximum dry weight (Schnyder and Baum, 1992).

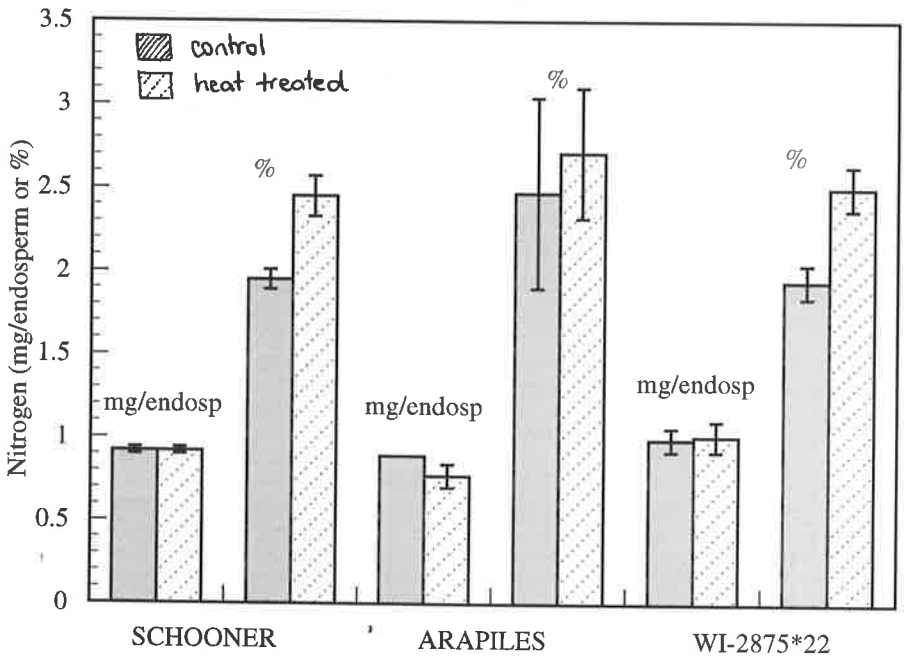


Fig 8.7 Differences in endosperm nitrogen content between control and heat treated Schooner, Arapiles and WI-2875*22 endosperms 42 days after anthesis, expressed as mg/endosperm and % dry weight.

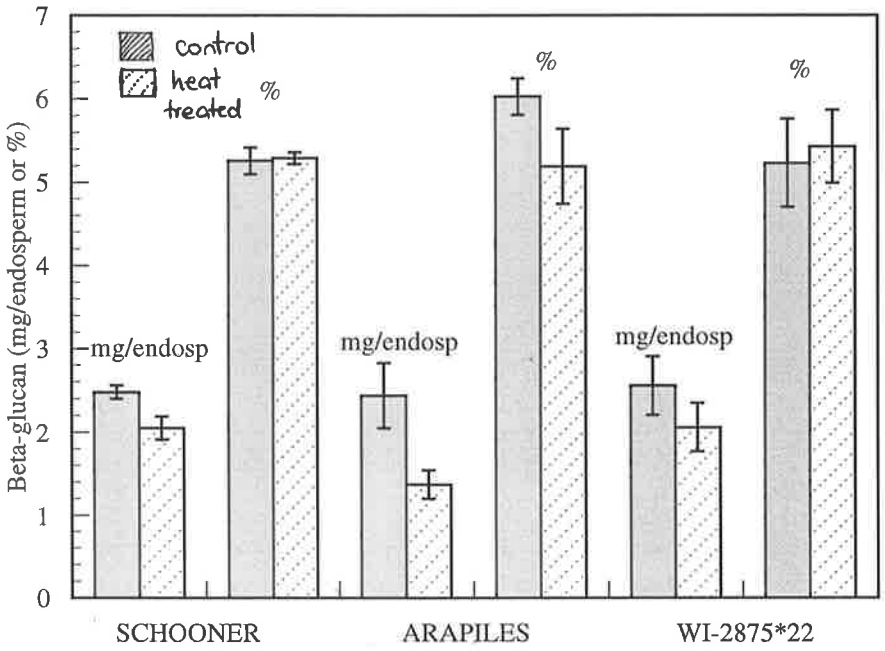


Fig 8.8 Differences in endosperm beta-glucan content between control and heat treated Schooner, Arapiles and WI-2875*22 endosperms 42 days after anthesis, expressed as mg/endosperm and % dry weight.

