Genetic and Environmental Control of Yellow Pigment in Durum Wheat (*Triticum turgidum Durum)* in Australia

Submitted by

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Acronyms Used in this Thesis

AACC = American Association of Cereal Chemists

ABA = Abscisic acid

AGT = Australian Grain Technologies

ANOVA = Analysis of Variance

BLUE = Best linear unbiased prediction

BSA = Bulk segregant analysis

CIELAB= Commission Internationale de l'Eclairage L* (brightness) a* (red colour) b* (yellow colour)

HPLC = High pressure liquid chromatography

MAS = Marker assisted selection

MEP = 2-C-methyl-D-erythritol 4-phosphate

MRT = Multiplex ready technology

NIR = Near infra-red reflectance spectroscopy

NSW = New South Wales

NVT = National Variety Trials

PPO = Polyphenol oxidase

QTL = Quantitative trait loci

ReML = Restricted Maximum likelihood Ratio

SA = South Australia

SARDI = South Australian Research and Development Institute

SSD = Single seed descent

SSR = Simple sequence repeat

TGW = One thousand grain weight

UA = University of Adelaide

YP = Yellow pigment

YP/grain = Yellow pigment content per grain

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Abstract

Environmental and genetic control of grain yellow pigment (YP) content of Australian durum cultivars has been investigated using large genotype x environment (including controlled environments) data sets and 2 large bi-parental recombinant inbred line durum wheat populations respectively.

Non-genetic variation in YP concentration was shown to be highly complex. This complexity was simplified by showing that final YP concentration is a function of the negative relationship between YP concentration and thousand grain weight (TGW), possibly due to starch dilution, and the total YP content synthesised per grain (YP/grain). Non-genetic variation in YP/grain was, not surprisingly, shown to be dependent on final TGW; however, it was also shown to be independent of TGW. Limited plant available water during grain filling resulted in both lower YP/grain and lower TGW; the net result was a modestly higher grain YP concentration. An hypothesis based on endosperm cell number that attempts to explain the observations is proposed and the implications of non-genetic control of YP for breeding programs discussed.

YP concentration and YP/grain were shown to be multi-genic traits and in both populations examined there was evidence of transgressive segregation. QTL on chromosomes 7AL, 7BL, 7BS, 6B, 1A and 3B in Wollaroi/Tamaroi and 7AL, 7BL, 7BS, 6BL, 6BS, 1A and 3B in WID22221/Tamaroi were additive and together explained >50% and >40% of the phenotypic variation respectively with 7AL and 7BS being the most important. Allelic variation at *Psy1-A1*, encoding phytoene synthase, was identified in both populations but contrary to expectation was not

associated with significant differences in YP phenotype. Rather the effect of chromosome arm 7AL appeared to be contributed by a QLT located proximal to *Psy1-A1*. In contrast, association between YP concentration and allelic variation at *Psy1-B1*, which has been identified in international germplasm, was confirmed in Australian durum wheat.

Chapter 1: Literature Review and General Introduction

1.1 General Introduction

There are two objectives of this chapter. Firstly, to provide background information that is relevant to yellow pigment (YP) in wheat. Secondly, to broadly review, through the study of published literature, the level of scientific understanding of the genetic and environmental control of YP in durum wheat (*Triticum durum*) at the time of commencement of this thesis in September 2005. The subsequent chapters of this thesis aim to build upon this understanding and will refer to more recent published literature. If scientific research published after commencement of this thesis were included in this review, the logic behind the direction taken by this thesis may not be so clear. Australian durum wheat production figures have been treated differently where a 2008 report has been referenced (Kneipp 2008) as production figures have had no influence on the scientific direction of the thesis.

Durum wheat is milled to produce semolina which is used primarily for pasta production (Troccoli et al. 2000). Durum wheat is often, depending on market conditions, the world's highest value cereal crop, and is an important food source for human consumption. Annual global durum production has been relatively stable at 30 to 35 million tonnes (Mt) from 1990 to 2005 (Pena and Pfeiffer 2005). Annual durum wheat production in Australia has averaged 427,000 tonnes over the five year period to the 2006-2007 season and peaked in the 2001-2002 season when production reached 796,000 tonnes (Kneipp 2008). Domestic demand for Australian durum is approximately 300,000 tonnes per annum and the remainder is exported, mostly to

Italy (Kneipp 2008). There are two main durum growing regions in Australia, one in northern NSW and the other in the mid north region of South Australia. Durum wheat from southern Australia has been generally regarded as having lower levels of YP than durum wheat from NSW (Mares 2005a).

The YP concentration of durum wheat is a very important quality trait for pasta production (Irvine 1971). This is due to a consumer preference for bright yellow coloured pasta (Troccoli et al. 2000). YP content can be measured from grain, flour or semolina and measured directly (AACC 1999). Alternatively YP concentration can be estimated based on the colour of the pasta, flour or semolina which is measured by reflectance spectroscopy and is the preferred method used by breeding programs as it is safe, cheap and fast (Pena and Pfeiffer 2005).

Yellow pigment concentration of durum wheat is strongly influenced by genotype and is highly heritable (Borrelli et al. 1999c; Braaten et al. 1962; Clarke et al. 2006; Elouafi et al. 2001; Johnston et al. 1983; Mares and Campbell 2001; Parker et al. 1998; Santra et al. 2005). A number of QTL have been found to be associated with variation in YP or flour colour in tetraploid (Elouafi et al. 2001; Hessler et al. 2002) and common bread wheat (Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998). However, no QTL have been found to be significantly associated with variation in YP concentration in adapted durum germplasm. Therefore improved understanding of the genetic control of YP concentration in Australian germplasm is required for Australian durum wheat breeders to be able to select for this important quality trait using marker assisted selection (MAS).

Many studies have reported a significant effect of the environment on YP concentration in wheat (Clarke et al. 2006; Hatcher et al. 2006; Irvine and Anderson 1953; Johnston et al. 1983; Lee et al. 1976; Mares and Campbell 2001; Matus-Cadiz et al. 2003; Rharrabti et al. 2003a, b; Zhou et al. 2005). However, the specific mechanism responsible for the variation in YP concentration between environments is not known. By reviewing the published literature it can be concluded that temperature (Clarke et al. 2006; Mangels 1932; Rharrabti et al. 2003b) and water availability (Clarke et al. 2006; Guler 2003; Guttieri et al. 2001; Pena and Pfeiffer 2005) may influence YP concentration in wheat or end product colour; however, this influence has been poorly characterised and these reports are far from conclusive and often contradict one another.

An improved understanding of the mechanisms responsible for variation in YP concentration between environments may lead to improved YP concentration selection methodologies or alternative methods of manipulating the YP concentration in wheat, perhaps including agronomic practices. The interaction between genotype and environment is also important in relation to breeding for improved YP concentration. Within the environments tested, Clarke et al. (2006) reported little to no genotype by environment interaction for YP concentration. There are no published studies of YP concentration variation across environments in Australian durum germplasm or the extent of any genotype x environment interaction. Similarly, it is not known if specific growing environments within Australia regularly produce durum with higher or lower concentrations of YP.

To address issues relating to YP concentration in the Australian, and particularly the South Australian, durum wheat industry through scientific research, this thesis aims to;

- Characterise the level of variation in YP across Australian wheat growing regions
- Compare the level of YP of durum wheat grown in traditional to non-traditional durum growing regions and also compare between Australian wheat growing region in the north and the south
- Define the relative consistency of performance of genotypes across environments (genotype by environment interaction) for YP concentration
- Characterise the level of genetic variation for YP concentration within durum wheat cultivars currently grown in Australia
- Characterise the relationship between flour colour and YP concentration to assess the suitability of measuring flour colour as an estimate of YP concentration for breeding and for research
- Attempt to identify any possible relationships between YP concentration of durum at an environment with specific environmental variables such as location, latitude, soil type, growing conditions and climatic factors
- Better characterise the relationship between grain YP concentration and grain weight
- Develop recombinant inbred line (RIL) populations of suitable size and homozygosity for genetic analysis of complexly inherited traits, including YP concentration

- Identify (and/or validate) QTL associated with YP concentration and/or YP content per grain (YP/grain) within the grain that will be suitable for MAS for improved YP concentration by Australian durum wheat breeders

1.2 Durum Wheat in the World

Durum wheat generally produces a higher yield of semolina during milling than common bread wheat due to the hardness of the grain (Dick and Matsuo 1988). Technically, the main difference between durum wheat semolina and wheat flour is particle size. Semolina particles are coarser (up to 500 μm and over) than flour (maximum 150 μm) (Kruger et al. 1996). Durum wheat possesses specific quality characteristics which optimize its use for particular end products (Dick and Matsuo 1988) and differentiate it from common bread wheat (*Triticum aestivum* L.) that is used for pan breads, flat breads, biscuits, cakes and Asian style noodles. Durum wheat throughout the Americas and Europe is predominately used for pasta (97-99%) and only approximately 2% for leavened breads and other uses (Bozzini 1988).

1.3 Durum Wheat Production in Australia

Annual durum wheat production in Australia has averaged 427,000 tonnes over the five year period to the 2006-2007 season (Kneipp 2008); an increase from about 8,000 tonnes in the late 1970's (Kneipp 2008) and less than 100,000 tonnes prior to 1995 (Kneipp 2008; Pagnotta et al. 2005). Production has decreased since a peak of 796,000 tonnes in the 2001-2002 season due to unfavourable seasons, disease and lower prices (Kneipp 2008). Production figures from season to season vary dramatically (Kneipp 2008) possibly due to durum in Australian being especially sensitive to drought, crown rot (*Fusarium pseudograminearum*) infection,

inconsistent premiums compared to bread wheat and the relatively small geographical locations in which durum wheat is produced which increases the impact of isolated drought. Australian durum wheat is exported to Italy (50% of exports), North Africa, South Africa, South America, Middle East and East Asia (Kneipp 2008). The majority of Australian durum wheat exported is therefore used for pasta production.

Wheat production in Australia generally follows the coast of south-west Western Australia and South Australia and the inland regions from the Great Dividing Range in eastern Australia (O'Brien et al. 2001). In terms of adaptation, O'Brien et al. (2001) explain that there are distinct environmental differences between the sub-tropical northern grain growing regions of eastern Australia and the more temperate, Mediterranean-like climate of the southern cropping region. Durum wheat in Australia is produced in both of these distinct environments and is confined to the higher production potential areas, particularly northern New South Wales (NSW) in the north, and the Mid North of South Australia (SA) in the south. Approximately 56% of Australia's durum wheat is produced in NSW and 41% in South Australia and the remainder in Queensland, Victoria and Western Australia (Kneipp 2008). Bellaroi, released in 2003, is the major variety grown in Australia (Kneipp 2008), most likely due to its popularity in the northern NSW region. In South Australia, Tamaroi, released in 1998, and Kalka, released in 2003, are currently (2006) the highest yielding and most popular varieties. Durum wheat from southern Australia has been generally regarded as having low levels of YP concentration (Mares 2005a). This could be due to the YP concentration of varieties grown in the south (Tamaroi and Kalka) compared to the varieties grown in the north (Bellaroi and Wollaroi) rather than a function of the South Australian environment not capable producing high

quality durum wheat with high concentrations of YP. There are no published reports suggesting intrinsic differences in grain YP concentration between growing regions in Australia.

1.4 Evolution of Wheat

Durum wheat is a tetraploid consisting of A and B genomes (AABB), while bread wheat is a hexaploid consisting of A, B and D genomes (AABBDD). Modern day cultivated durum wheat evolved from the hybridisation of diploid wild progenitors. *Triticum urartu*, (AA), related to *Triticum monococcum* (A^mA^m), and is believed to have hybridised with a diploid *Sitopsis* species related to *Aegilops speltoides*, (SS), to create the wild tetraploid, *Triticum turgidum spp. diccocoides* (AABB) (Feldman 2001). Chromosome pairing between A genome diploid *Aegilops* species (including *Aegilops speltoides*) and tetraploid wheats supports the theory that *Aegilops* species contributed the A-genome to modern day tetraploid and hexaploid wheats; however, a plausible B-genome parent has not been identified (Johnson and Dhaliwal 1978). There has been speculation that the B-genome donor may be an extinct species, or, that the B genome has been modified at the tetraploid level (Sarkar and Stebbins 1956; Sears 1948). From *Triticum turgidum spp. diccocoides* cultivated species arose including *Triticum turgidum* spp. *dicoccon* and spp. *durum* (macaroni or hard wheat). One or more of these tetraploids are believed to have hybridised with another wild diploid, *Aegilops tauchii* (DD), resulting in modern day hexaploid *Triticum aestivum* spp. *aestivum* (common or bread wheat), spp. *spelta* (dinkel or large spelt), spp. *compactum* (club wheat), spp. *sphaerococcum* (Indian dwarf or shot wheat) and spp. *macha* (Feldman). From this accepted model of the evolution of wheat it is clear that durum wheat can be regarded as a progenitor to modern day bread wheat.

1.5 Wheat Grain Structure

1.5.1 Introduction to Wheat Grain Structure

In a botanical sense, the tissues in a wheat grain can be grouped into three parts:

1) The embryo or embryonic axis, and the scutellum (often collectively referred to as "germ");
2) The endosperm (starchy endosperm and aleurone); and
3) The grain coat (pericarp and seed (grain) coat).

Prior to most end-use applications, durum grain is milled to produce semolina or flour that is composed primarily of starchy endosperm. During milling, the starchy endosperm is separated from the germ and both the grain coat and aleurone which are collected as pollard and bran respectively. Since the starchy endosperm is primarily used for pasta production the YP concentration of the starchy endosperm is an important factor influencing the colour of end products. As such, an understanding of the structure of the wheat grain and in particular of the starchy endosperm may be useful. The germ represents approximately 2.5-3.5% of the whole grain while the bran and endosperm represent approximately 14-16% and 82-84% of the whole grain respectively (Kent and Evers 1994; Simmonds 1989).

1.5.2 The Embryo

The embryonic axis represents the component responsible for the next plant generation and consists of primordial roots and shoots with leaf initials. Once milled the germ is generally able to be separated from the endosperm by sieving due to it being rolled flat after being broken loose during the milling process. Nevertheless,

during roller milling some of the germ lipids can be carried into the endosperm fraction thereby contaminating the flour. This can result in deleterious effects on the quality of end products if the contamination is excessive (Simmonds 1989).

1.5.3 The Endosperm

The endosperm consists of a central mass called the starchy endosperm and the aleurone, which is very small relative to the starchy endosperm. The starchy endosperm is made up of cells that are packed with nutrients which are stored in an insoluble form, with starch being the major component. Protein is the second most abundant nutrient within the starchy endosperm and its relative content is inversely proportional to the amount of carbohydrate starch. The protein per unit mass of endosperm tissue increases towards the periphery of the kernel (Kent and Evers 1994).

The size of the starchy endosperm cells decrease towards the outside of the kernel. The cell walls are mostly composed of arabinoxylans and mixed linked β-glucan (Fincher and Stone 2004) and increase in thickness toward the periphery of the kernel (Kent and Evers 1994). In wheat grain, YP is present in the amyloplasts (Fratianni et al. 2005; Hentschel et al. 2002; Panfili et al. 2004; Zandomeneghi et al. 2000; Zhou et al. 2004) which are specialised plastids for storage of starch granules (Kirk and Tilney-Bassett 1978). The starchy endosperm is made up of three types of cells; in order from the inner surface of the aleurone to the centre of the endosperm they are the *peripheral* or *sub-aleurone*, the *prismatic*, and the *central* cells (Mares and Stone 1973). The peripheral cells are formed last during grain development which results in

them being relatively short (20-60 μm) in the radial direction. During early stages of grain development, prior to differentiation of the aleurone layer, the peripheral and prismatic cells are formed by division of the cambial layer which subsequently differentiates into the aleurone. The central cells occupy the centre of the starchy endosperm, beneath the prismatic cells and around the crease area. They form shortly after fertilisation during the initial cellularisation of the endosperm (Simmonds 1989).

During milling the aleurone is usually separated from the starchy endosperm along with the grain coat. The aleurone of wheat is a single layer of cells ranging in thickness from 30-70 μm. Aleurone cells contain no starch but are high in protein and lipid and unlike the starchy endosperm remain as living cells throughout grain maturation and post-harvest storage (Kent and Evers 1994).

1.5.4 Grain Coat

The grain coat constitutes the exterior of the grain and contains approximately 25% cellulose (fibre), 25% pentosan and only 1-4% protein (Simmonds 1989). The grain coat represents maternal tissue, whereas the starchy endosperm, aleurone, embryo and scutellum are formed as a result of fertilisation and so are genetically influenced by both parents (Simmonds 1989). The implication of this during genetic analysis of segregating wheat genotypes is that the phenotype of the grain coat is determined by the genotype of the mother plant (Gale 1989). This may be important, particularly in early generation selection, if analysing YP concentration from whole grains or wholemeal flour which include the grain coat.

1.6 Fertilisation of the Wheat Flower and Genetic Makeup of Grain Fractions

The process of fertilisation of wheat commences when a pollen grain lands on the stigma of the wheat flower; this is referred to as pollination. The pollen grain germinates and produces a tube extending down between the style cells toward the ovary. The pollen cell then divides by mitosis and forms two sperm, the male gametes. The tip of the pollen tube then enters the ovary and releases the two sperm within the embryo sac. One sperm fertilises the egg to form a diploid ($2n$) zygote, the embryo, the other sperm combines with two polar nuclei in the centre of the large central cell of the embryo sac to form a triploid nucleus which continues to become the endosperm. This is called double fertilisation (Campbell 1996). This can be significant in hybrids for traits which are influenced by a dose effect because the endosperm has twice as many copies of the genes contributed from the female parent as it does from the male parent. Most of the YP in a wheat grain is in the endosperm (Law 2005) and so the triploid nature of the endosperm may need to be considered if analysing YP content of F_1 seeds where the two parents may have different alleles controlling grain YP content.

1.7 Yellow Pigment in Wheat

Yellow pigment concentration is a very important quality trait of durum wheat for pasta production (Irvine 1971). This is due to a consumer preference for bright yellow coloured pasta (Troccoli et al. 2000). By contrast, a creamy white coloured flour, or lack of yellow pigment, is often desirable in hexaploid bread wheat used for baking

bread (Mailhot and Patton 1988) and white salted noodles (Mares and Campbell 2001). This has led to selection pressure against high yellow pigment in bread wheat breeding and consequently has resulted in very low levels of yellow pigment in present day commercial bread wheat cultivars (Mares 2005a).

Yellow pigment in durum wheat grain is predominately associated with the presence of carotenoid compounds, classified into carotenes (polyunsaturated hydrocarbons) and xanthophylls with the latter being the most abundant pigment compounds in durum grain (Elouafi et al. 2001). The carotenoid biosynthetic pathway is well understood and detailed reviews have been published elsewhere (Cunningham 2002; Hirschberg 2001; Niyogi 1999; Sandmann 2002). Carotenes are polyunsaturated hydrocarbons, consisting of eight isoprene units that are cyclised at each end, while xanthophylls are hydroxylated carotenes (Coultate 1996). Lutein and zeaxanthin are xanthophyll compounds which are formed by the attachment of hydroxyl groups to each end of the α- or β-carotene molecules, respectively (Coultate 1996). Xanthophylls are involved with photoprotection of the photosynthetic apparatus in the chloroplast (Demmig-Adams and Adams 1996; Niyogi 1999) which may explain why high concentrations of xanthophyll have been found in the grain coat (Law 2005). Within non-photosynthetic material the roles of carotenoids are less clear but they are possibly related to seed dormancy since carotenoid production in the seed is important for abscisic acid (ABA) production and seed dormancy (Maluf et al. 1997), and/or could be related to seed protection since carotenoids contribute to the antioxidant system in wheat seeds, which limits free radical induced membrane deterioration and seed ageing (Calucci et al. 2004; Pinzino et al. 1999). During post-harvest storage of grain, the hydroxyl groups of lutein can be esterified with free fatty acids to form

lutein mono- and di-esters, although this appears to be more common in bread wheat than durum (Breithaupt et al. 2002). Chromatography has revealed that YP in durum is predominately due to free lutein (Lepage and Sims 1968) and Hentschel *et al*. (2002) found no esters present in the grain of eight German durum cultivars. In addition, research in Australia was unable to detect esters in Australian durum wheat cultivars (Mares 2005b).