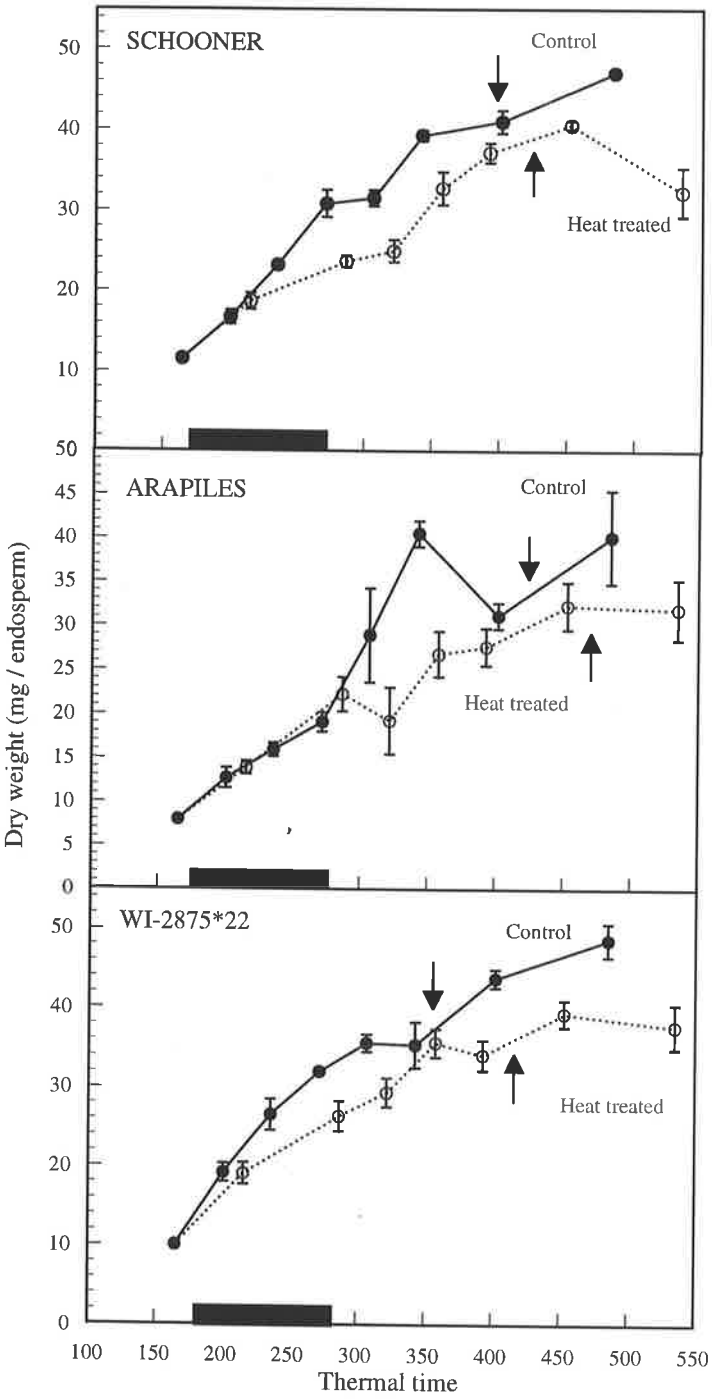


Fig 8.9 Accumulation of endosperm dry weight in Schooner, Arapiles and WI-2875*22, expressed in thermal time (degree days above base temp. of 7.1°C). Arrows indicate Relative Dry Matter of approximately 0.55gm per gm fresh weight (Schnyder and Baum, 1992). See section 8.3.7 for details.

The grain filling plot of heat treated Schooner endosperms did not diverge from that of control grain immediately following the imposition of moderately high temperature, but after exposure up to about 216°Cd after anthesis the curves diverged although the course of development appeared not to have been affected by heat. Control grain did not attain physiological maturity until 42 daa, while in heat treated grain this was achieved 35 daa (Fig 8.2). These points represent 485 and 453°Cd respectively, indicating that physiological maturity between treatments almost coincided in thermal time.

The grain filling profile of heat treated WI-2875*22 endosperms showed immediate divergence from control grain 18 daa. Even moderate temperatures affected the grain filling process in these grains, although the grain filling pattern remained similar to control grain. Physiological maturity was recorded 42 daa in control grain and 35 daa in heat treated grain, as for Schooner, and again coinciding in thermal time.

Deleterious effects of high temperature treatment were not apparent in Arapiles grain until 287°Cd after anthesis (21 daa, i.e. shortly after return to cooler conditions), and more than 100°Cd after the commencement of high temperature treatment. From this time the effects of the heat treatment became progressively more pronounced, although physiological maturity for the endosperms of both control and heat treated grain appeared to correspond to that of the other varieties.

From the data given in Fig 8.10 there were no indications of differences between treatments with respect to endosperm water content for either Schooner or Arapiles, and maximum endosperm water loss was observed between 35 and 42 daa in both control and heat treated grain of these varieties. Maximum water loss in endosperms of WI-2875*22 occurred between 30 and 35 daa under control conditions, while heat treated grain recorded the greatest loss of water between 35 and 42 daa.

Endosperm water content plotted on the basis of thermal time (Fig 8.11) revealed interesting differences between cultivars in response to heating. The high temperature treatment appeared to accelerate development in Arapiles so that until approximately 320°Cd (equivalent to 24 daa), these grains showed no change in the pattern of endosperm water content. Similarly, heat treated Schooner grains showed little divergence from the control pattern of endosperm water content until almost 400°Cd

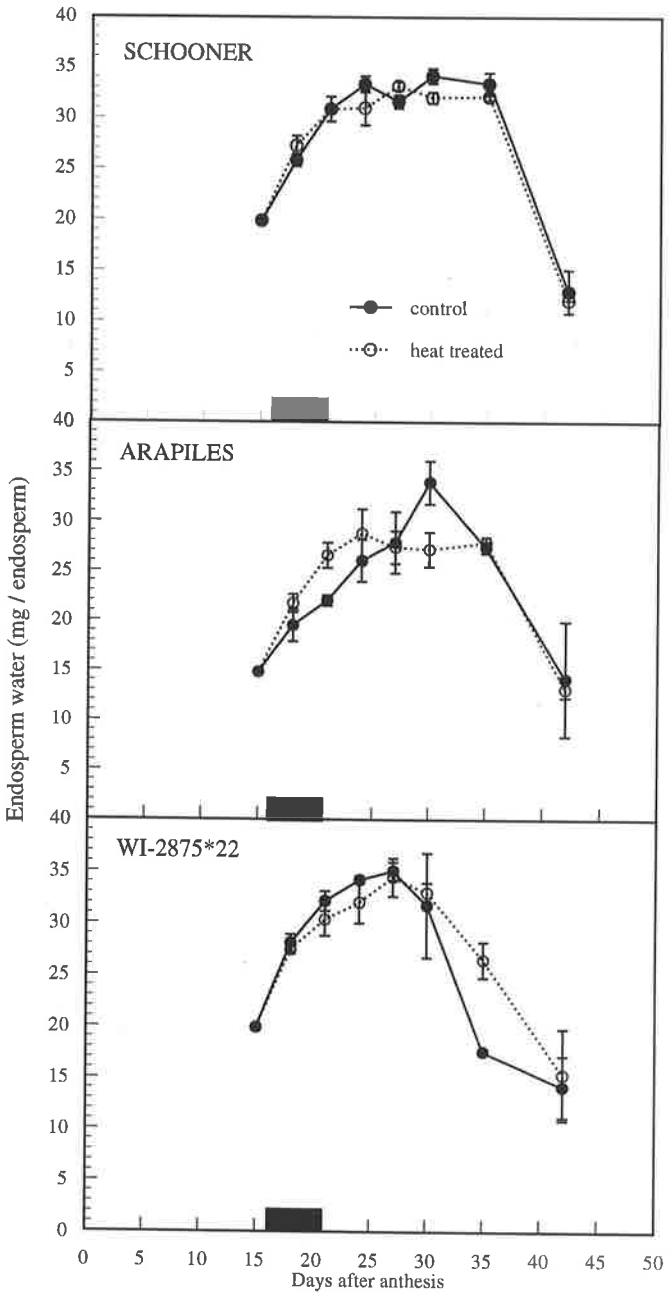


Fig 8.10 Changes in endosperm water content between control and heat treated grains for barley varieties, Schooner, Arapiles and WI-2875*22. Bar represents high temperature period.

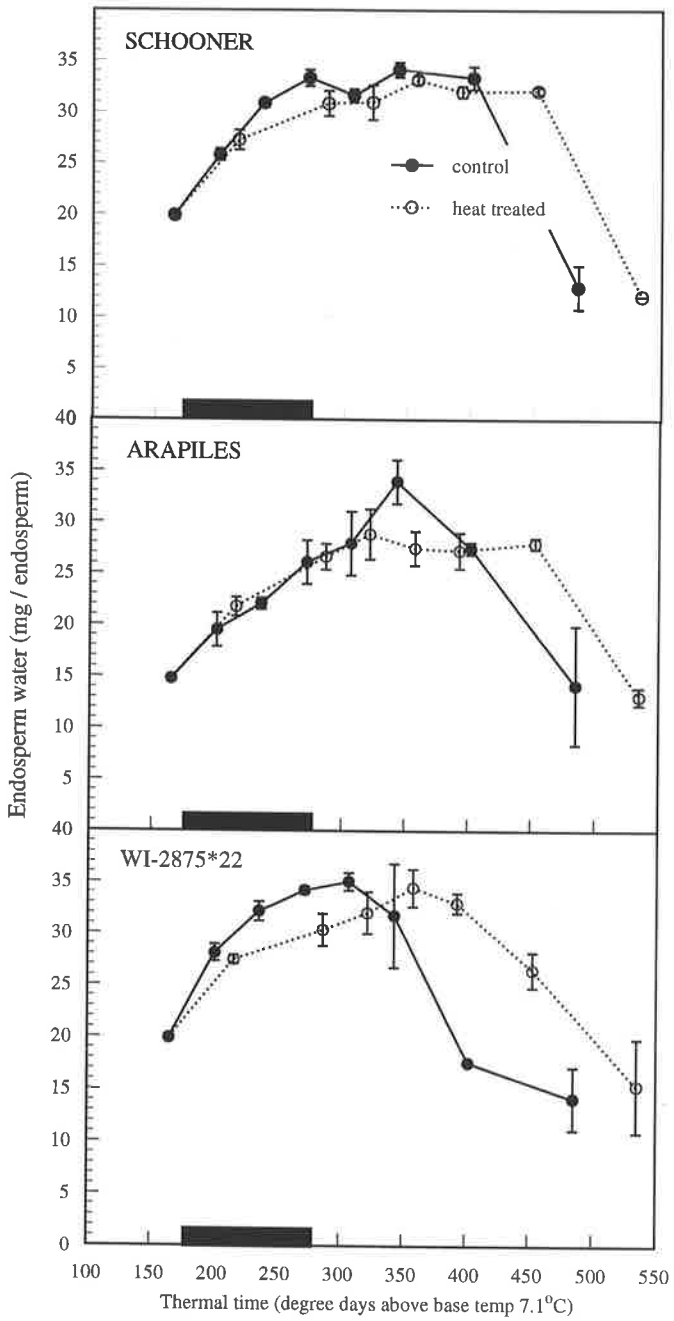


Fig 8.11 Changes in endosperm water content between control and heat treated grains for 3 barley varieties, plotted in thermal time

after anthesis. In contrast heat treated WI-2875*22 accumulated less endosperm water than control grain from 216°Cd after anthesis and this moisture plot continued to diverge from that of control grains throughout development. In all three varieties, the high temperature response was characterised by higher endosperm moisture content than control grains later in development.

8.3.8 **Effect of high temperature exposure on final whole grain composition**

The composition of harvest-ripe grain varied between varieties despite similarities in the percentage of storage material accumulated by all three varieties under both control and heat treated conditions (Table 8.10). Heat treated Schooner grain accumulated a greater proportion of starch and a reduced concentration of β-glucan than control grain. However, Schooner recorded the greatest increase in grain protein, calculated from nitrogen percentage using a factor of 6.25 (Tkachuk, 1969). Although WI-2875*22 suffered the greatest decrease in starch concentration and the greatest increase in β-glucan, it also recorded the lowest grain protein concentration of all varieties. β-glucan levels were consistently high for all varieties and rankings remained unchanged following high temperature exposure. Arapiles suffered the greatest reduction in whole grain dry weight due to exposure to high temperatures during grain filling (Table 8.3).

Table 8.10 Whole grain composition of harvest-ripe control and heat treated grain of Schooner, Arapiles and WI-2875*22. Nitrogen expressed as % protein by multiplying % nitrogen by a factor of 6.25 (Tkachuk, 1969).

Variety		Starch %	Nitrogen %	Protein %	β-glucan %	Total % (S,P,β-g)
Schooner	C	59.99	1.87	11.69	4.61	76.3
	H	***61.02***	***2.3***	***14.38***	***4.50***	***79.9***
Arapiles	C	59.34	2.17	13.56	4.90	77.8
	H	***57.03***	***2.3***	***14.38***	***5.20***	***76.6***
WI-2875*22	C	63.10	1.97	12.31	3.76	78.1
	H	***59.75***	***2.09***	***13.06***	***4.38***	***75.0***
Mean	C	60.81	2.00	12.81	4.42	77.4
	H	***59.27***	***2.23***	***13.90***	***4.69***	***77.2***

8.3.9 **Effect of high temperature treatment on final malting quality**

Elevated protein levels together with higher than usual grain β-glucan levels (WBMQEL Report, 1995) may have contributed to the lower than expected malt extract values recorded in this study. Malt extract values were similar within treatments and ranged from 74 - 78%. Protein levels following malting remained relatively high in all varieties (Table 8.11). The higher DP levels in Arapiles were associated with higher protein concentrations in grain which carried through to the malt. β-amylase, representing about 80% or more of DP in all varieties, was highest in Arapiles, both in control malt (87%) and heat treated malt (84%). While Schooner and Arapiles recorded little change in DP following high temperature treatment, this malting quality parameter was increased by 36% in WI-2875*22 (Table 8.11). This corresponded to an equivalent increase in malt β-amylase in this variety even though WI-2875*22 recorded a lower malt protein level than either Schooner or Arapiles.