Since flour and semolina are primarily composed of starchy endosperm, the YP concentration of this tissue largely determines the YP concentration in the end product. YP is found in all the grain tissues and whilst the concentration may be greatest in the germ, the endosperm contributes most of the YP due to its relative size. Genetic variation in xanthophyll content exists in bread and durum wheat grain, almost entirely due to differences in xanthophyll concentration in the endosperm, with only small differences in YP concentration in the bran and virtually no genetic variation for YP concentration observed in the germ (Law 2005).

In addition to increasing its marketability and pasta quality, higher lutein durum wheat may also have health benefits. Lutein has been shown to contribute to reducing age related macular degeneration (Berendschot et al. 2000; Landrum and Bone 2001, 2004; Olmedilla et al. 2001), which is the major cause of age related blindness (Humphries and Khachik 2003). Lutein is also an antioxidant (Landrum et al. 2002) which may offer further health benefits.

1.8 Assaying Yellow Pigment Concentration

There are a number of methods that can be used to determine or estimate the concentration of YP or its major component, lutein in durum wheat grain, flour or semolina. Analysis can be performed on ground whole grain (wholemeal), semolina (or flour), or the end product, usually pasta. YP can be extracted from semolina or wholemeal using water-saturated butanol according to AACC method 14-50.01 (AACC 1999). The pigment content is calculated directly from the absorbance at 435.8nm using a conversion factor representing the absorptivity of carotene and reported as carotenoid even though the bulk of the pigment is lutein. As a consequence the values obtained are relative rather than absolute. Alternatively, yellow pigment can be extracted with methanol instead of butanol (Mares 2005b).

Semolina or wholemeal can also be analysed by high-pressure liquid chromatography (HPLC) (Fratianni et al. 2005) to determine the absolute amounts of the individual component compounds. Authentic standards are used for identification and quantification of lutein and other carotenoids. Lutein generally has three absorbance peaks corresponding to VIS maxima (nm) 420, 446 and 472 (Köst and Zweig 1988). HPLC is more accurate than a spectrophotometer for determining lutein and total xanthophyll concentration, as the spectrophotometer is not able to distinguish between various compounds contributing to absorbance at the appropriate wavelengths. However, a high correlation ($r=0.98$, $p<0.01$) exists between HPLC measurements and measurements of pigments using the AACC method (Fratianni et al. 2005). Sample throughput is much lower with the HPLC than the spectrophotometer (Mares 2005a). Both methods are able to estimate lutein concentration on wholemeal from as

few as 10 grains (Mares 2005b). Both methods are destructive, time consuming, require expensive instrumentation, some technical capability, and perhaps more importantly accuracy relies on the ability to successfully extract all of the YP from the wholemeal, flour, or semolina.

The colour of semolina and pasta can also be measured directly by reflectance spectroscopy. Reflectance colorimeters, using the Commission Internationale de l'Eclairage (CIE) L*a*b* (CIELAB) system, have become common tools for indirect measurement of YP concentration of germplasm within breeding programs (Pena and Pfeiffer 2005). Reflectance spectroscopy provides values indicating brightness (L*), redness when positive (a*) and yellowness when positive (b*) which are derived from the three readings X (red value), Y (green value) and Z (blue value) (Feillet et al. 2000) which can be determined from a tristimulus colorimeter (Walsh et al. 1969). Since b* is a measure of yellowness it is the CIELAB b* value which is used to indirectly estimate YP concentration (Pena and Pfeiffer 2005). Instruments commonly used for these measurements include the "Minolta color meter" manufactured by Konica Minolta Holdings Inc. (Marunouchi, Chiyoda, Tokyo). Reflectance colorimeters offer benefits of safety, high throughput and a good correlation with chemical extraction methods (Acquistucci and Pasqui 1991; Johnston et al. 1980). Factors other than absorption by pigments, such as the physical nature of the sample matrix, can affect the amount of light reflected and the perception of colour. These limitations will be discussed in depth in the following section. This method is generally performed on semolina or flour but is otherwise non-destructive. The amount of semolina or flour that is required for analysis will depend on the instrument being used.

Near-Infrared Reflectance Spectroscopy (NIR) is a useful technique to estimate multiple characteristics such as protein, grain hardness and moisture content (Williams 1979; Williams and Thomson 1978). Modern near-infrared reflectance and near-infrared transmission spectrophotometers have extended the spectral range to include the visible region and can consequently be used to estimate YP concentration (McCaig et al. 1992). McCaig et al. (1992) showed that across a wide range of YP contents from 53 samples the correlation (r) value between YP concentration and YP estimated from NIR was 0.97. Most durum wheat breeding programs already measure protein using NIR spectroscopy (Clarke 2005), and therefore to include YP analysis using this method places minimal additional strain on resources, thus making it a very useful method for breeding programs. In order for this method to be successful it is critical that NIR procedures are calibrated to the laboratory reference method it is intended to replace. Errors can occur via inaccurate reference method data, day-to-day variation in the reference method, and inaccurate calibration between the reference method and the NIR method (McCaig et al. 1992). As for other reflectance spectroscopy methods, such as CIELAB measurements, colour measurements of flour, semolina, or pasta using NIR will be dependent on the physical nature of the sample matrix.

1.9 Expression of Yellow Pigment and Perception of Yellow Colour in Pasta and Semolina

Pasta colour is an important trait for consumer acceptance and marketing of pasta (Dick and Youngs 1988). Pasta colour potential is often estimated or predicted by measuring the colour of the semolina (Dick and Youngs 1988). The perceived colour of semolina can be affected by the size and shape of the semolina particles, the

amount of flour in the semolina sample, and the way the light is reflected off the semolina (Kill and Turnbull 2001). Semolina with fine granulations appears less yellow than semolina with larger granulations (Dick and Matsuo 1988). This can result in pasta with very similar colour being produced from semolina with significantly different perceived yellow colour (Dick and Matsuo 1988; Kill and Turnbull 2001). For this reason Kill and Turnbull (2001) recommend that any method of assessing semolina colour must take account of the physical effects of particle size and contamination and thereby concentrate on factors that influence the final colour of the pasta.

Semolina particle size can be influenced by milling processes (Hareland 1998) as well as physical grain characteristics such as grain hardness (Quaglia 1988) and grain vitreousness (Dexter and Matsuo 1981; Matsuo and Dexter 1980). A vitreous grain is a grain in which the endosperm has a glassy, translucent appearance rather than a mealy or floury appearance (Troccoli et al. 2000). Vitreousness of durum wheat is influenced predominantly by genotype but location and nitrogen fertiliser rate have also been shown to have a significant effect on vitreousness (Hadjichristodoulou and Della 1978). Protein content can also be an important factor influencing vitreousness, with higher protein wheat generally being more vitreous (Dexter and Matsuo 1981; Dexter et al. 1987; Hadjichristodoulou and Della 1978; Matsuo and Dexter 1980). Protein content has also been shown to be significantly positively correlated with pasta brownness (Grignac 1970; Kobrehel et al. 1974; Taha and Sagi 1987). The implications of pasta brownness and darkening on the expression and perception of YP are discussed later. Supporting the potential relationship between protein and flour colour, Mares and Campbell (2001) identified a QTL on chromosome 5B associated

with both protein and yellow flour colour (CIELAB b*). Protein content may be related to hardness although published literature is inconsistent on this point as multiple authors (Greenawa.Wt 1969; Groos et al. 2004; Stenvert and Kingswood 1977) have reported a positive relationship between protein and grain hardness while Moss et al. (1973) reported a negative relationship while Obuchowski and Bushuk (1980) reported no relationship as well as citing Newton et al. (1927) as making the same claim.

Grain hardness in bread wheat (*Triticum aestivum*) has been shown to be mostly associated with puroindoline proteins a and b which are controlled by the *Puroindoline* (Pin) genes, *Pina* and *Pinb*, that are tightly linked at a single locus called the Hardness (Ha) locus on chromosome 5DS (Capparelli et al. 2003; Doekes and Belderok 1976; Morris 2002). When either one of the puroindolines are absent or altered by mutation, then the result is hard texture. Durum wheat has no D genome and so lacks puroindolines and is very hard relative to bread wheat (Morris 2002). Further loci on chromosomes 2A, 2D, 5B and 6D have been reported to also be associated with grain hardness of wheat (Sourdille et al. 1996) but tend to have less influence than variation at the Ha locus.

Brightness, characterised by the CIELAB L* value (Kill and Turnbull 2001), has been found to be inversely related to yellow flour colour CIELAB b* in flour (Mares 2005b). This may be due to the bright white light overpowering the yellow light being reflected (Mares 2005a). Interestingly, darkening of end-products, which results in lower brightness (CIELAB L* value), has also been found to result in lower CIELAB b* value (Mares and Panozzo 1999; Mares and Campbell 2001). Black or brown

pigments absorb light throughout the visible spectrum which may be the reason for decreased brightness (CIELAB L*) and also decreased perception of YP, that absorb at a specific region of the spectrum, that results from black or brown pigments (Mares and Campbell 2001). By adding black dye to noodle sheets Mares et al. (2001) demonstrated that darkening resulted in a reduction in both CIELAB L* and CIELAB b* values. In a mapping study, Mares and Campbell (2001) identified two QTL which were associated with both darkening and a change in colour (CIELAB b*) of yellow alkaline noodle sheets. Darkening results from the additive effects of polyphenol oxidase (PPO) (Fuerst et al. 2006; Mares and Panozzo 1999) and non-PPO reactions (Mares and Panozzo 1999). PPO content is influenced by both genotype (Baik et al. 1994; Mares and Panozzo 1999) and the environment (Baik et al. 1994), whereas the non-PPO component of darkening shows limited genetic variation (Mares and Panozzo 1999). Variation in PPO has been reported to be under strong genetic control and particularly associated with QTL on homeologous regions of chromosomes 2A (Sun et al. 2005) and 2D (Mares and Campbell 2001). Brightness has also been shown to decrease in response to higher protein and ash contents, however the bases of these relations are not known (Feillet et al. 2000). Similar to darkening, speckiness, caused by the contamination of semolina with bran flakes, can also reduce the perception of YP. The amount of contamination with bran flakes is dependent on the ease of separation of the outer grain coat layers and aleurone from the endosperm during milling and sieving (Mares and Campbell 2001). The amount of bran contamination is correlated with the amount of red/brown colour which is characterised by the CIELAB a* value (Kill and Turnbull 2001).

Pasta browning has also been reported to mask yellow colour (Irvine and Anderson 1952) and reduce brightness (CIELAB L*) (Feillet et al. 2000). Although complex, the cause of pasta browning is generally a result of the combination of inherent brownness of the endosperm, impurity of the semolina, and of the Maillard reaction when pasta is dried at high temperatures. The Maillard reaction is the result of very complex reactions between free amino acids and reducing sugars producing brown 'melanoidin pigment' (Feillet et al. 2000). The intensity of the Maillard reaction has been shown to be influenced by the content of reducing sugars in paste semolina (Resmini and Pellegrino 1994) which in turn has been reported to be related to the semolina particle size (Sensidoni et al. 1996). Feillet et al. (2000) recommend using semolina with minimal starch damage and minimal amylolytic activity in order to reduce the intensity of the Maillard reaction.

During pasta manufacture YP can be degraded by lipoxygenase (LOX) (also called lipoxidase (D'Egidio 2000)) which is commonly found in durum grains (Joppa and Williams 1988). This reaction can be reduced by processing semolina in a vacuum or by adding oxidation inhibitors, such as L-ascorbic acid (Joppa and Williams 1988), β-Carotene or α-tocopherol (Borrelli et al. 1999b). An investigation by Borrelli *et al.* (1999a) found that the main factor involved in the loss of YP during processing was LOX, while ash content had a secondary and lesser role. YP loss was found to occur mostly during pasta processing and partly during milling. Irvine and Anderson (1953) reported that variations in LOX activity to be under genetic control, while only small differences were observed between environments. Lee et al. (1976) also reported significant genetic effects on LOX content and reported narrow sense heritability (h^2) of 37%. Reports of genetic variation in LOX content are supported by Hessler *et al.*

(2002) who identified a QTL associated with variation in LOX activity located on the homoeologous region of 4BS in a durum wheat population, and also by Mechelen *et al.* (1999) who mapped QTL associated with variation in LOX activity to chromosomes 4H and 7H in barley.

Grain size has been reported to be being negatively correlated with YP (Alvarez et al. 1999; Clarke et al. 2006; Markley 1937; Worzella 1942). This may be due to the dilution of YP with higher levels of starch in larger grains (Clarke et al. 2006; Hessler et al. 2002). QTL associated with variation in flour colour have also been shown to co-locate to QTL associated with variation in grain size suggesting that this QTL influences grain size which in turn indirectly affects YP concentration (Hessler et al. 2002; Mares and Campbell 2001). Similarly a QTL associated with variation in YP concentration was identified by Mares and Campbell (2001) on chromosome 4B coincident to the *Rht1* gene which corresponded to QTL associated with plant height and grain size.

In summary there are many factors which can influence the expression or perception of colour in semolina and/or pasta. The perceived colour of the end product, usually pasta, is the result of a combination of all of these many factors. Grain YP concentration determines the potential yellow colour of the end product. The other factors which also influence the colour of the end product are very important but may not be genetically or environmentally related to grain YP concentration. Therefore it could be considered preferable to study the genetic and environmental control of YP concentration in isolation of the many factors influencing the expression and/or perception of semolina or pasta colour to avoid confounding results. Measuring grain

YP concentration in combination with grain weight allows for calculations on amount of YP synthesised per grain (YP/grain) to account for any possible dilution effects of YP within the grain.

1.10 Genetic Control of Yellow Pigment in Wheat

1.10.1 General Genetic Control

Yellow pigment concentration of durum wheat semolina is strongly influenced by genotype and is highly heritable (Borrelli et al. 1999c; Braaten et al. 1962; Clarke et al. 2006; Elouafi et al. 2001; Johnston et al. 1983; Mares and Campbell 2001; Parker et al. 1998; Santra et al. 2005). Clarke et al. (2006) reported limited, or no genotype by environment interaction affecting YP concentration in seven durum wheat populations derived from crossing high pigment by low pigment parents grown in five field trials at two or more locations for two or more years. Furthermore, QTL associations with YP concentration have been reported to be generally stable across environments (Elouafi et al. 2001; Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998). Inheritance of YP concentration has been found to be complex, under polygenic control, and populations often display transgressive segregation (Clark and Smith 1928; Clarke et al. 2006; Elouafi et al. 2001; Johnston et al. 1983; Mares and Campbell 2001). Using classical quantitative genetic analysis, Clarke et al. (2006) found that the number of effective factors differing between parents for YP ranged from 3 to 27 and that the extent of the range varied across environments and also between populations. Reports of polygenic control of YP concentration (Clark and Smith 1928; Clarke et al. 2006; Johnston et al. 1983) are supported by QTL studies that have identified multiple QTL associated with variation in YP

concentration, none of which individually accounted for more than 60% of the observed genetic variance (Elouafi et al. 2001; Hessler et al. 2002; Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998). The genetic control of YP concentration has generally been reported to be under the influence of additive gene effect (Elouafi et al. 2001; Johnston et al. 1983; Lee et al. 1976; Mares and Campbell 2001; Santra et al. 2005). It was reported by Lee et al. (1976) that additive gene effects and no epistatic effects were identified in a diallel involving 10 durum parents from diverse origins. Using F_2 hybrids to determine the additive and non-additive genetic control of YP concentration, Lee et al. (1976) found that both additive and dominance genetic effects significantly influenced YP concentration. Additive genetic effects suggest that YP concentration is subject to an allelic dose effect. This can be important for phenotyping grain of early generation germplasm. Due to the double fertilisation phenomenon explained earlier, F_2 endosperm derived from an F_1 hybrid in turn derived from parent 1 with AA alleles and parent 2 with aa alleles will segregate in a 1:1:1:1 ratio of AAA, Aaa, AAa and aaa (Gale 1989). For traits that are influenced by an allelic dose effect, the phenotype of each of these classes will be different which can be particularly important when phenotyping in early generations for traits assessed on an individual grain basis. The implications of complex segregation due to double fertilisation can be avoided by ensuring that phenotyping be conducted on homozygous individuals.

Einkorn wheat (*T. monococcum*), derived from the original progenitor of durum wheat contributing the A genome, has been reported to have far greater grain concentrations of carotenoids, mostly lutein, than either durum or bread wheat (Degidio et al. 1993; Hidalgo et al. 2006; Leenhardt et al. 2006). This suggests that

there could be genes within the A genome of durum wheat contributing to high levels of grain YP concentration which is in agreement with other studies (Elouafi et al. 2001; Hessler et al. 2002; Mares and Campbell 2001; Parker et al. 1998). In addition to this, genetic studies and QTL analysis have revealed that there are also genes on the B genome involved in the control of YP concentration and/or flour colour (Elouafi et al. 2001; Kuchel et al. 2006b; Mares and Campbell 2001). Due to reduced selection pressure placed on einkorn wheat compared to durum and bread wheats there is likely to be more allelic variation within the species. This may provide an opportunity to utilise einkorn wheat as a genetic source of higher YP concentration. The successful use of einkorn wheat as a donor source for higher YP concentration would depend on successful recombination in the A genome between einkorn and durum wheat which generally do not recombine readily (Mares 2005a). The higher YP concentration found in the diploid einkorn wheat may be due to the fact that it has not undergone the polyploidisation process. Trait expression in diploids is less likely to be reduced by suppressor loci on homoeologous chromosomes which can result in gene silencing in polyploids (He et al. 2003). If this is the reason for high levels of carotenoids in einkorn wheat then this obviously reduces the opportunity for exploiting einkorn wheat as a source of higher YP concentration. Einkorn wheat generally has small and light grain size relative to durum wheat and bread wheat (Degidio et al. 1993; Troccoli and Codianni 2005). Therefore high concentrations of YP concentration in einkorn wheat may in part be due to less dilution of YP in small grain.

1.10.2 QTL Analysis

In hexaploid wheat, and to a lesser extent tetraploid wheat, a number of QTL influencing YP concentration or flour colour (CIELAB b*) have been identified, mostly on the group 3 and group 7 chromosomes (Elouafi et al. 2001; Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998) (Table 1.1). In addition, Joppa and Williams (1988) cite unpublished work by L.R. Joppa that suggested that genes influencing semolina colour were probably located on chromosomes 2A and 2B. None of the published QTL associated with YP concentration have been found to be coincident with QTL associated with factors previously reported to affect expression or perception of YP such as or grain hardness (which impacts semolina particle size), LOX or PPO which all have been shown to affect the colour of end products. There are no reports of QTL associated with YP concentration or flour colour in a durum wheat population. There have been limited studies which have identified QTL associated with actual YP concentration as opposed to estimated YP concentration using reflectance spectroscopy. Once QTL for high YP concentration have been identified breeders may wish to target these QTL in a marker assisted selection (MAS) strategy and following from this select for other traits such as enzyme activity and factors affecting perception of colour, separately, as these other traits seem to be under separate genetic control.