The high malt β-glucan concentrations may have been a consequence of initial high grain β-glucan values (Table 8.10) and the ranking of varieties with respect to this attribute remained unchanged following malting. High temperature exposure resulted in increased β-glucan catabolism, with all varieties recording a consistent reduction (50%) of this substrate during malting, compared with control grain (Table 8.11). Viscosity values, which were also high in both control and heat treated malt, were greatest for Arapiles.

Table 8.11 Results of malting quality analysis of Schooner, Arapiles and WI-2875*22 grain following control and high temperature treatment. Abbreviations: HWE, Hot water extract; DP, diastatic power; α-amyl, α-amylase; β-amyl, β-amylase; visc, viscosity; FAN, free amino nitrogen; MBG, malt β-glucan. Units for DP, α- and β-amylase are μmoles of maltose equivalents released/min/gm dry weight.

Variety		Malt protein %	HWE %	DP	α-amyl	β-amyl	Visc cP	FAN mg/l	MBG %
Schooner	C	11.40	77.61	384	66	318	6.05	109	2.38
	H	***14.98***	***74.45***	***357***	***83***	***274***	***4.45***	***136***	***2.25***
Arapiles	C	13.90	77.29	525	69	456	8.25	88	3.07
	H	***15.25***	***73.56***	***545***	***87***	***458***	***5.23***	***120***	***2.58***
WI-2875*22	C	12.38	77.53	274	40	234	5.93	81	2.64
	H	***13.26***	***74.46***	***373***	***59***	***314***	***5.21***	***121***	***2.31***
Mean	C	12.56	77.48	394	58	336	6.74	93	2.70
	H	***14.50***	***74.16***	***425***	***76***	***349***	***4.96***	***126***	***2.38***

8.4 Discussion

8.4.1 Grain filling in endosperm of Schooner, Arapiles and WI-2875*22 in response to high temperature during development

Arapiles did not perform as well as the other varieties in this study under control conditions. The number of grains per ear were consistently lower than in the other varieties and grain (and endosperm) dry weights were lower than those recorded in the field (Moody pers.comm.). Under control conditions, average individual grain weights for Schooner and WI-2875*22 compared favourably with field grown material from trials in 1995 at Horsham (irrigated site) with between 51 and 52 mg per grain (Dunn *et al.*, 1996; Logue, pers.comm.), and varietal rankings corresponded to long term yield results in South Australian field trials (South Australian Field Crops Evaluation Program, 1995).

The high temperature treatment, which extended for 11% of the total grain filling period, increased the average temperature by 1.4°C, from 18.9°C to 20.3°C. Schooner and WI-2875*22 showed similar patterns in the accumulation of dry matter under control conditions and in response to high temperatures in this study. This similarity between Schooner and WI-2875*22 has also been observed in field experiments (Logue, pers.comm.) and reflects their close genetic relationship (Section 3.1). Arapiles responded quite differently to high temperature, especially during the heating period (Fig 8.2), despite apparent similarity in growth patterns to the other varieties in the field when plants experienced periods of elevated temperature during grain filling (Long, pers.comm.).

All three varieties showed similarities between the accumulation of endosperm starch and dry weight under high temperature conditions, in line with our understanding of the contribution of starch to total grain dry matter (MacGregor and Fincher, 1993). Nitrogen content per endosperm was similar for Schooner and WI-2875*22 while starch content was reduced, indicating that in these varieties starch accumulation was more sensitive to heat treatment than nitrogen. This finding has been reported elsewhere in relation to high temperature (Bhullar and Jenner, 1985; Savin and Nicolas, 1996). Arapiles showed a marked contrast, with nitrogen synthesis reduced following high

temperature exposure. Obscured in part by the relatively poor performance of Arapiles under control conditions (when it accumulated 16-21% less starch than the other varieties), the reduction in nitrogen represents a significant deviation from the increase observed in Schooner and WI-2875*22, and the expected negative correlation between protein and yield (Jenner *et al.*, 1990). This provides evidence that protein and starch deposition in the barley grain are essentially independent events (Dhugga and Waines, 1989) and selection for acceptable levels of protein together with high yield (high starch) is feasible.

All three varieties responded with increased starch synthesis under moderately high temperatures (up to 32°C), which was sustained at a higher rate under high temperature conditions (35°C) in both Arapiles and WI-2875*22. Such high temperatures (35°C) have proved deleterious to starch synthesis in a number of other barley varieties (MacLeod and Duffus, 1988a), even with reduced daily exposure (Savin and Nicolas, 1996). Irreversible damage to starch synthetic enzymes (MacLeod and Duffus, 1988a), in particular soluble starch synthase (SSS) (Rijven, 1986) has been reported in grain subjected to temperatures above 30°C. There are several forms of this enzyme (Denyer *et al.*, 1994) and it is possible that one may be more resistant to the effects of heating than others, sustaining starch synthesis at high temperatures and conferring a degree of thermal tolerance. This may have been the case in the variety Arapiles. The severe and irreversible reduction in starch synthesis which occurred in Arapiles at the conclusion of the high temperature treatment was consistent with a 'knockdown' (Keeling *et al.*, 1993) or threshold response in the loss of SSS activity (Jenner, 1994), perhaps as a result of a combination of temperature and time of exposure. Starch synthesis in Schooner and WI-2875*22 on the other hand was maintained, albeit at a diminishing rate, following heat treatment. This response was consistent with a more rapid recovery of enzyme activity after high temperature exposure when the initial loss of catalytic activity immediately following the heating period was low (Hawker and Jenner, 1993).

While high temperature treatment reduced overall β-glucan accumulation in all 3 varieties, a result consistent with findings of Aman *et al.* (1989) and Coles *et al.* (1991) in barley grown under severe climatic conditions, the initial increase in the rate of β-glucan synthesis observed at high temperatures was also consistent with the proposition by Coles *et al.* (1991), of β-glucan as an integrator of transpiration rate. Aman *et al.*

(1989) attributed the reduction in β-glucan to under-development of the endosperm, since this component is deposited later in development. It is likely that earlier termination of β-glucan accumulation, reported in Chapter 4 (Section 4.3.3.3) is related to a shortened duration of grain filling generally observed under adverse climatic conditions (Sofield *et al.*, 1977a; Savin and Nicolas, 1996; Section 4.3.4). In the case of Arapiles, the apparent loss of β-glucan late in development may represent a change in endosperm metabolism due to cultural conditions (discussed in Section 6.4.2), but this requires further investigation.

8.4.2 **Effect of high temperature treatment on the concentration of storage products in harvest-ripe grain**

Final grain composition depended not only on the amount of storage component accumulated in the endosperm, but also on the contribution of the non-endosperm component to final grain dry weight. The rankings of varieties for grain storage components based on endosperm contents were often altered when expressed on a whole grain concentration basis. For example, the reduction in contribution to final grain weight of the non-endosperm component of heat treated Arapiles grains was a factor in determining the increased concentration of nitrogen in whole grains of this variety. While the endosperm of Arapiles grain accumulated less nitrogen under high temperature conditions than either Schooner or WI-2875*22 (Fig. 8.4), this variety recorded a higher concentration of nitrogen than WI-2875*22 and equivalent to Schooner in harvest-ripe grain. Expression of grain quality characteristics on the basis of the concentration of grain components may disguise potentially important relationships between the components of the grain. Indeed, the reduced accumulation of protein in Arapiles grains following high temperature represents an important genetic x environment interaction and warrants further investigation.

In addition, although the non-endosperm grain component remains largely ill-defined with respect to malting quality (MBIBTC, 1995), the role of these collective tissues (which include the embryo and husk) may be important in determining the performance of barley during malting. According to Henry (1990), the non-endosperm grain component may impose a limit on malting quality of a variety by reducing potential malt extract. Increased growth of the embryo, already reported to occur in heat treated Schooner grains (Chapter 6, Section 6.1), suggests that the contribution of the non-endosperm

component of the grain to total grain dry weight may be an important factor in determining the malting quality of grain which develops under adverse climatic conditions.

8.4.3 **Rate and duration of grain filling and changes in grain moisture**

The termination of grain filling was not (clearly) self-evident in this study. However, similarities in the patterns of dry matter accumulation for Schooner and WI-2875*22 under both control and heat treated conditions suggested that genotype affected the accumulation of grain dry matter. Savin and Nicolas (1996) also found evidence of genotypic differences between Schooner and Franklin with respect to the reduction in duration of grain filling under similar high temperature conditions. Average grain growth rates are difficult to interpret under the high temperature conditions applied in this study (Savin and Nicolas, 1996). However, recovery of grain dry weight accumulation rates following the heating period indicate that Schooner and WI-2875*22 (85-88% of control rate) were relatively more tolerant of heat than Arapiles (22% of control rate).

Schooner and WI-2875*22, however, did not follow the well-described developmental pattern of water content and dry weight accumulation generally recorded in cereals (Sofield *et al.*, 1977b) and the onset of rapid water loss did not coincide with maximum grain weight (Schnyder and Baum, 1992) under either control or heat treated conditions. Under control conditions dry matter accumulation appeared to continue after grain relative dry matter (RDM) content attained 0.55, the value Schnyder and Baum (1992) associated with final grain dry weight (see Fig 8.9, arrows). This was apparent in all three varieties all of which continued to accumulate endosperm dry matter between 36 and 42 daa (Fig 8.9), while endosperm moisture decreased (Fig 8.11). The evidence in this study indicates that the onset of rapid water loss may not have a decisive role in the cessation of dry matter accumulation.

Water content *per se* may not be the important attribute determining the termination of dry matter accumulation. Rather, the distribution of water within the developing grain (or endosperm itself) or the free energy associated with that water may be important. Matric potential, for example, a component of water potential, is the parameter used to account for the nature of the interaction between water and the surface of solids within or outside the cell. Such interactions alter the tendency of water molecules to react

chemically or to evaporate (Taiz and Zeigler, 1991). Interactions between water and the surface of starch granules within the endosperm cells of well developed barley grains, for example, may increase tension (negative hydrostatic pressure) and cause a reduction in water potential. Localised enzyme activity may thus continue under conditions of relatively low water content and water potential within the maturing grain.

8.4.4 **Effects of high temperature on malting quality**

Exposure to high temperature led to a reduction in hot water extract in all varieties, in line with results obtained for barley grown under field conditions (Eagles *et al.*, 1995) although not always the case if plants were cultivated under controlled enviroment conditions (Savin *et al.*, 1997a,b). High levels of starch and low protein concentrations were both associated with improved malt extract in this study. While protein has been correlated negatively with malt extract (Allan *et al.*, 1995; Eagles *et al.*, 1995), total starch content does not consistently show a positive relationship with malt extract (Allan *et al.*, 1995; Savin *et al.*, 1997a,b). Rather, associations have been found between malt extract and other starch qualities such as mean large granule diameter (Dunn *et al.*, 1996; Allan *et al.*, 1995) and starch quality (Swanston *et al.*, 1995). While varietal differences have been found in the sensitivity of starch accumulation to increased ambient temperatures (Tester *et al.*, 1991), Dunn *et al.* (1996) found that under some conditions environmental influences may also be greater than genotypic differences.