Table 1.1: Quantitative trait loci (QTL) associated with either yellow pigment (YP) or flour colour (FC) in wheat. % V.A.= Per cent of variation accounted for. N.R.= Not reported

Chromosome	Population	Trait	% V.A.	Reference
7AL	T. Dicoccoides/2*T. durum	YP	13	Elouafi et al. (2001)
	T. aestivum	YP + FC	27	Mares and Campbell (2001)
	T. aestivum	FC	N.R.	Mares and Campbell (2001)
	T. aestivum	FC	60	Parker et al. (1998)
7A	T. Dicoccoides/2*T. durum	YP	6	Elouafi et al. (2001)
7BL	T. Dicoccoides/2*T. durum	YP	53	Elouafi et al. (2001)
	T. aestivum	FC	10	Mares and Campbell (2001)
	T. aestivum	FC	48-61	Kuchel et al (2006)
3B	T. aestivum	YP + FC	20	Mares and Campbell (2001)
3A	T. aestivum	FC	17	Mares and Campbell (2001)
	T. aestivum	FC	13	Parker et al. (1998)
6A	T. aestivum	FC	13	Mares and Campbell (2001)
4B	T. aestivum	FC	7	Mares and Campbell (2001)

Variation in YP concentration and/or flour colour has been reported to be associated with QTL on chromosome 3BS in two bread wheat populations (Mares and Campbell 2001) and on chromosome 7AL also in two bread wheat populations (Mares and Campbell 2001; Parker et al. 1998) and a *Triticum dicoccoides* x durum population (Elouafi et al. 2001). A region on chromosome 3A, homoeologous to the 3BS QTL has been found to be associated with yellow flour colour in two separate bread wheat populations (Mares and Campbell 2001; Parker et al. 1998). Similarly, on chromosome 7BL, a homoeologous region to the 7AL QTL, has been found to be associated with yellow flour colour in two separate bread wheat populations (Table 1.1) (Kuchel et al. 2006b; Mares and Campbell 2001). The reported association of YP with the group 7 chromosomes concurs with reports by Alvarez et al. (1998) that carotene content is controlled by a QTL on chromosome $7H^{CH}$ in *Hordeum chilense*. The association of the three QTL identified in the *Triticum dicoccoides* x durum

population with variation in YP concentration were consistent across all environments tested exhibiting a strong genetic effect and only a weak QTL by environment effect (Elouafi et al. 2001).

Additional QTL associated with flour colour (CIELAB b*) in bread wheat populations have been reported by Mares and Campbell (2001) on chromosomes 2D, 4B, 5B, 5D and 6A and accounted for between 7% and 13% of the total variation in flour colour (CIELAB b*). However, unlike the QTL on groups 3 and 7, these QTL have generally only been identified in a single population and the association with flour colour was not necessarily stable across environments. The population in which QTL on chromosome 4B and 5B were found to be associated with flour colour (CIELAB b*) was assayed for YP concentration but there was no association found with YP concentration at these loci. However, the 5B QTL was found to be associated with protein content and the 4B QTL with plant height and grain size. The variation in flour colour at these loci was attributed to indirect effects of grain size and protein, rather than due to variation in YP synthesis (Mares and Campbell 2001). This highlights the importance of measuring YP concentration directly when identifying QTL associated with YP concentration.

In tetraploid wheat three QTL associated with YP concentration have been identified in a backcross population (population 600545/2*Ombrabi5derived from a cross between a *Triticum dicoccoides* landrace (600545) and a *Triticum durum* cultivar (Omrabi5), the durum cultivar Omrabi5 was the recurrent parent) (Elouafi et al. 2001). In this population a major QTL associated with YP concentration, accounting for 53% of the total phenotypic variation, was identified in a similar region on

chromosome 7BL to QTL identified in bread wheat by Kuchel et al. (2006b) and Mares and Campbell (2001). The other two QTL associated with YP concentration were both on chromosome 7A and accounted for 6% and 13% of the total phenotypic variation in YP concentration. The more significant of these, accounting for 13% of variation, mapped to a similar region to QTL identified in bread wheat by Parker et al. (1998) and Mares and Campbell (2001). All three QTL were consistent across all environments, exhibited a strong genetic effect and only a weak QTL by environment effect.

A translocation (distal region of 7EL) from *Lophopyrum ponticum (L. elongatum)* has been transferred to bread wheat, replacing chromosomes 7D in one case and 7A in another (Lukaszewski 2006). The hexaploid bread wheat which had 7A replaced was used for transferring the translocation into a durum wheat. The translocation was created for the purpose of introducing a novel leaf rust resistance gene (*Lr19*) into wheat. The *Lr19* gene was found to be linked to a gene, or genes, (*Y*) conferring high endosperm YP concentration (Lukaszewski 2006), supporting reports of association between YP concentration and the group 7 chromosomes. There is a PCR marker available to trace the alien chromosome segment (Lukaszewski 2006).

In conclusion, multiple QTL associated with YP concentration, or flour colour, have been identified and each individual QTL generally only explains a small proportion of the total variance in YP concentration. This supports the model of polygenic control of YP concentration of wheat grain. There has been relatively little research published on the genetic control of YP concentration in durum wheat. In particular, there have been no marker trait associations identified or validated in Australian durum

germplasm for YP concentration. Favourable QTL conferred by Omrabi5 identified by Elouafi et al. (2001) could potentially be transferred by MAS into low YP germplasm by durum breeders targeting higher YP concentration. However, this line was not selected for Australian conditions and is poorly adapted to the Australian environment (data not shown) and therefore may not be a suitable parent for breeding. Furthermore there is no indication if favourable QTL from Omrabi5 would elevate YP concentrations in Australian germplasm. QTL associated with YP concentration have been identified on the A and B genome in Australian bread wheat germplasm (Mares and Campbell 2001). However, due to the low levels of YP concentration in Australian bread wheat relative to durum wheat, Australian bread wheat is unlikely to be a suitable source of high YP concentration for durum breeding. The successful application of marker assisted selection (MAS) within breeding programs has been well documented (Jefferies et al. 2003; Kuchel et al. 2006b; Yousef and Juvik 2001; Yu et al. 2000). Therefore, there is an urgent need to identify and/or validate QTL associated with YP concentration in Australian germplasm to provide Australian durum breeders with the option to select for this crucial quality trait with MAS strategies.

1.11 Environmental Influences on Yellow Pigment

Selection for high YP concentration is an important and high priority breeding objective for durum wheat breeding programs (Clarke 2005; Di Fonzo et al. 2005; Elias and Manthey 2005; Ozberk et al. 2005; Pena and Pfeiffer 2005; Pfeiffer and Payne 2005; Royo 2005; Saulescu 2005). Assaying large numbers of genotypes for YP concentration can be expensive. In addition to the cost of assaying, selection for improved YP concentration within a breeding program will inevitably result in a

reduction in genetic variation for other economically important traits, such as yield, disease resistance and other quality traits. With so many resources being spent addressing the genetic control of YP concentration, it becomes critically important to understand the environmental control of YP concentration. Clarke et al. (2006) explain that clarification of environmental effects on YP and the mode of inheritance would aid planning of crosses, facilitate development of genetic markers, and contribute to formulations of strategies to increase pigment concentration of durum wheat. Within the environments tested Clarke et al. (2006) reported little to no genotype by environment interaction. Since there are no published studies of YP concentration variation across environments with Australian durum germplasm it is not known what the expected extent of genotype by environment interaction in Australia may be.

With a better understanding of the environmental influence on YP concentration it may be possible to manipulate YP concentration levels through agronomic practices, rather than through breeding. This would of course either allow breeding program resources to become more targeted toward improving genetic gain for other economically important traits, or, simply reduce breeding costs. The milling and pasta industry may also benefit from an improved understanding of the environmental control of YP concentration that might allow more strategic sourcing, and blending of grain to best suit certain end products. Similarly the wheat industry may benefit from identifying areas which may be conducive to producing high quality durum, thereby expanding the industry, or conversely by avoiding attempts to expand into areas that have a high chance of failure of producing high quality durum. Finally, through a better understanding of the environmental control of YP concentration, breeders may

find more targeted or more efficient methods for selecting genotypes superior for this critically important quality trait.

Many studies have reported a significant effect of the environment on YP concentration in wheat (Clarke et al. 2006; Hatcher et al. 2006; Irvine and Anderson 1953; Johnston et al. 1983; Lee et al. 1976; Mares and Campbell 2001; Matus-Cadiz et al. 2003; Rharrabti et al. 2003a, b; Zhou et al. 2005). However, the specific mechanism causing variation in YP concentration between environments is not known. Seven durum wheat populations were evaluated across a wide range of environments by Clarke et al. (2006). Although there was a significant effect of environment, Clarke et al. (2006) concluded that it was not clear how environment affected YP concentration, with the exception of a killing frost prior to maturity at one environment which appeared to affect pigment development. However, significant negative correlations between YP concentration and both grain weight and test weight were identified, both between genotypes and between environments. Other authors have also reported a negative correlation between YP concentration and either grain weight (Alvarez et al. 1999; Markley 1937; Worzella 1942) or test weight (Whiteside et al. 1934; Worzella 1942).

Yellow pigment was reported by Rharrabti et al. (2003b) to be positively correlated with seasonal maximum temperatures during the whole growing season which also reduced thousand grain weight (TGW). In contrast, Clarke et al. (2006) cite Mangels (1932) reporting higher YP concentration in cool seasons with ample moisture compared to years with hot, dry conditions. Temperature during grain filling was also positively correlated with YP concentration in experiments reported by Clarke et al.

(2006) while precipitation was found to be negatively correlated with YP concentration. Similarly, Guttieri et al. (2001) found that moisture stress in bread wheat was associated with higher alkaline noodle yellow colour (CIELAB b*) and lower TGW. In addition, Pena and Pfeiffer (2005) reported that moisture stress was associated with a 9% increase in yellow colour (CIELAB b* value). This contrasts somewhat with Guler (2003) who reported that irrigation at sowing and jointing was associated with higher YP concentration. Rharrabti et al. (2003b) found no effect of precipitation on YP concentration. It was suggested by Clarke et al. (2006) that the observed negative correlation between grain weight or test weight could be due to dilution effects of other grain constituents such as starch. It was also suggested that biotic or abiotic stress may influence YP concentration (Clarke et al. 2006). A QTL associated with both yellow flour colour and grain size, with opposite parents contributing the allele for the high phenotype for the respective traits, has been reported by Hessler et al. (2002). Similarly Mares and Campbell (2001) attributed a QTL associated with yellow flour colour to the indirect effects of grain size. Lutein development in bread wheat during grain filling was shown to occur up until 12-15 days post anthesis, after which the lutein concentration was shown to decline as the grain weight increased (Graham and Rosser 2000) which is consistent with reports of a negative correlation between grain weight and YP concentration. Despite the correlation observed between YP and TGW, Clarke et al. (2006) concluded that factors other than grain and test weight were also affecting pigment colour.

To summarise, there are many reports of significant effects of the environment on YP concentration (Clarke et al. 2006; Hatcher et al. 2006; Irvine and Anderson 1953; Johnston et al. 1983; Lee et al. 1976; Mares and Campbell 2001; Matus-Cadiz et al.

2003; Rharrabti et al. 2003a, b; Zhou et al. 2005). However, other than the relationship between grain size and YP concentration, the literature attempting to explain the environmental effects on YP concentration is inconclusive and the specific cause of variation in YP concentration across environments is generally unknown. Reports of a negative correlation between YP concentration and TGW are generally consistent across publications. Nevertheless, other factors are likely to be also affecting YP concentration (Clarke et al. 2006). Temperature and water stress have been reported to be implicated with variation in both YP concentration and TGW. Therefore further research is required to establish which specific environmental variables are causing variation in YP concentration particularly independent of the induced effects of variation in grain weight, and, if temperature and water stress are having a direct effect on YP concentration or an indirect effect through changes in grain weight.

1.12 Conclusion

Durum wheat is an important food source for the world with annual global production of approximately 35 Mt. Durum is also an important crop in Australia where on average approximately 400,000 tonnes are produced annually, but the production is highly variable between years. YP concentration is one of the most important quality traits of durum wheat and there is an urgent need to improve the YP concentration of durum wheat originating from South Australia to maintain and/or improve South Australia's durum grain and pasta competitiveness in international markets.

Effective and accurate methods of quantifying YP concentration, even on small flour samples, are available. In addition, cheap and high throughput methods of estimating YP concentration are available using reflectance spectroscopy as well as a non-destructive method utilising NIR. There are many factors which can influence both the expression of YP and the perception of YP in flour, semolina or pasta. It is expected that many of these traits may be under separate genetic and environmental control. It is suggested that for research requiring accurate data, YP concentration should be measured directly, rather than estimated from the colour of flour, semolina or pasta using reflectance spectroscopy or NIR.

Yellow pigment is generally a highly heritable but complex trait under additive genetic control. Multiple QTL associated with YP concentration have been identified, particularly in bread wheat, and mostly locate on the group 3 and 7 chromosomes. However, there have been no known significant marker-trait association studies focussed on YP concentration using purely durum wheat germplasm and there are currently no known QTL identified that Australian durum wheat breeders could sensibly target in a MAS strategy aiming to improve selection for YP concentration. This is urgently required in order to assist breeders aiming to improve this important quality trait in Australia and is a primary objective of this thesis.

Significant environmental effects on YP concentration have been reported. In addition a negative correlation between YP concentration and grain weight has been consistently reported in published literature. Differences in temperature and plant available water during grain filling have also been reported to be associated with variation in YP concentration. Further research is required to determine the effect of

specific environmental variables on YP concentration and if differences in temperature and plant available water affect YP synthesis directly, or, indirectly through their impact on grain weight. This thesis aims to address these knowledge gaps.

Chapter 2: General Methods

2.1 Introduction

This chapter describes methods for yellow pigment (YP) and yellow flour colour (CIELAB b*) analysis of flour and provides details on the genotypes and locations of field trials that were used throughout this thesis. Methods which are more specific to a specific experiment are described within each chapter.

2.2 General Methods

2.2.1 Flour Colour Analysis

Colour analysis of each flour sample was analysed by averaging three reflectance spectroscopy measurements using a Minolta colorimeter (Konica Minolta Chroma CR-400; Osaka, Japan). Flour samples were assessed for yellowness (b*), brightness (L*) and redness (a*) as defined by the Commission Internationale de l'Eclairage (CIE) to provide a CIELAB value. Only the CIELAB b* value for yellow flour colour has been reported throughout this thesis. Measurements were made within 14 days of milling.

2.2.2 Yellow Pigment Analysis

Flour production varied between experiments and so details are described in each chapter. Yellow pigment analysis of flour was performed using the standard American Association of Cereal Chemists (AACC) method 14-50.01 (AACC 1999) with the exception that methanol was used instead of water-saturated butanol as the extraction solvent as methanol is easy to use and less toxic than butanol. Comparison of solvents showed high correlation provided low or zero lutein ester content (Mares 2005a) and

there are no lutein esters in Australian durum wheat varieties (Mares 2005a). Three gram samples of flour were weighed into plastic scintillation vials and 15 ml of methanol added. The vials were gently shaken for 15 minutes on a mechanical rocker. The methanol / flour samples were poured from the plastic scintillation vials into glass tubes and spun on an Orbital 420 centrifuge at 3000 rpm for 10 minutes. The glass tubes were taken from the centrifuge and placed into a water bath at approximately 50°C for approximately 5 minutes. The supernatant was filtered using a cellulose acetate disposable syringe filter (Advantec; Bunkyo-Ku, Japan) and optical density at 440nm recorded using a Varian Cary 3 Bio UV-visible spectrophotometer after calibrating with pure methanol. YP concentration was expressed as mg/kg using a correction coefficient of 30.1 as recommended by the American Association of Cereal Chemists (AACC 1999). Extraction and quantification of YP from the 3g flour samples were generally conducted on a total of 22 flour samples at a time which will be referred to as a "batch". As a control, two 3g samples of control flour (bread wheat genotype Sunco) were included in each batch. For each flour sample, a minimum of two extracts were analysed in separate batches.

2.2.3 Yellow Pigment Content/ Grain Calculation

Yellow pigment expression was reported both as concentration, mg/kg, and as an absolute amount per grain, ng/grain. YP Concentration (mg/kg) was reported because it is relevant to the end-use of the grain and YP content per grain (ng/grain) was reported because it was anticipated that it would be less affected by environmental factors. Environmental factors influence final grain weight and can confound genetic effects for grain constituents that are synthesised early in grain development. The YP/grain

(ng/grain) was calculated by multiplying the estimated flour YP concentration (mg/kg), by the one thousand grain weight (TGW) (in grams) (Equation 1).

$$YP/seed(\text{ng})/\text{seed} = YP(\text{mg}/kg)..X...TGW(g) \qquad \text{Equation 1}$$

2.2.4 Grain Weight Calculation

One thousand grain weight was estimated by counting and weighing one thousand grains randomly sampled from a harvested bulk of each field plot. If one thousand grains were not available for a sample all the grains were counted and weighed and the TGW was calculated by extrapolating this data.

2.2.5 Genotypes

Grain samples sourced from yield trials (sections 2.2.6 and 3.2.1) of 11 genotypes comprising 10 of the most widely grown Australian durum wheat varieties, plus an unreleased but highly adapted durum wheat breeding line (WID22221), were selected for analysis of variation in YP concentration across environments (Table 2.1). WID22221 was chosen as preliminary data showed this line to produce very high concentrations of YP (data not shown). Wherever possible, grain from the complete set of all 11 genotypes were analysed for YP concentration. However, this was not always possible which resulted in an unbalanced data set which was considered during the statistical analysis of the results (section 3.2.4).

Table 2.1: Genotypes used in the GxE analysis for yellow pigment concentration. Pedigree, adaptation regions, and number of site year combinations sampled are presented

Genotype	Pedigree	Primary region of adaptation[A]	Years sampled (no. of sites in parenthesis)
Tamaroi	Altar-84/4/Tam-1-B-17/Kamilaroi/3/Wells/56111//Guillemot	SA	2003 (12), 2005 (13), 2006 (6), 2007 (11)
Kalka	Wollaroi/3/Linghzi/ Yallaroi//RH880009	SA	2003 (12), 2005 (13), 2006 (9), 2007 (11)
Yallaroi	Guillemot Sel. No. 3/Kamilaroi sib.	NSW	2003 (12), 2005 (13), 2006 (8), 2007 (11)
Wollaroi	Tam 1-B-17/Kamilaroi sib.// Rokel Sel./Kamilaroi sib	NSW	2005 (13), 2006 (9)
Gunderoi	Pedigree not known. Breeding code CID522121	NSW	2003 (12), 2005 (2), 2006 (9)
Bellaroi	Yallaroi//Tam-1-B-17/Kamilaroi/4/Tam-1-B-7/Kamilaroi/3/Rokel Sel.//Kamilaroi/5/Sterna/Sula Sel.	NSW	2003 (12), 2005 (13), 2006 (9)
Arrivato	Tetraprelude/Waitohi	NSW	2003 (12), 2005 (2), 2006 (9)
Jandaroi	920777/111586	QLD/NSW	2007 (11)
Hyperno	Tamroi/Kalka sib	QLD/NSW/SA	2006 (8), 2007 (9)
Saintly	Kalka sib//Kalka sib/2*Tamaroi	QLD/NSW/SA	2006 (4), 2007 (11)
WID22221	(W/2/6/3*W9/6/-2a)*WD98002/6)/7/2	SA	2006 (5)

[A] NSW=New South Wales, SA=South Australia, QLD=Queensland

For analysis of genetic control of variation in YP concentration and QTL analysis, two bi-parental populations of recombinant inbred lines (RILs) were developed. The first population was developed between the parents Wollaroi and Tamaroi by single seed decent (SSD) to identify and/or validate QTL associated with genetic variance in YP concentration, to study genotype x environmental (G x E) interactions, and to investigate the genotypic relationship between TGW and both YP concentration and YP/grain. These two parents represent the extremes of genetic variation for YP amongst Australian durum cultivars. Wollaroi is a semi-dwarf (*Rht1*) line that produces very high concentrations of flour YP, is early to midseason in maturity and is adapted to Australia's northern durum growing regions in New South Wales and Queensland. Tamaroi, generally has moderate concentration of flour YP compared to many durum cultivars internationally, however is amongst the lowest of all Australian cultivars. Tamaroi is a semi-dwarf (*Rht1*) line adapted to Australia's southern durum growing regions in South Australia (SA) and has similar maturity to Wollaroi, often about two to four days later flowering than Wollaroi. Both lines were developed by the New South Wales (NSW) Department of Primary Industries Durum breeding program led by Dr Ray Hare. Two hundred and ninety individual F_2 seeds derived from F_1 hybrid seed, with parents Wollaroi and Tamaroi, were self-pollinated to produce F_3 seed and a single seed from each of the 290 F_3 plants was grown and allowed to self-pollinate. This procedure was repeated for each generation until the F_5 generation. After the F_5 generation each RIL was regarded as being generally homozygous and homogeneous. Individuals of this population are hereafter referred to as being F_5 derived. For each generation, each RIL was allowed to self-pollinate sufficiently distant from one another to minimise the chance of outcrossing. Both parents display very poor anther extrusion and a very cleistogamous phenotype and so

considering the phenotype of the parents and the growing conditions, the risk of outcrossing was anticipated to be very low. Care was taken to ensure there was no selection during population development. Generations were progressed at a rate of three generations per year by utilising controlled environment facilities where temperature and day length were controlled. Seeds were pre-germinated in petri dishes at four degrees Celsius to break any potential dormancy and to help prevent selection against any unexpected vernalisation requirements. There were no large differences within the population for height or maturity.

The second population of RILs was developed also by SSD and derived from F_1 hybrid seed developed by hybridising WID22221 with Tamaroi. The aim of developing this population was to validate results produced from the Wollaroi/Tamaroi population in a different background and to validate previously published results using Australian tetraploid germplasm grown in Australia. WID22221 is a semi-dwarf (*Rht1*) breeder's line, produces very high grain concentrations of YP, is adapted to the durum growing regions of SA, is of medium maturity very similar to Tamaroi and was developed by Australian Grain Technologies (AGT) and the University of Adelaide (UA) and selected by Dr Jason Reinheimer. The processes used for population development were the same as the Wollaroi/Tamaroi population. The WID22221/Tamaroi population consists of four subpopulations from four separate individual F1 seeds to guard against any potential effects of selfing. From these four F_1 seeds 73, 188, 55 and 23 F_5 derived progeny were developed and multiplied for testing. The total population therefore consists of 339 F_5 derived RILs.

2.2.6 Environments Sampled for Non-genetic Control of Yellow Pigment

Grain for analysis of YP variation across environments was obtained from replicated field yield trials (section 3.2.1) from 22 sites over five years between 2003 and 2007 inclusive (Table 2.2). Within each trial at a location each genotype was replicated three times. Grain samples from all three replicates were bulked together for YP analysis. From the 22 locations over the five year period there were 46 site x year combinations, hereafter referred to as "environments". The two distinct durum wheat growing regions in Australia, North (Northern NSW) and South (the Mid North and Yorke Peninsula regions of South Australia), were sampled as well as other sites from more marginal durum growing regions, such as the Eyre Peninsula and Murray Mallee in South Australia, the western Mallee in Victoria and the Riverina in southern NSW.

2.2.7 Trials for Genetic and QTL analysis

2.2.7.1 General overview of experiments for genetic and QTL analysis

Trials were conducted over five seasons at two field locations and one trial was conducted in a glasshouse. Wollaroi/Tamaroi RILs were trialled at four field experiments during 2006 and 2007. WID22221/Tamaroi RILs were trialled in one glasshouse experiment and one field experiment in 2007/2008 and 2008 respectively. Phenology data was not recorded on individuals of either population.

Table 2.2: Sites sampled for grain YP concentration, region of site and number of genotypes sampled from each site for each year between 2003 and 2007. NSW=New South Wales, SA=South Australia, VIC=Victoria, YP=Yorke Peninsula, EP=Eyre Peninsula

Site	Region	Years sampled (number of genotypes sampled for each year in parentheses)
Biniguy	Northern NSW	2006 (8)
Booleroo	Northern Mid North, SA	2003(6), 2005(5), 2006(8), 2007(6)
Cummins	Lower EP, SA	2003(6), 2005(5), 2007(6)
Horsham	Western Mallee, VIC	2005(7)
Kimba	Upper Mid EP, SA	2003(6),
Minnipa	Western EP, SA	2003(6), 2005(5), 2007(6)
Mintaro	Lower Mid North, SA	2003(6), 2005(5), 2006(9), 2007(6)
Narrabri	Northern NSW	2005(7), 2006(7),
Palmer	Murray Mallee, SA	2004 (6)
Paskeville	Lower Mid North, SA	2003(6), 2005(5), 2007(6)
Quirindi	Northern NSW	2006(7)
Roseworthy	Lower Mid North, SA	2006(9)
Rudall	Mid EP, SA	2005(5), 2007(6)
Spalding	Mid North, SA	2003(6), 2005(5), 2007(6)
Tamarang	Northern NSW	2006(8)
Turretfield	Lower Mid North, SA	2003(6), 2005(5), 2007(6),
Ungarra	Mid EP, SA	2003(6), 2005(5), 2007(6)
Urania	Mid YP, SA	2003(6), 2005(5), 2007(6)
Winulta	Upper YP, SA	2006(9)
Wokurna	Upper YP, SA	2003(6), 2005(5), 2007(6)
Wunkar	Murray Malley, SA	2003(6),
Yenda	Riverina, southern NSW	2006(11)

2.2.7.2 Turretfield 2006 - Wollaroi/Tamaroi RILs

Wollaroi/Tamaroi RILs (F_5 derived F_6 ($F_{5:6}$) seed) were hand sown at Turretfield (longitude 138.83, latitude -34.5) in the mid-north of South Australia. The trial design was a completely randomised design with repeating grid checks (Wollaroi and Tamaroi) alternating every 20 plots. For all trials, seed of test lines (RILs) and seed of check lines were sourced from a common seed multiplication trial to eliminate variation due to seed source. Each RIL was grown in a single replication consisting of approximately 100 seeds hand sown in a single 1 meter strip (plot) with parental checks every 20 plots. Plots were sown such that there were 8 plots sown side by side (8 rows) and 44 plots sown end on end (44 columns). Therefore the spatial layout of the field trial consisted of a total of 352 plots sown in 8 rows and 44 columns. A gap of approximately 150 mm was left between rows while columns were approximately 200 mm apart. The trial was sown on the 15th June 2006. The trial was kept weed free for the duration of the trial. The trial was hand harvested using a sickle after harvest maturity in early December and grain threshed using a mechanical, custom made harvester provided by the Australian Grain Technologies' durum breeding program.

2.2.7.3 Waite 2006 / 2007- Wollaroi/Tamaroi RILs

Wollaroi/Tamaroi RILs were grown at the Waite campus, University of Adelaide (longitude 138.63, latitude -34.97) over the summer of 2006 / 2007 with the same trial design as for the Turretfield 2006 trial but with a different randomisation. Seed was $F_{5:7}$. The trial was sown by hand on the 22nd December 2006. The trial was kept weed free for the duration of the trial. The trial was harvested by hand at harvest maturity in mid May 2007 and grain threshed using a mechanical harvester.

2.2.7.4 Turretfield 2007- Wollaroi/Tamaroi RILs

Wollaroi/Tamaroi RILs ($F_{5:8}$ seed) were grown at Turretfield in 2007. The trial design was as for the Turretfield 2006 trial but with a different randomisation. The trial was sown by hand on the 25th June 2007.

2.2.7.5 Waite 2007 - Wollaroi/Tamaroi RILs

Wollaroi/Tamaroi RILs ($F_{5:9}$ seed) were grown Waite Campus as a completely randomised block design. Each of the two blocks were as for the other trials in terms of plot size and replication of checks and a single replication of each individual of the Wollaroi/Tamaroi population. Each block was randomised differently and can be regarded as separate replicate. This trial was sown by hand on 29th June 2007.

2.2.7.6 Waite 2007 / 2008 - WID22221/Tamaroi RILs

One plant of each of the 339 WID22221/Tamaroi RILs and the parents, WID22221 and Tamaroi, were grown in 23 free draining trays in sterilised coco peat soil over the summer of 2007/2008. The seeds were sown on January 1st 2008 and plants were grown in a glasshouse at the Waite campus. The glasshouse was cooled by evaporative air conditioners that were regulated by a thermostat to operate at temperatures above 15 degrees Celsius. The primary objective was to multiply seed for trials in the 2008 winter/spring season. Flour colour analysis was conducted on 222 individuals of the population which yielded sufficient seed for both future trials and milling for flour colour analysis. Each plant was hand harvested and threshed using a mechanical thresher.

2.2.7.7 Turretfield 2008 - WID22221/Tamaroi RILs

WID22221/Tamaroi RILs were trialled at Turretfield in 2008. The trial design was as for the Wollaroi/Tamaroi trial at Turretfield in 2006, however WID22221/Tamaroi RILs were sown instead of Wollaroi/Tamaroi RILs and the checks every 20 plots were WID22221 and Tamaroi. The trial was sown in 6 rows and 60 columns and was sown on 17th June 2008.

2.2.8 Statistical Analysis

Analyses of variance (ANOVA), simple linear and multiple linear regression analysis, and Restricted Maximum Likelihood (ReML) analysis to calculate best linear unbiased estimates (BLUEs) were performed using Genstat 11 software (Rothamstead Experimental Station, Harpenden, England). Significant differences between means from various treatments were determined using a Tukey test with JMP software (JMP 5.0.1.2 (1989–2003, SAS Institute Inc., Cary, NC, USA). Normality of frequency distributions were statistically tested using an Anderson-Darling test for normality using JMP software (JMP 5.0.1.2, 1989–2003, SAS Institute Inc., Cary, NC, USA).

Part I – Analysis of Non-Genetic Control of Yellow Pigment Concentration in Durum Wheat

Chapter 3: Yellow Pigment Variation across Environments

3.1 Introduction

Consumers prefer pasta which is coloured bright yellow (Troccoli et al. 2000) which is why yellow pigment (YP) concentration is one of the most important quality traits of durum wheat for pasta production (Irvine 1971). Selection for high levels of YP concentration is an important breeding objective for durum wheat breeding programs (Clarke 2005; Di Fonzo et al. 2005; Elias and Manthey 2005; Ozberk et al. 2005; Pena and Pfeiffer 2005; Pfeiffer and Payne 2005; Royo 2005; Saulescu 2005). Breeding programs commonly select for high YP concentration indirectly by using reflectance colorimeters on milled flour (Pena and Pfeiffer 2005) which offer benefits of safety, high throughput, and a good correlation between chemical extraction and reflectance measurement methods (Acquistucci and Pasqui 1991; Johnston et al. 1980). Modern near-infrared reflectance spectrophotometer (NIR) instruments have now extended the spectral range to include the visible region which can be used to estimate yellow pigment concentration (McCaig et al. 1992). NIR reflectance spectrophotometry offers breeding programs the benefit of being non-destructive, allowing for early generation phenotyping when seed quantities are low. NIR reflectance spectrophotometry is also very high throughput and can simultaneously generate other important data such as protein and moisture content and grain hardness. However, if visible reflectance spectroscopy is being used to select for high YP concentration then the ability of this method to predict YP concentration needs to be validated to ensure measurements are not being confounded by variation in other variables such as protein, hardness, vitreousness, brightness (L*), redness (a*) or activity of enzymes such as lipoxygenase or polyphenol oxidase (PPO). Although

results from reflectance spectroscopy are reportedly closely correlated to wet chemistry methods for determining YP concentration (Acquistucci and Pasqui 1991; Johnston et al. 1980), there are no published reports on the reliability of this method using Australian germplasm grown in Australia. Whilst activity of enzymes and semolina particle size are important factors contributing to end product quality, they are likely to be under separate genetic control to YP concentration and may not be as heritable. Therefore, for the purpose of genetic selection it may be more efficient to first select directly for YP concentration and then for each of these other factors separately.

Many quality traits of wheat, including YP concentration, have been shown to be strongly influenced by environment (Clarke et al. 2006; Hatcher et al. 2006; Irvine and Anderson 1953; Johnston et al. 1983; Lee et al. 1976; Mares and Campbell 2001; Matus-Cadiz et al. 2003; Rharrabti et al. 2003a, b; Zhou et al. 2005). Moisture stress and/or irrigation have been reported to be associated with variation in YP concentration or flour colour (CIELAB b*) in one direction or another (Guler 2003; Guttieri et al. 2001; Pena and Pfeiffer 2005). Reports on the effect of moisture stress and/or irrigation are contradictory to one another suggesting further research is required. YP concentration has been reported to be associated with temperature during the growing season (Clarke et al. 2006; Mangels 1932; Rharrabti et al. 2003a) and also negatively correlated with grain weight (Clarke et al. 2005; Hessler et al. 2002; Mares and Campbell 2001). It has been suggested that the association between YP concentration and grain weight may be due to starch dilution (Clarke et al. 2005; Hessler et al. 2002; Mares and Campbell 2001), but underlying causes of variation in YP concentration across environments are not well understood.

Within wheat breeding programs it can be useful to understand the mechanism causing variation in a target trait across environments and what specific variables are contributing to this variation so that the most efficient and effective selection strategy can be adopted. From a broader industry perspective it is also useful to understand causes of variation in YP across environments for the purpose of identifying regions that may be suitable for growing and sourcing high quality durum wheat. There are no published studies comparing Australia's two durum growing regions (in the north and in the south) for production of high quality durum in terms of YP concentration. Similarly there are no published studies which have investigated the potential for non-traditional durum growing regions in Australia to produce high quality durum high in YP content.

The objective of the research presented in this thesis was to improve understanding of the specific causes of variation in YP concentration of durum wheat across environments in Australia. This research involved making comparisons between grain YP concentration and various environmental parameters over a wide range of environments. The outcomes of this research could also assist in;

(i) Determining the accuracy of measuring YP concentration indirectly with the use of reflectance spectroscopy

(ii) Determining the level of variation in flour YP concentration between environments in Australia;

(iii) Determining the range in variation among Australian cultivars for YP concentration

(iv) Determining if there is any difference in flour YP concentration originating from grain grown in Australia's northern durum growing region relative to that grown in Australia's southern durum growing region

(v) Determining if the flour YP concentration originating from grain grown in traditional durum growing regions is different to grain produced in non-traditional durum growing regions

3.2 Methods

3.2.1 Multiple Environment Field Trials

Grain samples of genotypes (Table 2.1, Chapter 2) were collected from field plot trials conducted by South Australian Research and Development Institute (SARDI) (www.sardi.sa.gov.au), National Variety Trials (NVT) (www.nvtonline.com.au), and Australian Grain Technologies (AGT) (www.ausgraintech.com). Grain samples were analysed for YP concentration, flour colour (CIELAB) reflectance spectrometry values, and thousand grain weight (TGW). The purpose of the trials conducted by SARDI, NVT and AGT was to evaluate yield and physical grain quality of elite breeding lines and commercial cultivars over a range of environments. Yield data for each genotype grown at each location was obtained and used in the analysis to compare yield data to YP concentration and also to TGW. Each genotype was replicated three times within each site and each replicate was represented by a single plot. A composite sample of 150g from each of the three plots for each genotype from each site was provided for analysis. Genotypes and environments sampled were as explained in Chapter 2 (Tables 2.1 and 2.2 respectively).

3.2.2 Milling

3.2.2.1 2003, 2006 and 2007 Grain Samples

For each grain sample moisture content was measured on 100 crushed grains using a Shizuoka Seiki (model CTR-800E; Shizuoka-Ken, Japan) single grain moisture tester. An 80g sample of grain from each sample was raised to 12% moisture (w/w) by adding water two days before milling and then up to 15% moisture (w/w) by adding water the day before milling. The conditioned grain was milled using a temperature-controlled Brabender Quadrumat Junior mill (Duisburg, Germany). Temperature was maintained between 22°C and 26°C. Only flour was used for analysis of colour and yellow pigment, with the bran and germ discarded. Between milling and analysis of colour and pigment concentration the flour was stored in the dark at room temperature. Flour colour and yellow pigment were assessed as per methods described in Chapter 2.

3.2.2.2 2004 and 2005 Grain Samples

For the 2004 and 2005 grain samples 10 grains from each sample for each replication were weighed and then pulverised into wholemeal with a 3M Espe Rotomix™ capsule mixer (3M Espe, USA). Grain was loaded into a capsule together with a steel ball bearing and placed on the Espe rotational/centrifugal capsule mixing unit for ten seconds to reduce the grain to a uniform, homogeneous powder. For all other samples, methods outlined in 3.2.2.1 for the 2003, 2006 and 2007 samples were adopted.

3.2.3 Weather Data

Weather data for each trial site was obtained from the Australian Bureau of Meteorology recording station closest to the experimental site or interpolated from Bureau of Meteorology sites using the Silo data base (http://www.nrw.qld.gov.au/silo/ppd/index.html) for the period from September 20th to November 4th which broadly represents the grain filling period for these sites in these seasons. As grain for YP analysis was obtained opportunistically from breeding trials and independent variety trials post-harvest, flowering dates were not recorded and so more precise dating of the grain filling period was not possible. Similarly, phenology data for individual genotypes was also not available. The following climatic variables were recorded for the period between September 20th and November 4th, except for the whole season average daily maximum temperature which was represented by the data available through the period from June 1 to November 4;

Average maximum temperature

Average minimum temperature

Average net solar radiation

Total cumulative rainfall

Relative humidity at maximum temperature

Relative humidity at minimum temperature

Vapour pressure

Evaporation and

Whole growing season average daily maximum temperature

3.2.4 Data and Statistical Analysis

Laboratory analytical data was analysed with a Restricted Maximum Likelihood (ReML) analysis to calculate best linear unbiased estimates (BLUEs) of YP concentration for each genotype that was sampled from each environment. Genotype was entered into the model as a fixed term. To account for laboratory error, batch number, day of analysis and day of milling were included in the model as random terms in the event that the variance attributable to these terms was at least as large as the standard error, which was rare. Because only a single composite sample was provided of each genotype from each environment from field trials, the standard error associated with YP concentrations of each genotype from each environment could not be calculated. To examine the effect of specific environmental characteristics and plant traits on YP concentration, simple linear and multiple linear regression analysis was performed. Analysis of variance (ANOVA) of the effect of environment and genotype on YP concentration (Table 3.2) was performed using an unbalanced treatment structure. YP concentration was fitted to the model as the response variate and environment and genotype were fitted as the explanatory variates (factors). Mean YP concentration of each genotype was estimated with ANOVA. To account for the unbalanced data set, site and year were blocked as nuisance factors. Similarly, mean YP concentration and standard error of site and region (south vs. north and also traditional vs. non-traditional) were estimated by ANOVA blocking genotype and year as nuisance factors. Correlations between environments were calculated in Microsoft Excel. The mean correlation value between a set of environments was calculated by adding the correlation value of each pair of environments and then dividing that number by the number of pairs of environments. Amount of phenotypic

variance explained or accounted for by any factor or variable was calculated with a regression analysis.