WI-2875*22, which produced a lower protein concentration than Schooner, showed improved diastatic power, indicating that it may be possible to develop cultivars with low inherent protein without an associated loss of DP. It is known that β-amylase is the main contributor to DP (Arends *et al.*, 1995), and it appears from these results that selection for high β-amylase alone may be more useful in breeding for high DP than α-amylase activity in the malt.

While β-glucan content has been shown to be influenced by both genotype and environment (Aman *et al.*, 1989), WI-2875*22 showed increased levels of this endosperm component following high temperature treatment and Schooner recorded reduced grain β-glucan levels as in previous studies (Savin *et al.*, 1997a; Table 4.3). The greater increase in non-endosperm component in WI-2875*22 compared to Schooner (9.2 cf 5.2%) may in part have accounted for the increase in concentration of β-glucan

(see Section 8.4.2). All varieties exhibited increased β-glucan degradation in response to high temperature. While this may have been due to increased β-glucanase activities which have been correlated with low malt β-glucan levels by other workers (Stuart *et al.*, 1988; Barber *et al.*, 1994) this has not always been found to be the case (see Chapter 7, Table 7.2). The enhanced degradation of β-glucan may have been due to heat induced changes in the structure or organisation of endosperm cell walls, making them more accessible to breakdown. The association between heat treatment, low viscosity and reduced malt β-glucan represents a beneficial response associated with barley cultivation under adverse conditions. Low viscosity and low malt β-glucan reduce the likelihood of encountering processing problems, in particular filtration problems after extraction of malt (Bathgate, 1975) and filtration or haze problems in beer production (Bathgate and Dalgleish, 1975).

8.5 **Conclusions**

Schooner, Arapiles and WI-2875*22 showed varying responses to high temperature exposure with respect to the accumulation of dry weight and endosperm components, starch, protein and β-glucan. The reduction in starch accumulation represents the most significant detrimental effect of high temperature and made the greatest contribution to the reduction in final grain weight.

The effect of heat on the non-endosperm component of the grain shows both genotypic and environmental variation. Changes in the contribution made by the non-endosperm components to final grain weight have the potential to alter the relative concentration of important grain storage components. In some cases this may mask important high temperature effects on key endosperm components, such as in the case of protein in heat treated Arapiles grain. In this case the apparent increase in nitrogen observed in heat treated grain was not due to a higher absolute level of nitrogen but rather to a reduction in the non-endosperm component of the grain. The reduction in total grain nitrogen observed in heat treated Arapiles grains represents a potentially important heat stable quality characteristic when selecting for improved malting quality under conditions of high temperature exposure during grain development.

Evidence in this study indicated that water loss may not have a decisive role in the termination of grain filling. Continued accumulation of endosperm dry matter at low moisture levels suggested that water distribution and/or components of water potential may be more important than overall water content in the cessation of grain growth.

Chapter 9

General discussion

Characterisation of the response of barley grain to a period of high temperature exposure during development has been undertaken in this project. The research presented in this thesis has contributed to the understanding of the relationships between the environment (high temperature) and grain components during development, and the effects on germination and malting. This can benefit breeders by providing guidance desirable heat stable quality characters in potential malting barleys. The development of new varieties of malting quality barley is vital to secure a substantial share of the international market for Australian malt and malting barley.

Starch is the single most important grain component contributing to final grain dry weight, and the reduction in starch accumulation was the major factor lowering barley grain weight following high temperature exposure. Starch is the major source of soluble carbohydrate during mashing (after malting). The lower accumulation of starch observed in barley grains exposed to a brief period of high temperatures was associated with reduced synthesis and localised starch degradation within the barley endosperm. The catalytic activity of individual enzymes of the committed pathway of starch synthesis (Fig 2.2) varied throughout development. Sucrose synthase, UDPglucose pyrophosphorylase, ADPglucose pyrophosphorylase (ADPGp), soluble starch synthase (SSS) and granule bound starch synthase showed reduced catalytic activity following brief exposure to elevated temperatures. One enzyme (ADPGp) showed an initial increase in catalytic activity following exposure to moderately high temperatures (up to 32°C). The discovery that some starch synthetic enzymes incurred a reduction in activity only after the heating period had ended should be considered when planning future studies of thermo-tolerance in starch synthetic enzymes. Some degree of recovery of catalytic activity following the heating period was exhibited by all enzymes investigated in this study, although in some cases evidence of recovery was not observed until 12 days after the completion of the heating period. It appears that the rate of recovery of starch synthetic enzyme activity lost following brief exposure to heat may be critical in determining the overall tolerance of barley varieties to high temperatures during grain filling.

Evidence from this study suggests that it is likely that the activity of soluble starch synthase (SSS) in the barley endosperm has an important role in the accumulation of starch following exposure to heat in barley as in wheat (Jenner *et al.*, 1993; Keeling *et al.*, 1993). More detailed research is required to determine the extent of control of the flux in the pathway from sucrose to starch of this enzyme, both during and following high temperature exposure.

Evidence that starch may continue to be actively synthesised in barley endosperms of some barley varieties under high temperature conditions (35°C) warrants further investigation. Information regarding the catalytic activity of individual starch synthetic enzymes in grains of Arapiles and WI-2875*22, for example, may provide valuable information about the limitations to starch accumulation under high temperature conditions. Thermo-tolerance with respect to starch synthesis may also lead to improvements in the stability of both yield and potential malt extract in new malting quality varieties.

Starch degradation during grain development has been reported in cereals (MacGregor and Dushnicky, 1989b). High α-amylase activity has been associated with late-maturity α-amylase development in wheat (Mares *et al.*, 1994). Evidence of late maturity α-amylase has not been reported in barley. Further research is required to understand more fully the mechanisms which led to starch degradation in barley grains developing under controlled environment conditions in this study. For example, the structural changes in these grains did not conform to patterns of early germination (Gibbons, 1980; MacGregor and Dushnicky, 1989). However, starch degradation leading to reduced starch levels produces grain changed both in composition and structure. This lessens not only the overall malt quality by reducing potential malt extract, but also the predictability of performance of these grains during malting. Such changes to grain structure influence water uptake and distribution and subsequent endosperm modification during malting (Schildbach *et al.*, 1990; Schildbach and Rath, 1994).