3.3 Results

3.3.1 Estimation of Yellow Pigment Concentration by Reflectance Spectroscopy

There was a highly significant (P<0.001) correlation between CIELAB b* values and the YP concentration estimates based on pigment extraction by methanol and quantification by spectrophotometer. The CIELAB b* analysis explained 86 per cent of the observed variation in traditional wet chemistry method (Figure 3.1 and Table 3.1). The correlation between CIELAB b* values and the YP concentration estimates was stronger in the years that YP concentration was estimated from 3g of flour (2003, 2006 and 2007) rather than from 10 ground grains (2004 and 2005) (Table 3.1).

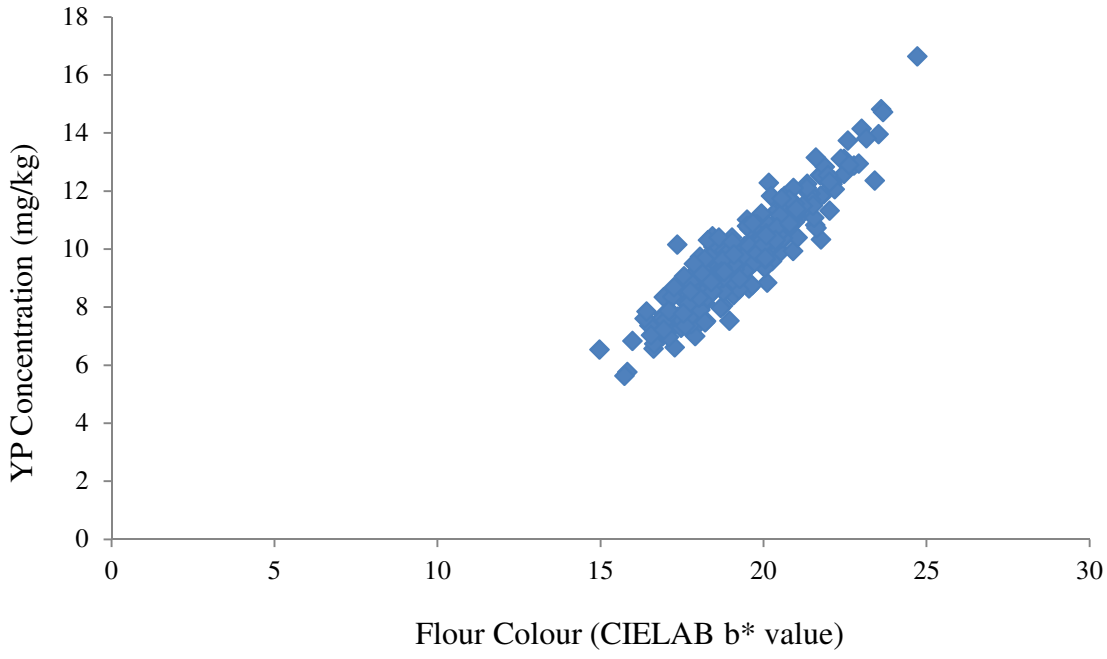

Figure 3.1: Relationship between flour colour (CIELAB b*) and yellow pigment (YP) concentration for 11 genotypes grown across 21 sites over seasons 2003 and 2005- 2007 (r = 0.93, n=255

Table 3.1: Correlation (r) between flour colour (CIELAB b* values) and flour yellow pigment concentration (mg/kg) for all durum genotypes tested across all years and number of samples (N) for each year

Year	r	N
2003	0.95	23
2004	0.68	83
2005	0.87	61
2006	0.95	83
2007	0.93	88

3.3.2 Characterisation of Genotypes and Environments for Yellow Pigment Concentration across Sites and Years

Both Genotype and Environment had a significant ($p<0.001$) effect on YP concentration (Table 3.2). The means square from ANOVA indicates the relative contribution of Genotype and Environment to the observed variance in YP concentration. Within this set of Genotypes and Environments, genotype (mean square 59.26) contributed a greater portion of variance in YP concentration than Environment (mean square 15.86) (Table 3.2). Since there was just a single grain sample for each genotype from each environment, the interaction effect between environment and genotype was not included in the analysis due to a lack of degrees of freedom.

Table 3.2: Results from analysis of variance of yellow pigment concentration presenting sum of squares, means squares and significance of effect (p value) associated with Environment and Genotype across all field trials

Source	Sum of Squares	Means Squares	p value
Environment	919.98	15.86	<.001
Genotype	592.62	59.26	<.001

There was a very large range in YP concentration across sites, even within a single season; for example, in samples from South Australia in the 2006 season the YP concentration of the genotype Kalka ranged from 5.64 mg/kg at Winulta to 11.43 mg/kg at Mintaro (Figure 3.2). The range in YP concentrations was even larger between seasons (Table 3.5 and Figure 3.3). YP concentration for Kalka at Mintaro ranged from 4.53 mg/kg in 2004 to 11.43 mg/kg in 2006. For comparison, the largest range between genotypes at any site was at Narrabri in 2006 where Arrivato produced 5.76 mg/kg YP concentration and Hyperno 13.74 mg/kg YP concentration.

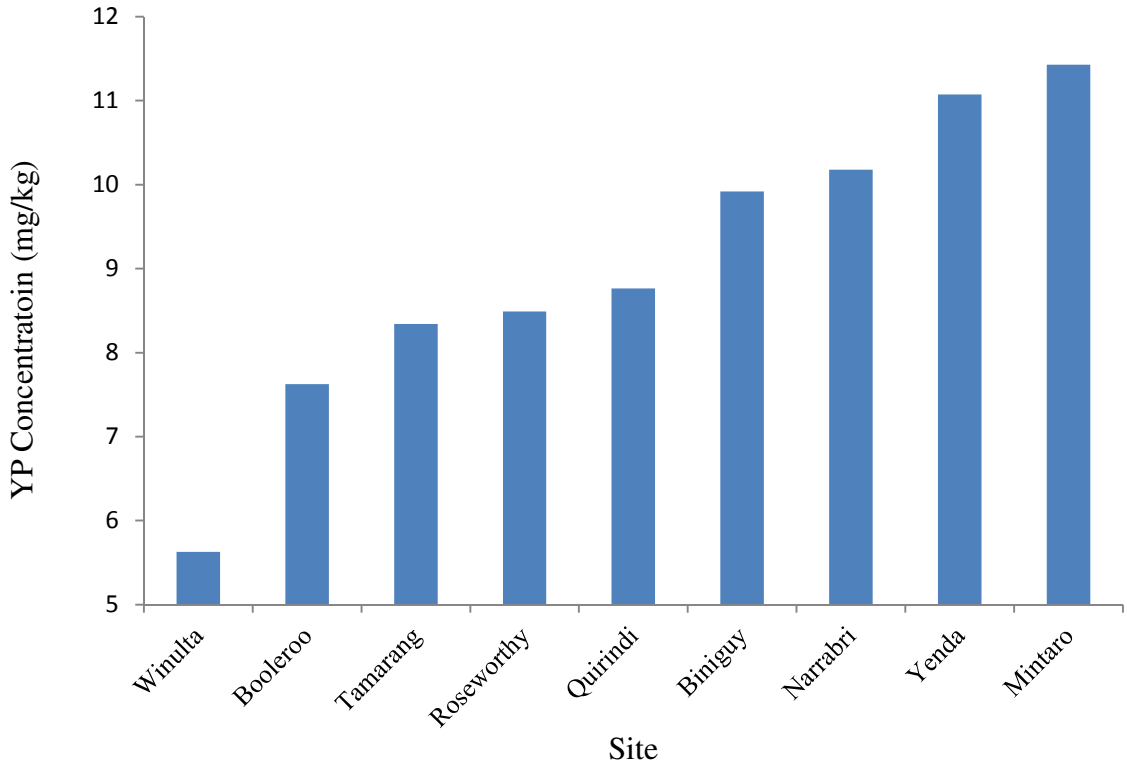

Figure 3.2: Yellow pigment (YP) concentration of the genotype Kalka from a selection of sites in 2006

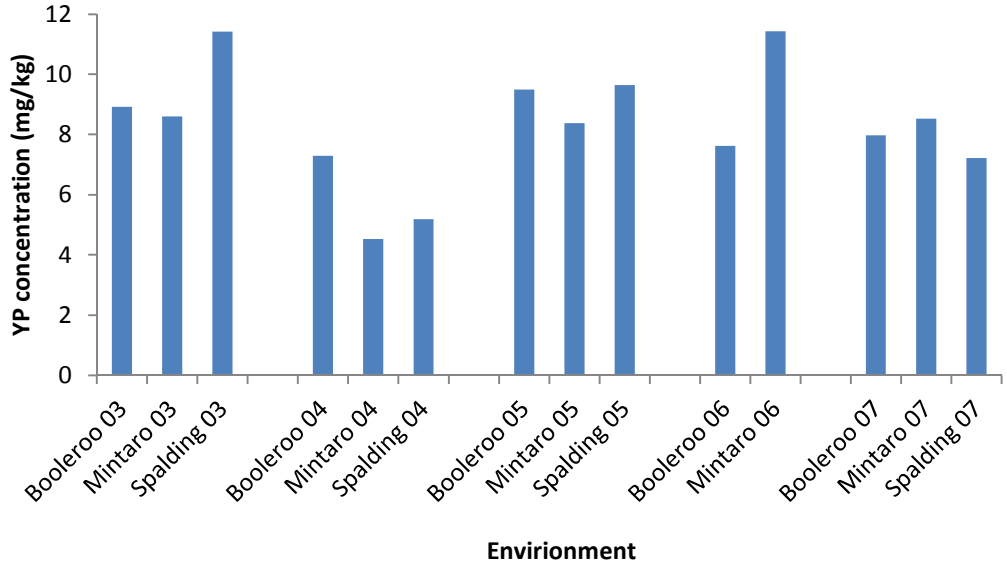

Figure 3.3: Yellow pigment (YP) concentration of the genotype Kalka from a range of environments including Booleroo, Mintaro and Spalding in years 2003 to 2007

Site mean YP concentration of grain samples were very inconsistent across years and YP concentrations of grain samples from various sites were inconsistent relative to one another (Table 3.3 and Figure 3.3). For example, the YP concentration of Kalka in 2004 was higher at Booleroo than at Mintaro but this was reversed in 2006 and a similar reversal in ranking occurred between Booleroo and Spalding in 2003 and 2004 (Figure 3.3). Despite the strong environmental effect on YP concentration, differences between genotypes in YP concentration were generally very well conserved across years (Table 3.4) and sites, indicating very limited genotype by environment interaction. The YP concentration data from each individual site correlated strongly with data from each of the other sites. A correlation matrix for all possible pairs of the 59 environments has not been presented due to the size of such a matrix; however, the average correlation (r) value between each pair of environments was r = 0.88.

Although YP concentration data of each site correlated strongly with all the other sites, YP concentration data from some sites, as might be expected, aligned more closely with data from the remaining 46 sites than others; there was no simple reason identified for this and it was not related to site mean YP concentration and may have simply been due to random variation in the data.

Table 3.3: Site mean flour yellow pigment concentration (mg/kg) from all sites sampled from years 2003-2007. Genotype and year were blocked as nuisance factors in the analysis of variance. Each site has been classified as representing either Australia's southern or northern wheat growing regions and has also been classified as being either a traditional (T), non-traditional (N) or marginal (N/T) durum growing region

Region	Site	2003	2004	2005	2006	2007	All data
South (N)	Yenda				11.69		10.05
South (T)	Spalding	11.14	7.14	10.18		9.04	9.70
South (N)	Minnipa	9.21	7.84	9.22		10.85	9.64
South (T)	Paskeville	9.81	6.74	9.96		10.05	9.51
South (N)	Lock		7.87	9.23		9.36	9.34
North (T)	Biniguy				11.14		9.32
South (N/T)	Turretfield	9.79	6.28	10.02		9.27	9.24
South (T)	Wokurna	9.75	6.48	8.14		10.44	9.09
South (T)	Mintaro	8.40	5.63	9.79	11.18	9.93	8.99
South (N/T)	Booleroo	8.88	8.11	10.10	8.82	9.57	8.93
South (N)	Cummins	9.14	6.56	8.61		9.74	8.87
North (T)	Quirindi				10.22		8.43
South (N)	Ungarra	8.47	5.90	8.77		9.03	8.37
South (N)	Wunkar	8.24	6.38				8.36
South (T)	Urania	8.49	5.67	8.66		9.25	8.35
South (N)	Kimba	8.07	6.49				8.26
North (T)	Narrabri			7.15	10.91		8.18
South (N/T)	Roseworthy				9.39		7.85
South (N)	Palmer		5.59				7.71
North (T)	Tamarang				9.03		7.21
South (N)	Horsham			6.73			6.76
South (T)	Winulta				7.84		6.30

Table 3.4: Genotype mean yellow pigment concentration (mg/kg) across all sites for each year from 2003 to 2007 estimated with ANOVA. Site and year were blocked as nuisance factors to account for the unbalanced dataset

Genotype	2003	2004	2005	2006	2007	All data
Hyperno				12.15	11.94	11.26
WID22221		8.29		13.45		11.11
Wollaroi		8.83		10.84		10.34
Jandaroi					10.87	10.31
Bellaroi	11.62	6.47	11.64	11.36		9.86
Saintly				9.63	10.32	9.49
Yallaroi	9.19		9.19	9.39	9.34	8.57
Kalka	9.17	5.92	9.17	9.06	7.79	8.00
Gunderoi	8.29		8.29	8.66		7.48
Tamaroi	8.34	5.01	8.34	8.54	8.15	7.40
Arrivato	8.08	5.36	8.08	8.22		7.37

There was no significant difference in YP concentrations observed between Australia's two durum growing regions, in the north and the south (Tables 3.3 and 3.5) or between traditional and non-traditional durum growing regions (Tables 3.3 and 3.6). Comparisons from multiple sites between the northern and southern regions can only be made for the 2006 season due to the selection of sites which were able to be sampled in each year (Table 2.2, Chapter 2). In 2006 more sites from the south were sampled than from the north which corresponded with a greater range in YP concentration among sites in the south than in the north. Genotypes Hyperno, WID22221 and Wollaroi consistently produced the highest YP concentrations while Arrivato, Tamaroi, Gunderoi and Kalka consistently produced the lowest (Table 3.4).

Table 3.5: Mean yellow pigment (YP) concentration of sites from the northern or southern growing regions of Australia in 2006 (Mean YP conc. 2006) and mean YP concentration across all environments (Mean YP conc. All Data). Genotype and year were blocked as nuisance factors in the analysis of variance to account for the unbalanced dataset. Standard error of the mean (S.E.) are shown

Region	Mean YP conc. 2006	S.E.	Mean YP conc. All Data	S.E.
North	10.32	0.473	8.29	0.43
South	9.79	0.723	8.63	0.24

Table 3.6: Mean yellow pigment concentrations (YP conc.) of traditional and non-traditional durum growing sites. Genotype and year were blocked as nuisance factors in the analysis of variance to account for the unbalanced dataset. Standard error of the mean (S.E.) are shown

	Mean YP conc.	S.E.
Traditional	8.51	0.34
Non-traditional	8.60	0.34

3.3.3 Relationship between Environmental Variables and Variation in Yellow Pigment Concentration

There was no significant association between any of the climatic variables measured and YP concentration using the whole data set. Similarly, using multiple linear regressions there was also no significant association between any group of climatic variables and YP concentration. Interaction effects involving genotype were not able to be tested due to limited replication resulting in a lack of degrees of freedom. There was a significant year effect on YP concentration (Table 3.7) and therefore regression analysis between YP concentration and environmental variables was performed both between and within years. Grain yield was also not associated with YP concentration

(data not shown). In some seasons, for some varieties, there was a significant relationship between YP concentration and either thousand grain weight (TGW), or maximum temperature during grain filling, or both (Table 3.8). A multiple linear regression model including TGW and maximum temperature during the estimated grain filling period explained up to 85 per cent of the variation in YP concentration within a genotype and year (Table 3.8). Significant correlations between YP concentration and TGW were negative, except for the genotype Yallaroi in 2006, and were positive between YP concentration and mean temperature during grain filling. Despite TGW being significantly negatively correlated with YP concentration in some situations, this relationship did not explain all the variance in YP concentration (Table 3.8) and there were a range of YP concentrations from grain samples with similar TGW. An example of this is displayed graphically for two varieties in Figure 3.4.

Table 3.7: Results from analysis of variance of yellow pigment concentration across all 59 environments presenting sum of squares, means squares and significance of effect (p value) associated with Genotype, Site and Year

Source	Sum of Squares	Means Squares	p value
Genotype	664.0	66.4	<.001
Site	276.0	13.1	<.001
Year	442.2	110.5	<.001

Table 3.8: Significance (p value) of associations between yellow pigment (YP) concentration and thousand grain weight (TGW) and maximum temperature during grain filling (Temp. Max.) calculated from a multiple linear regression analysis. Percentage of variation in YP concentration explained by the model (% Variation) and number of samples (N) are presented. ns=Not significant, na=Not applicable due to no significant terms in the model

Genotype	Year	p (TGW)	p (Temp. Max.)	% Variation	N
Arrivato	2004	**<.001***	ns	**85.2**	13
Bellaroi	2004	ns	ns	na	14
Kalka	2004	**0.005***	ns	**45.4**	13
Tamaroi	2004	**<.001***	**0.044***	**74.2**	14
Wollaroi	2004	**<.001***	ns	**78.3**	14
Bellaroi	2005	ns	**0.009***	41.4	13
Kalka	2005	ns	ns	na	13
Tamaroi	2005	ns	ns	na	13
Wollaroi	2005	**0.045***	**0.012***	**49.7**	13
Yallaroi	2005	ns	**0.045***	**21.2**	13
Arrivato	2006	ns	**0.043***	**56.6**	9
Bellaroi	2006	ns	ns	na	9
Kalka	2006	ns	ns	na	9
Tamaroi	2006	ns	ns	na	6
Wollaroi	2006	ns	ns	na	9
Yallaroi	2006	**0.043***	**0.024***	**67**	8
Kalka	2007	ns	ns	na	11
Tamaroi	2007	ns	ns	na	11
Yallaroi	2007	ns	ns	na	11

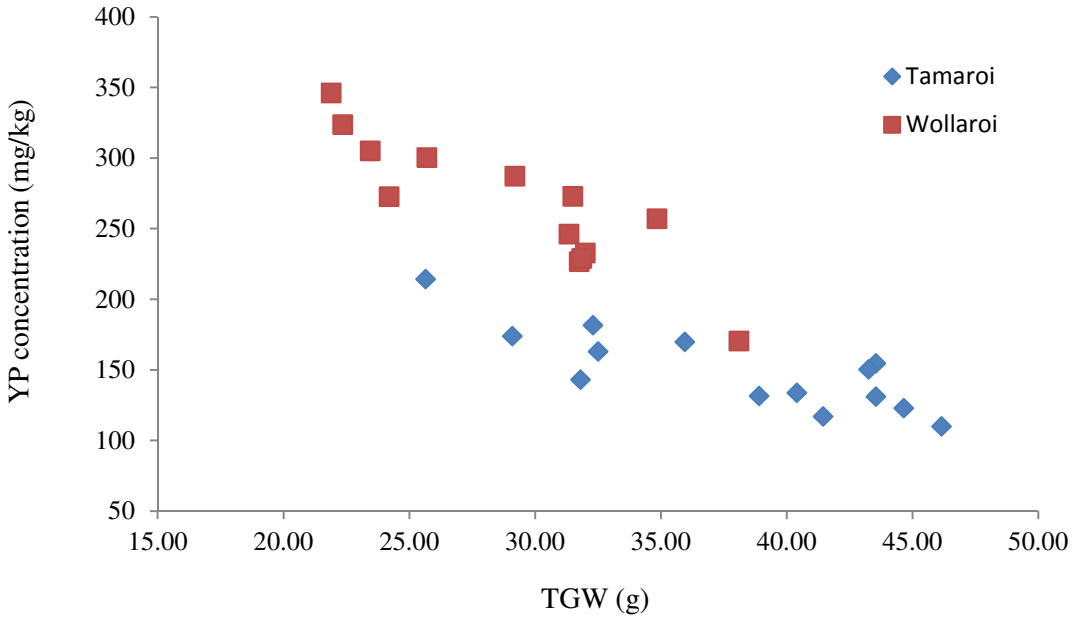

Figure 3.4: Relationship between one thousand grain weight (TGW) and flour yellow pigment (YP) concentration across fourteen field locations in the 2004 season for the genotypes Tamaroi (♦) and Wollaroi (■)

3.3.4 Relationship between Climatic Variables and Grain Weight

Although both TGW and maximum temperature were both shown to be correlated with YP concentration (Table 3.8), TGW and maximum temperature were themselves significantly ($p<0.001$) negatively correlated with one another (Table 3.9). In addition, many of the climatic variables were significantly ($p<0.05$) correlated with one another (Table 3.9).

Table 3.9: Correlation (r) value between climatic variables and between each climatic variable and one thousand grain weight (TGW) measured during the grain filing period across 46 environments over five years. Significance levels are indicated in superscript where A = p<0.001, B = p<0.01 and C = p<0.05 and ns = non-significant. Evap = Evaporation potential, Yield = Site mean yield, Precip = Precipitation, Max. = Maximum, Min = Minimum, Temp = Temperature, RHmaxT = Relative humidity at daily maximum temperature, RHminT = Relative humidity at daily minimum temperature

Variable	Evap	RHminT	RHmaxT	Min. Temp.	Max. Temp.	Precip.	Sunlight	TGW
Grain Yield	-0.41^{B}	ns	0.25^{C}	ns	ns	0.29^{C}	ns	0.46^{A}
TGW	-0.66^{A}	0.45^{A}	0.67^{A}	ns	-0.70^{A}	0.72^{A}	-0.68^{A}	
Sunlight	0.57^{A}	-0.37^{B}	-0.72^{A}	ns	0.77^{A}	-0.65^{A}		
Precip.	-0.57^{A}	0.53^{A}	0.82^{A}	0.40^{B}	-0.43^{B}			
Max. Temp.	0.68^{A}	-0.39^{B}	-0.64^{A}	0.44^{B}				
Min. Temp.	ns	ns	ns					
RHmaxT	-0.77^{A}	0.78^{A}						
RHminT	-0.66^{A}							

3.4 Discussion

3.4.1 Measurement and Estimation of Yellow Pigment Concentration

The very strong correlation between flour YP concentration and yellow flour colour (CIELAB b* value) (Figure 2.1) can give Australian breeders confidence that selecting for YP using flour colour will be effective and not excessively confounded by other variables such as protein, particle size, a*, L* or enzyme activity. The correlation between YP concentration and yellow flour colour was slightly weaker in 2004 and 2005 suggesting that either the very small sample size (10 grains) reduced

the accuracy of YP concentration estimate, or alternatively that there was a year effect influencing the relationship between flour YP concentration and yellow flour colour. As flour colour measurements are based on visible reflectance spectroscopy, it is expected to be closely correlated with measurements using NIR spectroscopy in the visible wavelength range. McCaig et al. (1992) showed that YP concentration and NIR estimates to be very closely correlated for a diverse range of durum wheat grown in Canada. NIR offers the benefits of being non-destructive as it can give an estimate based on wholegrain and can simultaneously provide estimates for multiple important traits such as protein content, grain hardness, and moisture content and there is practically no additional cost to add an additional trait once a calibration has been developed.

3.4.2 Yellow Pigment Comparisons between Sites and Regions

Inconsistent site effects between years indicates that YP concentration is influenced by environmental factors that are variable across years, such as the specific weather in a given season, more so than factors that are less variable across years, such as soil type, altitude or latitude. Therefore it is logical to consider and analyse climatic data when studying non-genetic causes of variation in YP concentration. From these data, neither the northern or southern wheat growing regions of Australia produced significantly different concentrations of YP. The highly variable and inconsistent site mean YP concentrations across years makes it impractical to predict which growing regions may be more or less likely to produce higher levels of flour YP concentration within any given season. Historical climatic data could be used to establish most probable environment types within regions; however, without a clearer understanding

of the mechanism of environmental influence on YP concentration, this is unlikely to improve the predictability of YP concentration within even a well characterised environment. Millers and pasta producers would benefit from having an indication of the level of YP concentration that can be expected from a given site or region prior to harvest. This information would assist millers and pasta producers to determine where to best source their grain or semolina and how to best mix or blend semolina to achieve a high quality end product. However, these data indicate that making such predictions is very difficult.

There was no significant difference between sites where durum wheat has traditionally been grown and sites where it has not. This, in addition to the lack of consistency in YP concentration of sites across years, suggests that extending the durum growing region would not result in durum wheat with lower (or higher) concentration of YP. However, if the durum growing regions were to be extended, other factors under environmental control which relate to the perception of YP such as protein, hardness and vitreousness would need to be considered. In addition, other factors such as adaptation and protein would obviously need to be considered regardless of their effect (or lack thereof) on YP concentration. Australia's traditional durum growing regions are in regions which have a reliable climate allowing growers to achieve high protein (usually through applying high rates of nitrogen fertiliser) required for high quality durum without risk of terminal drought and or heat stress reducing grain quality or yield. In these regions durum wheat is also relatively well adapted yielding at least as high as bread wheat and in some regions durum wheat is even higher yielding. Finding areas able to satisfy these criteria is likely to be a more

significant constraint to the expansion of the durum growing area than ability to achieve high concentrations of YP concentration.

There was a significant effect of genotype across all the data and a larger proportion of the total variance in YP was attributed to genotype than environment. The level of interaction between genotype and environment, or broad sense heritability, unfortunately could not be calculated as only composites of each genotype at each site were available for analysis. Without replication within a site the error associated with the mean YP concentration of each genotype at any site could not be calculated. However, on average, genotype YP concentration data between sites were all highly correlated with one another. Without replication within sites, looking at the correlation between sites provides a method of assessing the interaction between genotype and the environment; and the high level of correlation within this data set suggests that there is very minimal genotype by environment interaction. The average correlation between sites ($r = 0.88$) was higher than expected considering firstly the size of the data set (46 environments across 5 years and two growing regions) and secondly that there was no field replication or spatial adjustment to reduce error due to field variation. This observation is consistent with previous studies reporting minimal genotype by environment interaction for YP concentration and/or flour colour (Clarke et al. 2006; Elouafi et al. 2001; Parker et al. 1998; Patil et al. 2008). Limited genotype by environment interaction means that expensive multisite testing of genotypes for YP concentration is not required by breeding programs. In addition, it did not seem to matter which sites were used for YP analysis as there was no clear pattern between sites that correlated better or worse with other sites. This makes

selection for YP within a breeding program relatively easy and straight forward compared to other complex traits such as grain yield.

3.4.3 Environmental Control of Yellow Pigment Concentration

There was a highly significant effect of environment on YP concentration. The cause of this variation across environments was not able to be identified. For some varieties, in some seasons, there was a significant correlation between YP concentration and TGW, or maximum temperature during grain filling, or both TGW and maximum temperature during grain filling in the case of Tamaroi in 2004 and Wollaroi in 2005. Temperature and TGW are not necessarily independent of one another and were shown to be negatively correlated with one another. A negative relationship between temperature and grain weight during grain development has been reported previously (Wiegand and Cuellar 1981). Furthermore, many of the environmental variables were significantly correlated with one another and with TGW, such as precipitation, amount and intensity of sunlight, maximum daily temperature and TGW for example. It is therefore very difficult to confidently ascertain the direct effect of each variable on YP concentration.

An association between grain weight and YP concentration has also been reported by other authors (Alvarez et al. 1999; Clarke et al. 2006; Hessler et al. 2002; Mares and Campbell 2001; Markley 1937; Worzella 1942). The positive correlation between YP concentration and temperature identified in this study contrasts with reports from Mangels (1932) but concurs with research published by Clarke et al. (2006). These results are similar to those reported by Rharrabti et al. (2003a) that YP concentration

was positively correlated with maximum temperature over the whole growing season. However, in the current study whole season temperature was not as strongly associated with YP concentration as temperature during the grain filling period, indicating that conditions during grain development are more important than pre anthesis conditions. Wardlaw (1994) showed that temperature prior to grain development can affect the response of the grain to increases in temperature which is one possible explanation for the association between whole season temperature and YP concentration reported by Rharrabti et al. (2003a). Within each genotype and between sites there was a significant amount of variation in YP concentration that could not be explained by TGW and between sites there was variation in YP concentration from grain samples with very similar TGW. These data suggest there were environmental effects on synthesis of YP content per grain (YP/grain) independent of TGW.

This study has developed a very large data set and therefore the inability to identify the environmental causes of variation in YP suggests the cause is highly complex. Dissecting the environmental control of a complex trait from field data is difficult as there may be many variables simultaneously having an effect on the trait. For this reason more structured and targeted experiments need to be conducted. Rainfall during grain filling was not associated with YP concentration despite a number of studies reporting that water relations were associated with YP concentration or flour colour (Clarke et al. 2006; Guttieri et al. 2001; Mangels 1932). However, in the current study temperature was found to be associated with YP concentration in some situations and plant available water could be considered to be related to temperature. Water limitation may exacerbate the effects of heat stress due to a reduction in the

plant cooling effects from transpiration. Higher temperatures may in turn exacerbate water stress by increasing the rates of transpiration. Experiments conducted under controlled conditions that manipulated temperature and plant available water during grain development, would be expected to conclusively determine the impact of these variables on YP concentration. Given the association between TGW and YP concentration observed in some situations, and the negative relationship between temperature and TGW, then TGW should also be considered when interpreting treatment effects on YP concentration. Utilising controlled environments avoids the confounding effects of multiple variables simultaneously impacting on the trait of interest, which is often the case in field trials.

3.4.4 Conclusion

Measuring flour colour by reflectance spectroscopy is an accurate method of estimating the YP concentration of flour. Therefore this method is suitable for selecting genotypes with increased YP concentration within a breeding program. Genotypes expressed similar levels of YP concentration relative to one another across environments indicating limited genotype by environment interaction for this trait.

Significant variation between environments for flour YP concentration was observed and the cause of this variation appears complex. As a consequence it is difficult to determine which specific environmental conditions result in higher or lower concentrations of YP. There was no discernible pattern among environments and the variation in YP concentration. There also did not seem to be any consistent differences in YP concentration between traditional and non-traditional durum

growing regions or between the northern and the southern durum growing regions. Temperature during grain filling and TGW both appear to at least be implicated in the environmental influences on YP concentration; however further research is required to fully understand the effect of these variables. Targeted experiments under controlled environment conditions have been suggested and will be the objective of the following chapter.

Chapter 4: Non Genetic Control of Yellow Pigment

4.1 Introduction

In the previous chapter, it was concluded that the effect of the environment on yellow pigment (YP) concentration in durum wheat is complex with significant variation across sites and seasons. While there was consistency in the relative concentrations among varieties, there was no consistency among the sites. Non-genetic causes of variation in YP concentration could not be identified from the field data sources in Chapter 3, and therefore targeted experiments under controlled conditions may better be able to determine the major environmental influences on YP concentration. Data presented in Chapter 3 and also in published literature shows that variation in YP concentration is associated with both temperature (Clarke et al. 2006; Mangels 1932; Rharrabti et al. 2003a) and grain weight (Alvarez et al. 1999; Clarke et al. 2005; Hessler et al. 2002; Mares and Campbell 2001; Markley 1937; Worzella 1942). Plant available water can influence grain weight, and, as explained in Chapter 3, can be regarded as not being independent of temperature. Furthermore, multiple authors have reported effects of moisture stress, irrigation, or moisture availability, influencing YP concentration or flour colour. It has been suggested that the relationship between YP concentration and grain weight may be due to starch dilution (Clarke et al. 2006). Grain weight is not independent of temperature (Chowdhury and Wardlaw 1978; Marcellos and Single 1972; McDonald et al. 1983; Sofield et al. 1977; Tashiro and Wardlaw 1989; Wiegand and Cuellar 1980) or moisture stress (Day and Intalap 1970; Guttieri et al. 2001; Pierre et al. 2008). If changes in grain weight are associated with variation in YP concentration, then grain weight must be considered when investigating the effects of temperature and moisture on YP concentration. If grain weight is to be considered then it could be useful to also consider yellow pigment

content per grain (YP/grain), as this would provide an indication of the total amount of YP synthesised, independent of any potential dilution effects associated with TGW.

The aims of this Chapter are to use controlled environments and irrigation to assist in determining the effect of temperature and plant available water during grain development on YP concentration and YP synthesis per grain, while also considering the treatment effects on grain weight. This Chapter also aims to determine the physiological relationship between grain weight and both YP concentration and YP synthesis per grain within an environment. This was achieved by;

(i) Investigating variation in YP concentration and YP/grain between early and late tillers across a range of controlled environments, and,

(ii) Investigating variation in YP concentration and YP/grain from grain differing in size due to physiological processes during plant growth and not environment or genotype

4.2 Methods

4.2.1 Experiment 1: Effects of Temperature and Plant Available Water

4.2.1.1 Plant Material and Pre-treatment Conditions

Experiment one was designed to investigate the post anthesis effect of temperature and irrigation levels on YP concentration and on YP/grain of two durum genotypes. Durum wheat plants of genotypes Wollaroi and Tamaroi were grown in 254 mm diameter free-draining pots filled with sterilised coco peat potting mix. Descriptions of Wollaroi and Tamaroi are provided in Chapter 2 (section 2.2.5). The soil was

added to the pots to within 30mm of the surface. Every second day the soil was irrigated to saturation point to ensure the plants were not water stressed. Prior to the imposition of the treatments the plants were grown, four per pot, in an air conditioned glasshouse cooled by evaporative air conditioners. Pots were well spaced to allow ample light interception by the plants and airflow around the pots. On all plants the three main stems were tagged at anthesis and the flowering dates of each of these stems were recorded.

4.2.1.2 Treatments

A three way factorial experiment was designed where two genotypes, two temperature treatments and two watering regime treatments were used. Sixteen single plant replicates were sown into four pots (four plants per pot) for each genotype x temperature x watering regime treatment combination. Due to poor establishment a few plants were culled from some treatment combinations. Grain from each individual plant was harvested and analysed separately and in doing so represented a separate replicate. Treatments were applied from five days post anthesis through to physiological maturity.

Two temperature treatments were achieved using controlled temperature growth rooms. The plants were either grown in the "warm" growth room at 32/22°C (day/night temperature) and an average temperature of 27.6ºC and average relative humidity of 62% or in the "cool" growth room at 27/18°C and an average temperature of 22ºC and average relative humidity of 67.7% (Table 4.1). In both growth rooms the day length was 14 hours and light was supplied by a 1:1 ratio of metal halide and high pressure sodium lamps. At general spike height, the lamps provided approximately 250 $\mu mol.second^{-1}.m^{-2}$.

Table 4.1: Temperature and humidity conditions for the two growth rooms used in Experiment 1

	Temperature (ºC)			Relative humidity (%)		
Growth room	Average	Average daily min	Average daily max	Average	Average daily max.	Average daily min.
Cool	22	17.3	25.9	62	80.9	48
Warm	27.6	21.7	32	67.7	86.8	39.4

The two watering treatments were water unlimited and water limited. The water unlimited treatment was achieved by watering pots daily to saturation. For the water limited treatment each pot was watered to saturation and allowed to drain prior to the beginning of the treatment, then, during the treatment period each pot was watered every second day to half the weight of its saturated weight. Water use was measured for the water limited treatment by weighing each pot before and after watering. Within each treatment combination there were sixteen plants sown in four pots (four plants per pot). Pots were sown during summer on the 7th of February 2008.

4.2.1.3 Harvest and Measurements

Once all grains from a plant had reached physiological maturity each plant was harvested, threshed by hand and grain placed into an aerated oven at 37°C for 5 days to dry. Plants were regarded as being at physiological maturity once the spikes, leaves and stems, including the nodes, had senesced and there was no visible green colour remaining. Grains originating from each individual plant were counted, weighed, and milled to whole meal flour using a cyclone mill. Analysis of YP concentration, YP/grain and thousand grain weight (TGW) were estimated as explained in Chapter 2.

4.2.2 Experiment 2: Effects of Timing and Pattern of Water Deficit on Yellow Pigment Concentration and Yellow Pigment per Grain

Experiment 2 examined the effect of increasing water deficits during the period between early head emergence and early grain fill under high temperatures using the cultivar Tamaroi. The experiment was sown on the 7th February 2008 with identical pre-treatment conditions, harvest methods and YP analysis as Experiment 1. At Zadock stage 47 when the flag leaf sheath was opening, that is at split boot, two watering treatments were applied: a well-watered treatment achieved by watering the pots daily to saturation point and a water-stressed treatment which was achieved by withholding water completely from Zadock growth stage 47 for a period of 17 days, after which time the pots were saturated and remained saturated until harvest. At the time of the beginning of the treatment (Zadock growth stage 47) the pots were transferred to the warm growth room (32°/22°C) where they remained until harvest. Seventeen days into the treatment period (approximately 10 days post anthesis) the pots in the water deficit treatment were rewatered and were then watered regularly (water unlimited) until harvest. Within each treatment there were sixteen single plant replicates sown into four pots (four plants per pot). Pre-treatment, harvest and measurements were as for Experiment 1.

4.2.3 Experiment 3: Effect of Tiller Management and Variation in Yellow Pigment and Grain Weight between Early and Late Tillers

Experiment 3 was designed to determine if grains differing for TGW due to tiller hierarchy, or alteration of the source/sink balance, display the same relationship between TGW and YP concentration and between TGW and YP/grain as that shown

when climatic variables induce differences in TGW. This experiment was conducted concurrently with Experiment 1 using the genotype Wollaroi. Experiment 3 was a 3 way factorial experiment where the durum wheat genotype Wollaroi was grown under two different temperature treatments which were achieved using the same growth rooms as described in Experiment 1 under well-watered and water-limited conditions achieved in the same way as for Experiment 1 and three "grain origin" treatments. The three "grain origin" treatments were;

I. Grain sourced from the three most advanced tillers with restricted tillering (all later tillers pruned)
II. Grain sourced from the three most advanced tillers with no restriction of tillering
III. Grain sourced from the fourth oldest and all remaining younger later tillers

Grain from the three most advanced (oldest) tillers of each plant (hereafter referred to as "advanced tillers") were tagged at anthesis and compared to grain from fourth and all later (younger) tillers (hereafter referred to as "later tillers") within each treatment combination. To examine the effects of differences in source sink balance on TGW and YP concentration, a selection of plants were pruned back to the three main stems. The plants that were pruned are referred to as "restricted" tillering while the unpruned plants are referred to as "unrestricted". The pre-treatment conditions, harvest methods and YP analysis were as for Experiment 1. Within each treatment combination there were a minimum of eight individual plants (four plants per pot).