Most reports have concluded that grain nitrogen had not been significantly changed by a short period of high temperature in either wheat (Stone and Nicolas, 1995) or barley (Savin and Nicolas, 1996). However, Stone *et al.* (1996) observed significantly lower grain protein levels in Oxley, a wheat variety showing sensitivity to even brief exposure to high temperatures. Genotypic variation has also been revealed in the accumulation of nitrogen under high temperature conditions in the present study, highlighting the possibility of selecting for barley varieties which accumulate less nitrogen. The possibility of alterations to the composition of

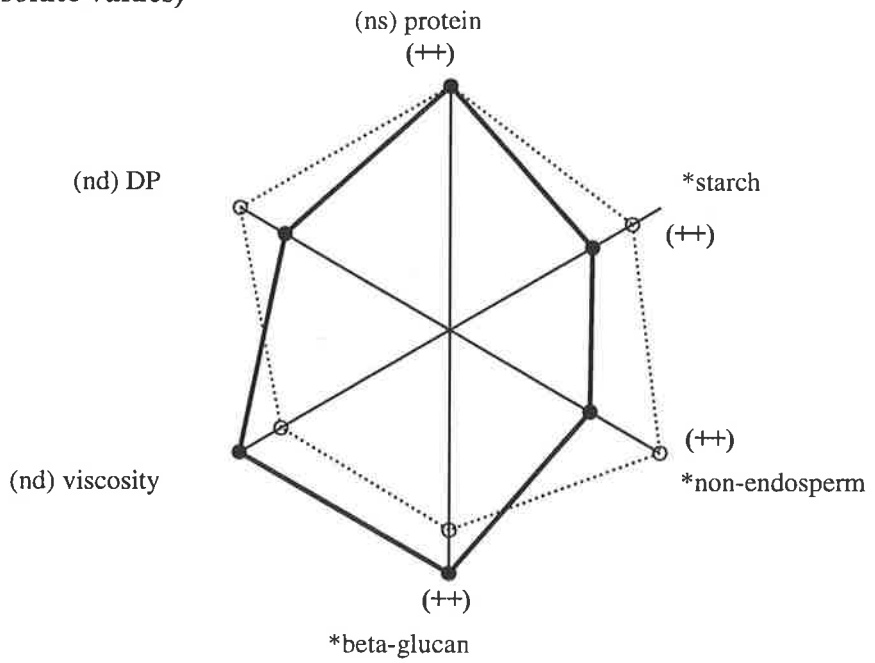
protein and non-protein endosperm nitrogen as a consequence of heat treatment has also been postulated in this study. It is particularly interesting to observe that Stone *et al.* (1996) report that while significant changes to final grain protein levels may not always eventuate following exposure to heat in wheat, protein composition has been found to be altered. Changes to nitrogen metabolism, nitrogen concentration and the induction of heat shock proteins have been documented in relation to high temperature exposure (Paulsen, 1994), although any involvement of these in inheritance of high temperature tolerance has yet to be established (Vierling, 1990).

Heat inducibility of particular genes may alter the functional quality of resultant grain. The increased rate of β-amylase synthesis at high temperatures observed in barley grains in this study suggests that specific proteins may be synthesised more rapidly in barley grains under high temperature conditions. This illustrates the potential for improved diastatic power in grain experiencing high temperatures during grain filling and suggests that further examination of protein accumulation under high temperature conditions may afford benefits for improved malting quality, although yield reductions due to reduced starch may still counteract such benefits. Alterations to protein composition following high temperature exposure during grain growth have been associated with changes in wheat quality for baking (Blumenthal *et al.*, 1995; Stone *et al.*, 1996).

Exposure to high temperature during grain filling appeared to have both beneficial and detrimental effects on malting quality. This appeared to be largely due to the opposing effects of heat on starch, protein and β-glucan synthesis (see Fig. 9.1a). The theoretical relationship between these grain components and malt extract has been illustrated by Savin (1995). Good quality malt extract can be expressed in terms of high fermentable sugar (starch) content, optimal protein content, low β-glucan content and optimal enzyme activity (including diastatic power and β-glucanase). The net effect of heat on potential malt extract is influenced by the effect of heat on these individual components.

Final grain composition depends not only on the amount of storage component, but also on the contribution of non-endosperm dry matter to overall grain dry weight, and this has been shown to vary with temperature during development. Fig 9.1 illustrates the influence of grain attributes on malting quality, with components expressed in absolute values and as percent dry weight. In this study, for example, evidence was found for genetic independence in such

(a) malt quality parameters (absolute values)



(b) malt quality parameters (% dry weight)

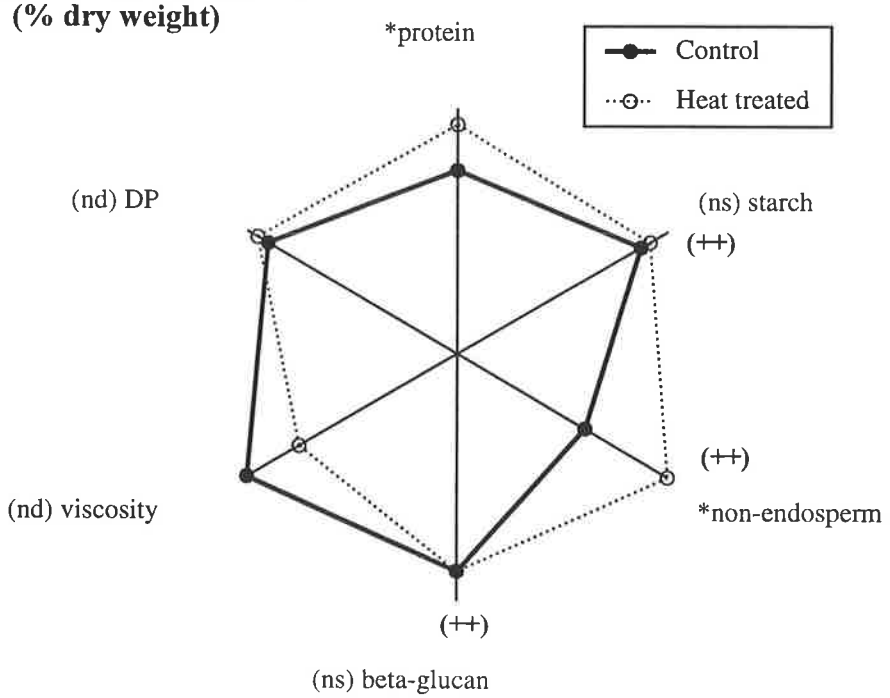


Fig 9.1 Effects of high temperature on whole grain malt quality parameters and their influence on malt extract improvement for Schooner barley. For each attribute a decrease in value signifies a beneficial influence on malt extract (varietal expt data).
* indicates significant difference between treatments; (ns), not significant, (nd) not determined
(++) indicates attributes which showed evidence of differences between cultivars

characters as starch concentration (Schooner recorded an increase with high temperature exposure, while other varieties recorded reductions), protein accumulation (reduced by high temperature in Arapiles, when expressed as mg per endosperm,) and diastatic power (increased in WI2875*22 despite similar protein level to Schooner). Fig 9.1(a) shows that high temperature had detrimental effects on both starch and the non-endosperm component of the grain in absolute terms. In this study genotypic variation has been observed in both attributes, illustrating the potential to improve both characteristics and therefore improve malt extract. When endosperm components are expressed on a percent dry weight basis, common practice for defining malting quality (MBIBTC, 1995), the effects of high temperatures were no longer apparent in changed starch and β-glucan concentration, while endosperm protein concentration was detrimentally affected (decreased following heat treatment). Hence, expression of endosperm components on a concetration basis has the potential to mask important relationships between grain components (e.g. starch and protein in Fig 9.1 a, b). Results from this study suggest that when quality attributes are expressed on a concentration basis, that the contribution of the non-endosperm grain component may prevent selection of the critical grain and malt characteristics which lead to improved malting quality.

While the important contributors to malt extract remain largely undefined (MacLeod *et al.*, 1993), the current investigation of changes in malting quality due to exposure to elevated temperatures has shown that selection for high temperature stability in individual grain components, such as starch and protein may be more appropriate in the search for high temperature tolerant malting barley varieties, than selection simply on the basis of potential malt extract.

Several important findings of this study resulted from structural observations of the grains throughout development until harvest-ripeness and following malting. Firstly, changes in the distribution of endosperm storage products were observed in heat treated grain. Altered distribution of endosperm components (such as starch, protein and β-glucan) has the capacity to change water uptake and distribution during early germination and to reduce the predictability of malting performance. Secondly, degradation of endosperm components may also occur during grain development. The possibility that endosperm degradation may take place in field grown plants as in those produced in growth cabinets cannot be ruled out and requires further investigation.

Finally, the discovery that increased growth of the embryo occurs in Schooner grain following high temperature exposure provides an indication that non-endosperm tissues may also vary in response to high temperature. Indeed the possibility that there is both genetic and environmental variability in the accumulation of non-endosperm tissues, besides the embryo, has also been shown (see Chapter 8, Table 8.3). The concentrations of important endosperm components have been shown to be detrimentally affected by a greater contribution by non-endosperm tissue to final grain weight. This was illustrated by the high temperature effects on the accumulation of grain nitrogen by Arapiles. In this case, a potentially beneficial quality outcome (reduction in the accumulation of endosperm nitrogen) would be overlooked by a breeder selecting on the basis of grain protein concentration. This exposes problems in the assessment of barley quality based on the measurement of the concentration of grain storage components (MBIBTC, 1995). Thus an increased focus may need to be placed on the non-endosperm component of grain in order to determine the optimum contribution to final grain weight. Moreover, the expression of grain composition on a per grain basis provides an alternative and less ambiguous method for defining quality, by eliminating the effect of any genetic variation in the non-endosperm component of the barley grain.

Microscopic observations of grain grown under control conditions revealed changes in grain structure prior to harvest ripeness reminiscent of grains during early germination, which raises doubts about the general view that grain growth and germination are mutually exclusive events (Bewley and Black, 1994; Kermode, 1995). Partial degradation of the crushed cell layer was consistent with the production of cell wall hydrolysing enzymes by the scutellum during the first 12 hours of germination (MacGregor *et al.*, 1994). Thus it appeared that endosperm degradation processes were occurring during development and while the accumulation of storage reserves continued in other regions of the barley grain. Maturation drying is reported as the normal terminal event in the development of seeds and this facilitates the 'switch' from developmental to germinative mode (Bewley and Black, 1994). However, Finklestein and Crouch (1984) have found evidence that embryogenic and germination-specific processes may occur simultaneously in immature embryos. This suggests that severe perturbation of the structural and metabolic processes of the seed's cells, as a result of drying, may not be required to elicit germination specific changes in developing grain. Subtle changes in abscisic acid (ABA) sensitivity and minor fluctuation in osmotic potential may effect changes in gene expression within specific tissues at a molecular level. Do changes in membranes (during drying or upon rehydration) play a role through the availability and/or sensitivity of receptors

to these cues? Elucidation of the precise mechanisms involved in the temporal change from development to germinative mode, remains a challenging research area. The possibility of a continuum between development and germination, remains unexplained by current theories regarding interactions between the embryo and seed environment.

The value of structural observation of grain has been highlighted by the present study. In 1991 a European Brewing Convention (EBC) working party on quality stated that, *"The current trend in barley quality assessment is away from empirical methods such as micromalting and towards direct measurements of barley structure, function and composition."* However, microscopic methods are not currently used by barley breeders in Australia and rarely by researchers involved in malting barley quality improvement. Quantitative analysis of grain structure provides a limitation to role of microscopic methods in a breeding program. While these methods have the potential to reveal information to the barley breeder which greatly influences the performance of grain during malting, as indicated by the distribution of grain components within the grain and the structure of the endosperm tissue prior to imbibition and germination, it is possible that its greatest use may lie in defining better potential parents, rather than as a routine tool.

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