4.2.4 Experiment 4: Effects of Temperature Variation and Water Deficits

Experiment 4 was essentially a repeat of Experiment 1 in which plants were exposed to two different growing environments and watering treatments during grain filling, but Experiment 4 was conducted in two glasshouses rather than in growth rooms. The temperature treatments were imposed by growing the plants in two separate glasshouses (differing in temperature) during the treatment period and the water treatments were as for Experiment 1. One glasshouse was cooled by evaporative air conditioning; this treatment is referred to as "air conditioned". The other glasshouse had no air-conditioning, this treatment is referred to as "non-air conditioned". The experiment was conducted over summer and early autumn and atypically high air temperatures were experienced during the treatment period. Consequently, the average maximum temperatures for both temperature treatments were higher than desired. The air-conditioned glasshouse was cooled to an average temperature of 18.6°C and had an average relative humidity of 73.6% (Table 4.2). The non-air conditioned glasshouse had an average maximum temperature of 23.6°C and had an average relative humidity of 56.4% (Table 4.2).

Table 4.2: Temperature and humidity conditions for the air conditioned (AC) and non-air conditioned glasshouse (Non-AC) in Experiment 4

Glasshouse	Temperature (°C)			Relative humidity (%)		
	Average	Daily min.	Daily max.	Average	Daily Min.	Daily Max.
AC	18.6	11.5	36.1	73.6	93.6	35
Non-AC	23.6	15.9	44.1	56.4	73.6	26.4

4.2.5 Experiment 5: Physiological Effects of Grain Size on Yellow Pigment

Experiment 5 was a field experiment to characterise the relationship between TGW and YP concentration and YP/grain independent of genotype or environmental conditions external to the plant. Grain samples provided by The South Australian Research and Development Institute (SARDI) from the National Variety Trials (NVT) were collected from a single plot from five separate sites. Due to poor availability of grain at the time of sampling, a different genotype was sampled from each site rather than the whole range of genotypes from each site. This was not regarded as a problem because experiment 5 was not designed to compare the relationship between TGW and YP concentration between genotypes or between environments. Each grain sample was divided into five different grain size categories, hereafter named "screening category", by passing the grain through a number of sieves of different aperture width. Each screening category is named according to the aperture width of the sieve used to create each screening category, which for the five screening categories were; <2.0 mm, 2.0 - 2.2 mm, 2.2 – 2.5 mm, 2.5 - 2.8 mm and >2.8 mm. Each sample was then milled into whole meal flour using a cyclone mill. Thousand grain weight (TGW) and YP analysis were as explained in Chapter 2.

4.2.6 Measurements

4.2.6.1 Temperature and Humidity

The temperature and humidity were continually measured in the glasshouses and growth rooms in all experiments using Tiny Tag™ data loggers with measurements taken and logged every five minutes during the treatment period.

4.2.6.2 Yellow Pigment Analysis

Yellow pigment analysis was as described in Chapter 2 except one gram of flour and 5ml of methanol was used instead of 3g of flour and 15ml of methanol. Due to limited flour availability per sample, a single extraction was performed per wholemeal flour sample.

4.2.7 Statistical Analyses

Results for Experiments 1 and 2 were statistically analysed by ANOVA. Genotype, growth room, glasshouse, water treatment and grain source were fitted as factors. TGW, YP concentration and YP/grain data of grain from each individual plant for each treatment combination were included in the model as response variables. Significant differences between means from various treatments were determined using a Tukey test. Significant differences between means of water transpired and evaporated from pots in the water limited treatments were also calculated using a Tukey test. Graphical representations of TGW plotted against YP concentration and of TGW plotted against YP/grain with linear regression lines were created using Microsoft Excel.

4.3 Results

4.3.1 Experiment 1

4.3.1.1 Temperature and Humidity

An average temperature difference of 5.6ºC during the day and 4.4ºC during the night was achieved between the “warm” and “cool” growth rooms (Table 4.1). There was

also a difference in relative humidity between the growth rooms which may have been due to an unrelated experiment running concurrently in the "cool" growth room which included a de-humidifier. The "cool" growth room had a relative humidity range between 48% and 81% and average of 62% and the "warm" growth room had a relative humidity range between 39.4% and 86.8% and average of 68% (Table 4.). The temperature difference between growth rooms is similar to the difference in temperatures recorded during grain development between environments described in Chapter 3.

4.3.1.2 Water Use

There was no significant difference in amount of water used by plants between the "cool" growth room and the "warm" growth room within the water limited treatment (Table 4.2) indicating that there were no significant differences in evapotranspiration between growth rooms. Therefore it is not expected that there was a difference in water availability between growth rooms.

Table 4.3: Average litres (L) of water transpired and evaporated from pots in the water limited treatment in each environment in Experiments 1 and 4. Amounts of water connected by the same letter are not significantly different according to a Tukey test

Growth Environment	Treatment	Water loss due to Evapotranspiration (L)
Glasshouse	Warm	11.78^{A}
Glasshouse	Cool	4.34^{C}
Growth Room	Warm	9.42^{AB}
Growth Room	Cool	8.61^{B}

4.3.1.3 Yellow Pigment Concentration

There was no significant three way interaction observed between genotype, water treatment and growth room on flour YP concentration (Table 4.4). There was no significant difference between growth rooms in YP concentration under well watered conditions. However, under water limited conditions the "warm" growth room was associated with significantly lower YP concentration (Table 4.5 and 4.6). As expected from the field data presented in Chapter 3, within a given treatment combination, Wollaroi produced higher YP concentration than Tamaroi (Table 4.5). YP concentrations were significantly higher when plants were grown in the "cool" growth room (Tables 4.5 and 4.6) with the greatest difference in YP concentration between growth rooms expressed by Tamaroi (Table 4.5).

Table 4.4: Significance (p) values of each treatment combination on yellow pigment concentration (YP conc.), thousand grain weight (TGW) and yellow pigment per grain (YP/grain) in Experiment 1

Treatment	YP Conc.	TGW	YP/grain
Genotype	<0.001	ns	<0.001
Water	<0.001	<0.001	<0.001
Growth Room	<0.001	0.002	ns
Genotype*Water	ns	ns	ns
Genotype*Growth Room	0.008	0.092	ns
Water*Growth Room	<0.001	0.004	ns
Genotype*Water*Growth Room	ns	ns	ns

Table 4.5: Mean yellow pigment (YP) concentration of Wollaroi and Tamaori durum wheat grown in either the "cool" or "warm" growth room in Experiment 1. Levels of YP concentration connected by same letter are not significantly different as determined by a Tukey test

Environment	Genotype	Mean YP concentration (mg/kg) for treatment combination
Cool	Wollaroi	12.04^{A}
Cool	Tamaroi	10.84^{AB}
Warm	Wollaroi	9.93^{B}
Warm	Tamaroi	6.62^{C}

There was a significant ($p<0.001$) negative relationship between TGW and YP concentration for both Wollaroi and Tamaroi (Figure 4.1). Separate lines of best fit were plotted for Wollaroi and Tamaroi (Figure 4.1) as there was a significant effect on YP concentration (Table 4.4).

Table 4.6: Mean yellow pigment (YP) concentration, one thousand grain weight (TGW) and YP per grain (YP/grain) of durum wheat grown in the "warm" and "cool" growth rooms with different irrigation (water) treatments in Experiment 1. Values with the same letter within a column are not significantly different as determined by a Tukey test

Water treatment	Temperature treatment	YP concentration (mg/kg)	TGW (g)	YP (ng) /grain
Well watered	Warm	8.13^{A}	43.4^{A}	353^{A}
	Cool	9.33^{A}	42.8^{A}	399^{A}
Water-limited	Warm	8.43^{A}	31.7^{B}	267^{B}
	Cool	13.24^{B}	20.5^{C}	271^{B}

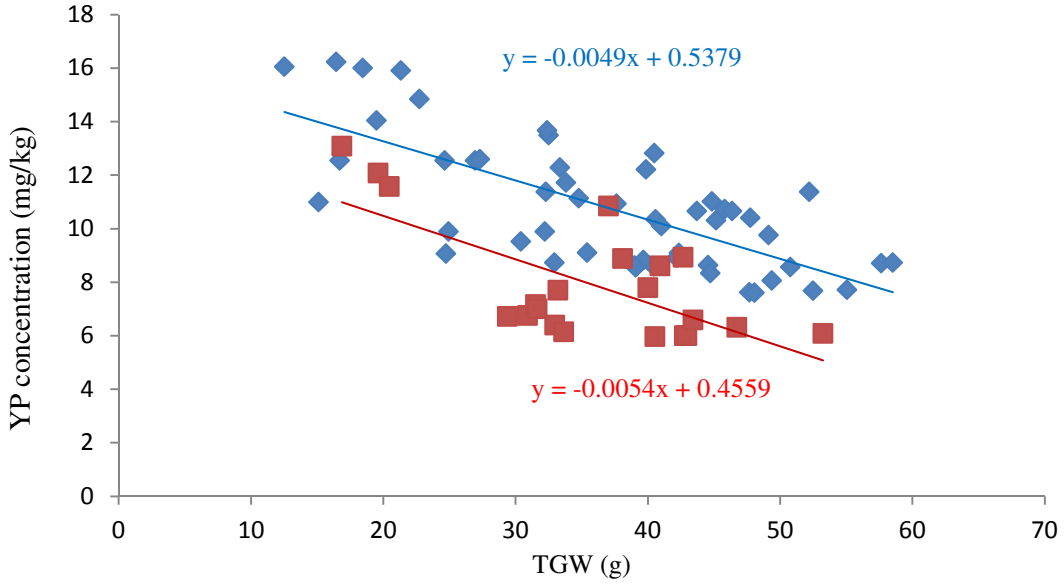

Figure 4.1: Relationship between flour yellow pigment (YP) concentration and thousand grain weight (TGW) showing significant (p<0.001) negative correlation for both Wollaroi (r = -0.72; N=52; ♦) and Tamaroi (r = -0.68; N=21; ■) between YP concentration and TGW. All data points within and across treatment combinations in Experiment 1 are presented

4.3.1.4 Thousand Grain Weight

There was no significant three way interaction among genotype, water treatment and environment on TGW (Table 4.4). Limited water resulted in significantly ($p<0.001$) reduced TGW in both growth rooms (Tables 4.4 and 4.6). Under water limited conditions, growing plants in the "cool" growth room resulted in significantly ($p<0.01$) lower TGW than growing plants in the "warm" growth room (Table 4.6). Under well watered conditions there was no significant difference in TGW of plants from the two growth rooms.

4.3.1.5 Yellow Pigment Content per Grain

For YP/grain, genotype and watering treatment were the only significant ($p<0.001$) treatment effects (Table 4.4). The water unlimited treatment was associated with significantly ($P<0.001$) higher YP/grain than the water limited treatment (3.76 and 2.74 ng/grain respectively) and Wollaroi produced significantly ($p<0.001$) higher YP/grain than Tamaroi (3.81 and 2.69 ng/grain respectively). There was a significant ($p<0.01$) positive correlation between YP/grain and TGW in both genotypes (Figure 4.2). A separate regression was fitted for each genotype as there was a significant effect of genotype on YP/grain.

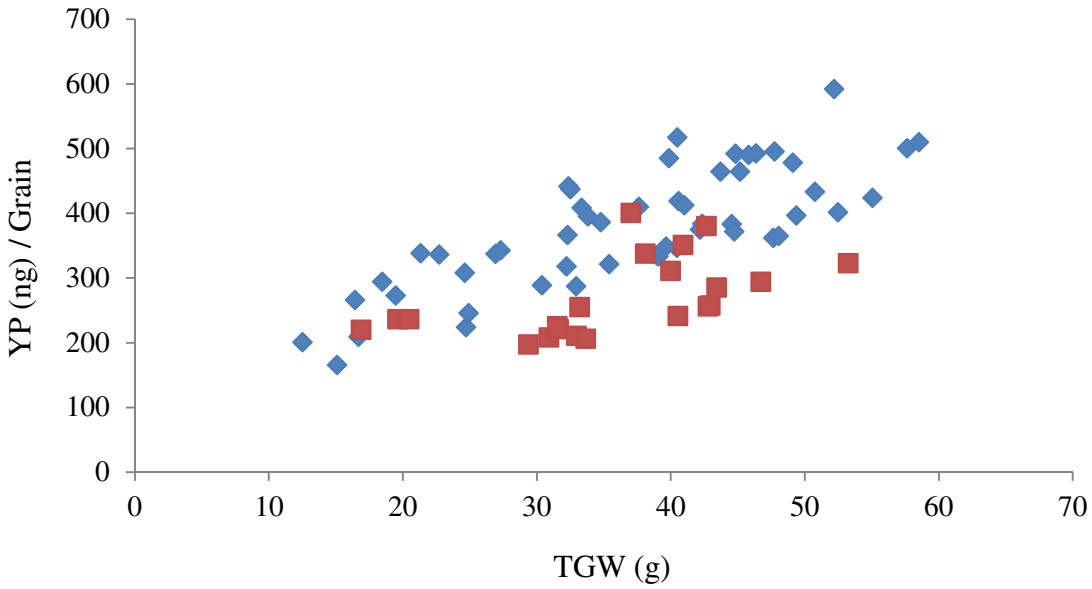

Figure 4.2: Relationship between yellow pigment per grain (YP(ng)/grain) and one thousand grain weight (TGW) for Wollaroi (r = 0.72; N=52; ♦) and Tamaroi (r = 0.52; N=21; ■) within and across all treatment combinations in Experiment 1

4.3.2 Experiment 2

Water stress during early grain fill resulted in significantly ($p<0.001$) lower TGW and YP/grain and significantly ($p<0.001$) higher YP concentration (Table 4.7).

Table 4.7: Mean yellow pigment (YP) concentration, thousand grain weight (TGW) and yellow pigment per grain (YP(ng)/grain) values for both water treatments in Experiment 1. Response variable values not connected by the same letter are significantly different according to a Tukey test ($^{A, B}$)

Water treatment	YP concentration (mg/kg)	TGW (g)	YP (ng) / grain
Water Stress	7.83^{A}	37.9^{A}	321^{A}
Water limited	14.45^{B}	10.4^{B}	167^{B}

As in Experiment 1, there was a significant ($P<0.001$) negative relationship between TGW and YP concentration (Figure 4.3). The range in TGW and YP concentration was mostly due to differences between the two water treatments which created two "groups" of data (Figure 4.3).

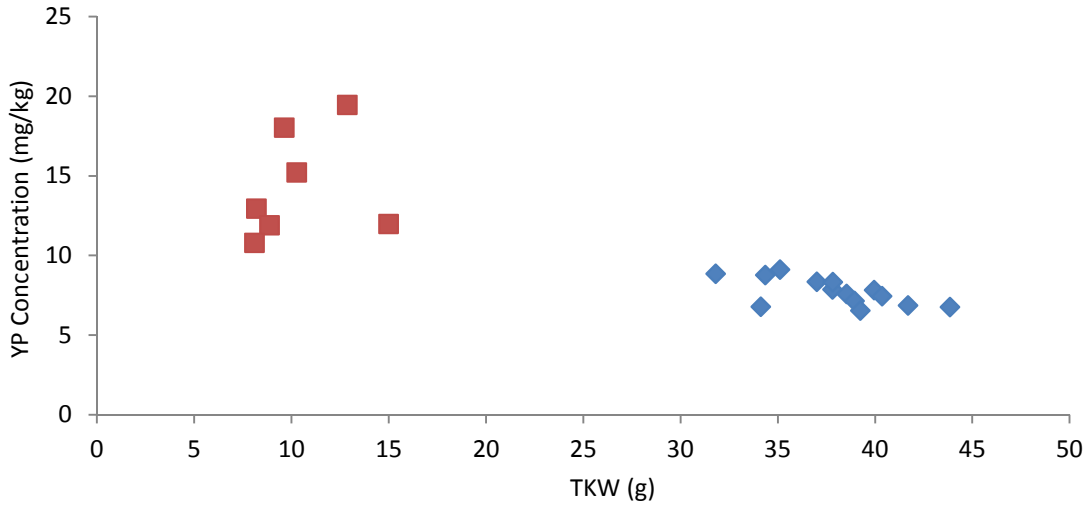

Figure 4.3: Relationship between yellow pigment (YP) concentration and one thousand grain weight (TGW) of Tamaroi showing a significant ($p<0.001$) negative correlation ($r= -0.84$; $N=21$). All data within and across treatment combinations in Experiment 2 are presented

4.3.3 Experiment 3

The plants that grew with unrestricted tillering produced an average of 6 tillers per plant (data not shown). Pruning all but the three most "advanced tillers" did not significantly affect grain YP concentration, TGW, or the YP/grain (Table 4.9). There was a significant effect of "grain origin" on YP concentration, TGW and YP/grain (Tables 4.8 and 4.9). Grain sourced from the "later tillers" had lower TGW and lower YP/grain and higher YP concentration than grain sourced from "advanced tillers" (Table 4.9). There was a highly significant ($p<0.001$) negative correlation ($r = -0.80$, $N=12$) between YP concentration and TGW (Figure 4.4).

Table 4.8: Significance (p) values of each treatment combination on yellow pigment concentration (YP conc.), thousand grain weight (TGW) and yellow pigment per grain (YP/grain) in Experiment 3

Treatment	YP Conc.	TGW	YP/grain
Water	<0.001	<0.001	<.001
Growth Room	0.009	ns	ns
Grain Origin	0.004	<0.001	0.001
Water *Water	0.001	0.007	ns
Water *Growth Room	ns	ns	ns
Growth Room *Growth Room	ns	ns	ns
Water * Growth Room * Grain Origin	ns	ns	ns

Table 4.9: Mean grain yellow pigment (YP) concentration, thousand grain weight (TGW) and yellow pigment per grain (YP(ng)/grain) resulting from different tiller management treatments and from advanced or later tillers ("grain origin"). Means with the same letter within a column are not significantly different according to a Tukey test

Tillering treatment	Grain Origin	YP concentration (mg/kg)	TGW (g)	YP (ng) /grain
Restricted	Advanced tillers	9.87^{A}	43.58^{A}	430^{A}
Unrestricted	Advanced tillers	10.23^{A}	40.25^{A}	412^{A}
Unrestricted	Later tillers	11.56^{B}	30.92^{B}	357^{B}

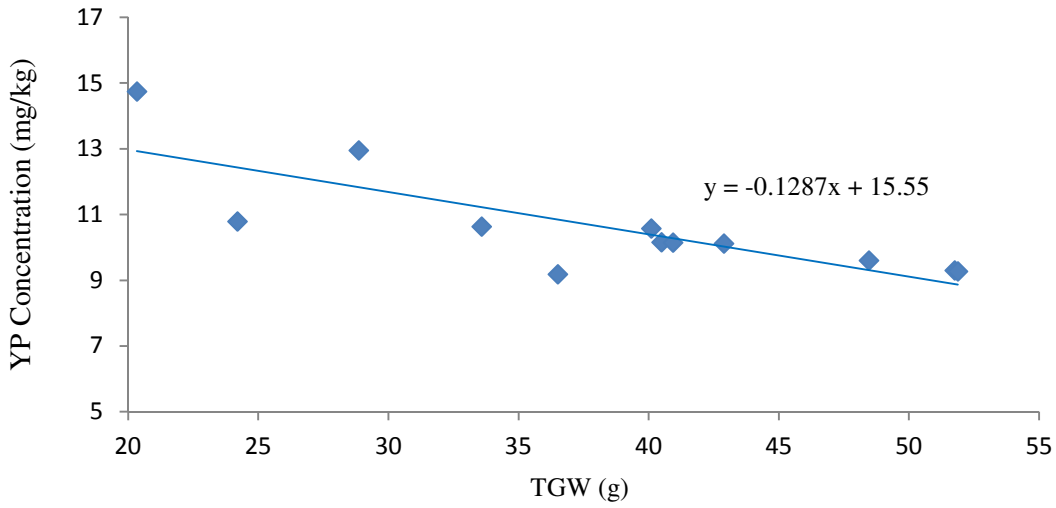

Figure 4.4: Relationship between mean yellow pigment (YP) concentration and mean thousand grain weight (TGW) showing a significant ($p<0.001$) negative correlation ($r = -0.80$) for the genotype Wollaroi for each treatment combination in Experiment 3

4.3.4 Experiment 4

4.3.4.1 Temperature and Humidity

The air conditioned glasshouse had on average an 8.0ºC cooler maximum temperature and 4.4ºC cooler minimum temperature and was more humid than the glasshouse without air conditioning. Due to the heat wave in March 2008 the evaporative air conditioners were running continuously and most likely contributed to the high humidity associated with this treatment (Table 4.2).

4.3.4.2 Water use

Unlike the water limited treatment in Experiment 1, there was a significant difference in water use of plants between the air-conditioned glasshouse and the non-air-conditioned glasshouse (Table 4.2). Therefore it could be concluded that plants under the water limited treatment in the non-air-conditioned glasshouse experienced more severe water stress than plants under the water limited treatment in the air conditioned glasshouse.

4.3.4.3 YP Concentration

There was a significant three way interaction between treatment effects genotype, water treatment and glasshouse on YP concentration (Table 4.10). The non-air conditioned glasshouse was associated with higher YP concentration, except for the genotype Tamaroi when grown under water limited conditions (Table 4.11). Restricting water supply resulted in higher YP concentration of both Tamaroi and Wallaroi in both the air conditioned and non-air conditioned glasshouses (Table 4.11).

Table 4.10: Significance (p) values of each treatment combination on yellow pigment concentration (YP conc.), thousand grain weight (TGW) and yellow pigment per grain (YP/grain) in Experiment 4

Treatment	YP Conc.	TGW	YP/grain
Genotype	<0.001	ns	<0.001
Water	<0.001	<0.001	<0.001
Glasshouse	<0.001	<0.001	<0.001
Genotype*Water	ns	0.001	ns
Genotype* Glasshouse	<0.001	<0.001	ns
Water* Glasshouse	ns	0.003	<0.001
Genotype*Water* Glasshouse	<0.001	<0.001	0.072

Table 4.11: Effect of glasshouse (air conditioned (AC) or non-air conditioned (Non-AC)) and water treatments on thousand grain weight (TGW) and (YP) concentration of Tamaroi and Wallaroi. Values with the same letter are not significantly different according to a Tukey test

Genotype	Glasshouse	Water treatment	TGW (g)	YP concentration (mg/kg)
Tamaroi	AC	Water unlimited	60.79^{A}	4.21^{D}
		Water limited	31.38^{D}	9.03^{AB}
	Non-AC	Water unlimited	49.23^{BC}	5.72^{C}
		Water limited	24.81^{DE}	6.62^{BC}
Wallaroi	AC	Water unlimited	57.94^{AB}	5.42^{CD}
		Water limited	50.46^{BC}	6.62^{C}
	Non-AC	Water unlimited	43.85^{C}	8.13^{B}
		Water limited	17.98^{E}	11.14^{A}

There was a significant ($p<0.001$) negative correlation between TGW and YP concentration in both Wollaroi and Tamaroi in Experiment 4 (Figure 4.5). Two separate regression lines were fitted as there was a significant genotype effect on YP concentration.

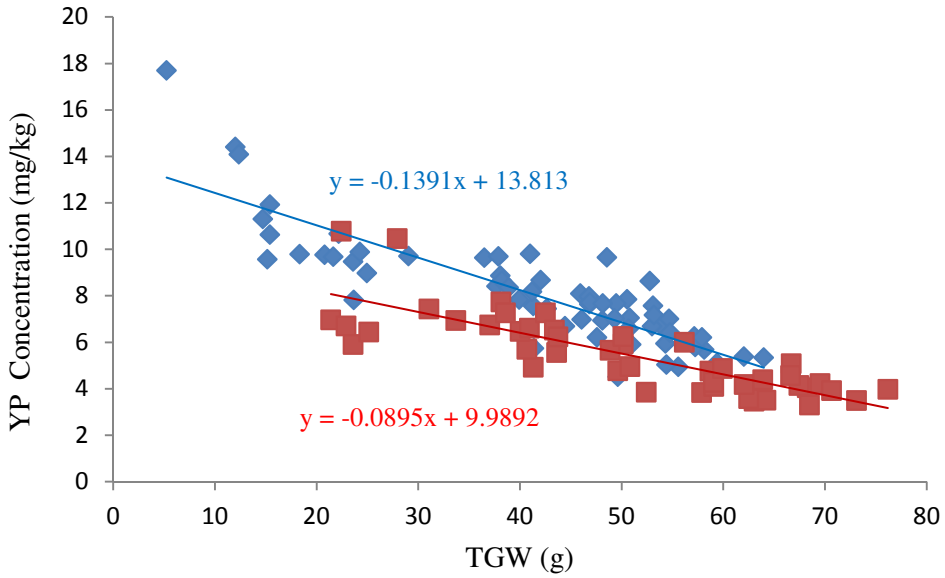

Figure 4.5: Relationship between flour yellow pigment (YP) concentration and thousand grain weight (TGW) for Wollaroi (r = -0.86; N=64; ♦) and Tamaroi (r = -0.82; N=45; ■) within and across all treatment combinations in Experiment 4

4.3.4.4 Thousand Grain Weight

There was a significant three way interaction between genotype, glasshouse and water treatment on TGW (Table 4.10). Both the non-air conditioned glasshouse and limited watering were associated with lower TGW (Table 4.11). The TGW of Wollaroi and Tamaroi showed a differential response to limited watering when grown in the non-air conditioned glasshouse. With limited watering, the TGW of Tamaroi was significantly ($p<0.01$) lower, approximately 50 per cent lower, than the water unlimited treatment, regardless of the glasshouse the plants were growing in (Table 4.11). With limited watering, the TGW of Wollaroi was also significantly ($p<0.01$) lower, but only in the non-air conditioned glasshouse (Table 4.11).

Under water limited conditions, the TGW of Wollaroi and Tamaroi also showed a differential response to the glasshouse the plants were grown in. When grown in the non-air conditioned glasshouse Wollaroi plants had lower TGW than Wollaroi plants grown in the air conditioned glasshouse, regardless of the watering treatment. However, Tamaroi plants grown in the non-air conditioned glasshouse also resulted in lower TGW compared to Tamaroi plants grown in the air conditioned glasshouse but only under water limited conditions (Table 4.11).

4.3.4.5 YP/grain

There was no significant three way interaction of the treatments on YP/grain (Table 4.11). The treatment combination of plants grown under water limited conditions in the non-air conditioned glasshouse resulted in significantly ($p<0.001$) lower YP/grain than any other treatment combination (Table 4.12). There was a significant ($p<0.001$) positive correlation between YP/grain and TGW (Figure 4.6).

Table 4.12: Means of yellow pigment per grain (YP(ng)/grain) from the air conditioned (AC) and non-air conditioned (Non-AC) glasshouses under water limited and water unlimited treatment combinations. Mean day/night temperatures in degrees Celcius (Temperature) associated with each glasshouse are presented. Levels of YP/grain connected by same letter are not significantly different according to a Tukey test

Glasshouse	Temperature (ºC)	Water treatment	YP (ng)/grain
Non-AC	44/16	Well watered	347^{A}
Non-AC	44/16	Water limited	195^{B}
AC	37/12	Water limited	330^{A}
AC	37/12	Well watered	319^{A}

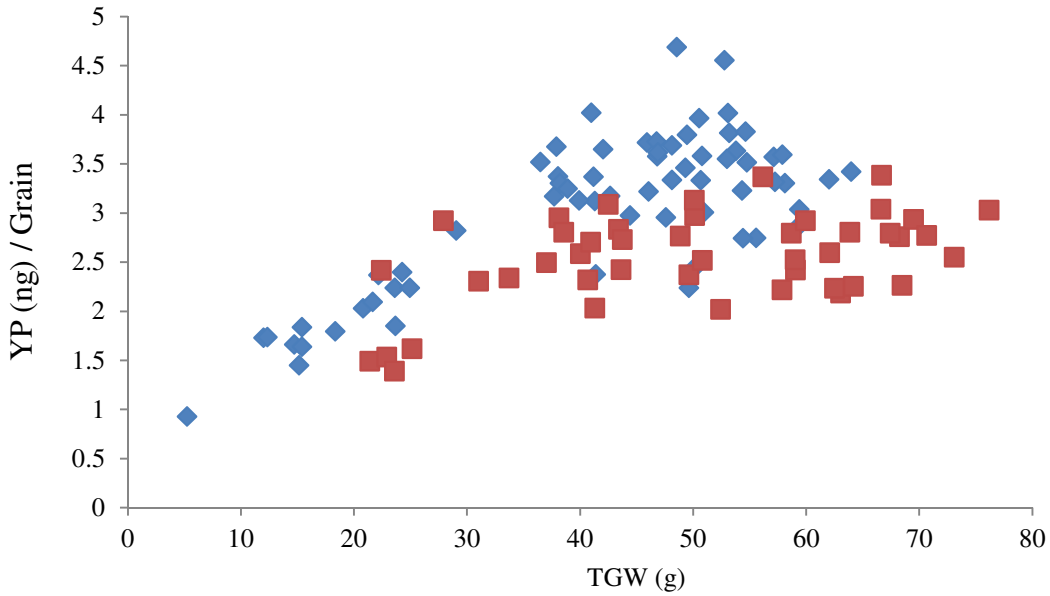

Figure 4.6: Relationship between yellow pigment per grain (YP(ng)/grain) and thousand grain weight (TGW) for Wollaroi (r =0.77, N=64; ♦) and Tamaroi (r = 0.49; N=45; ■) within and across treatment combinations in Experiment 4

4.3.5 Experiment 5

The YP concentration declined linearly as grain size increased (Table 4.13 and Figure 4.7) for each genotype. There was a limited amount of Wollaroi grain at the lowest grain sizes and so neither YP concentrations nor YP/grain values were determined for these grain sizes. The YP/grain increased linearly with increasing grain size in each genotype (Table 4.14 and Figure 4.8). Plotted regression lines of YP/grain against TGW for each genotype (Figure 4.8) were plotted so as to intercept the axis at x=0, y=0 as this makes sense biologically as no YP is possible if there is no grain.

Table 4.13: Average thousand grain weight (TGW) and yellow pigment (YP) concentration from each screening category for each genotype

Screening Category (mm)	TGW (g)	YP concentration (mg/kg) for each genotype				
		Hyperno	WID22221	Saintly	Bellaroi	Wollaroi
<2.0	14.6	10.17	8.61	8.7	8.4	-
2.0-2.2	22.8	9.96	8.64	8.37	7.8	7.53
2.2-2.5	29.5	9.21	7.98	7.89	7.46	7.25
2.5-2.8	36.5	8.73	7.62	7.31	7.19	6.5
>2.8	45.5	8.4	7.16	6.92	7.13	5.87

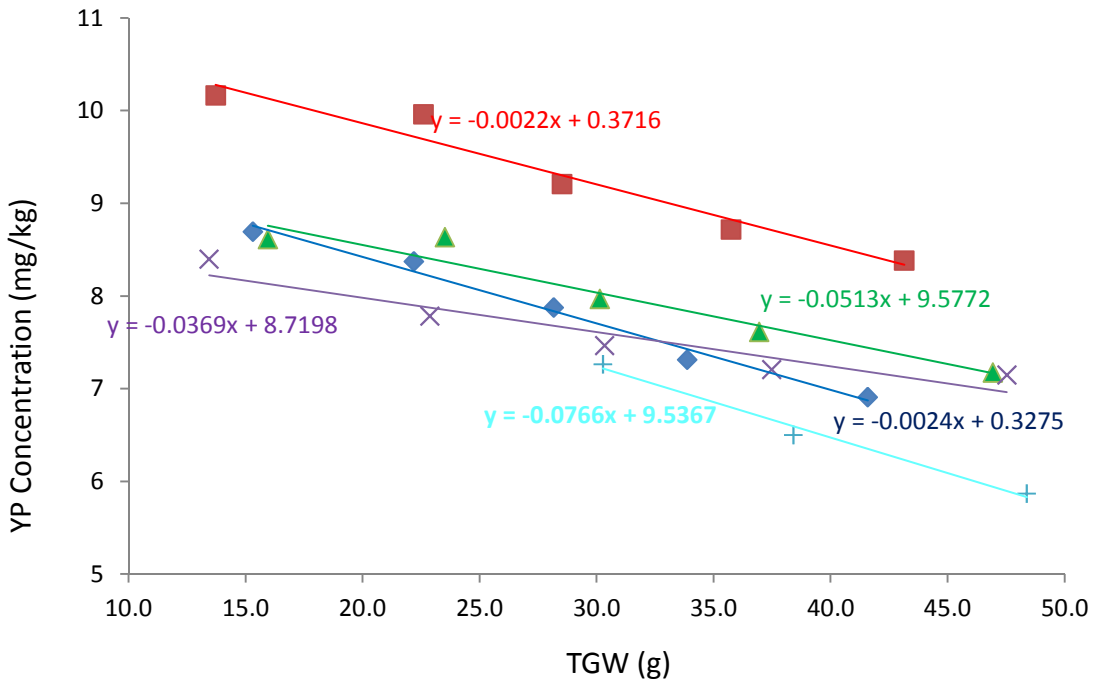

Figure 4.7: Relationship between flour yellow pigment (YP) concentration and thousand grain weight (TGW) for varieties Saintly (r = -0.99; N=5; ♦), Hyperno (r = -0.98; N=5; ■), Bellaroi (r = -0.95; N=5; X), WID22221 (r = -0.97; N=5; ▲) and Wollaroi (r = -0.99; N=3; +)

Table 4.14: Average thousand grain weight (TGW) and yellow pigment per grain (YP(ng)/grain) from each screening category for each genotype

Screening Category (mm)	TGW (g)	YP(ng)/grain for each genotype				
		Hyperno	WID22221	Saintly	Bellaroi	Wollaroi
<2.0	14.6	138	138	132	114	-
2.0-2.2	22.8	225	201	186	177	-
2.2-2.5	29.5	261	240	222	225	219
2.5-2.8	36.5	312	279	246	270	249
>2.8	45.5	360	336	285	339	282

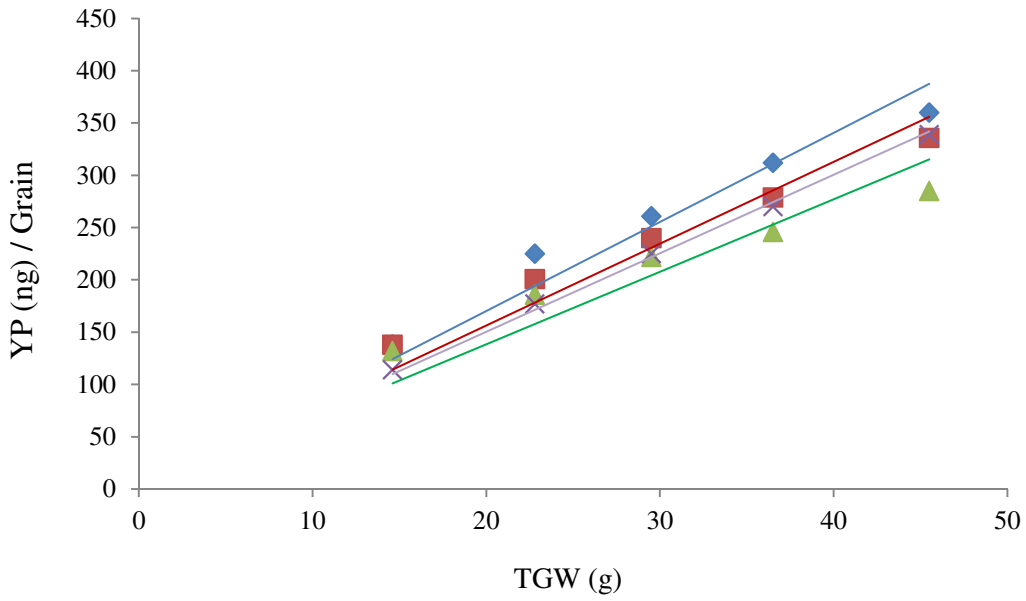

Figure 4.8: Relationship between yellow pigment per grain (YP(ng)/grain) and thousand grain weight (TGW) in Experiment 5 for varieties Hyperno (♦; y=0.28x; r = 0.93; N=5), WID22221 (■; y=0.26x; r = 0.97; N=5), Bellaroi (X; y=0.25x; r = 1; N=5) and Saintly (▲; y=0.23x; r = 0.78; N=5)

4.4 Discussion

4.4.1 Environmental effects on YP

Limiting water during either the grain filling period or pre anthesis and early grain fill period resulted in lower YP/grain. However, limiting water at these growth stages also reduced TGW. The net result of limiting water during grain filling was higher YP concentration. There was an exception to this in the "warm" growth room in Experiment 1 where the lower YP/grain resulting from the limited water treatment (3.53mg/kg / 2.67mg/kg) was of a similar magnitude (in percentage terms) to the decrease in TGW (43.4g / 31.7g) (Table 4.6), resulting in no significant effect of water treatment on YP concentration.

The glasshouse or growth room that the plants were grown in generally had no significant effect on YP/grain. There was one exception in Experiment 4 where the non-air conditioned glasshouse resulted in lower YP/grain within the water limited treatments. This was likely the result of a difference in water stress of plants between glasshouses. Plants in the limited water treatment in the air conditioned glasshouse were not as dry and were not dry for as long compared to those in the non-air conditioned glasshouse; as indicated by the amount of water lost through evapotranspiration (Table 4.3). There were differences in temperature and humidity between growth rooms in Experiments 1 and 3 and between glasshouses within the well watered treatments in Experiment 4 which was not associated with variation in YP/grain.

In both Experiment 1 (using growth rooms) and Experiment 4 (using glasshouses), variation in temperature and humidity were significantly associated with variation in TGW and also with YP concentration, consistent with the negative relationship observed between TGW and YP concentration elsewhere. As explained in the previous paragraph there were no differences in YP/grain associated with these treatments. Instances where there was no association between TGW and growth room, such as in Experiment 1 under the "water unlimited" treatment, there was also no association between YP concentration and growth room. It is therefore likely that the differences in YP concentration between growth rooms or glasshouses are due to an indirect effect of variation in TGW associated with the difference in temperature and humidity between growth rooms/ glasshouses.

4.4.2 Variation in Yellow Pigment Independent of Genotype or Extraneous Environmental Conditions

A significant positive correlation was observed between YP/grain and TGW. In Experiments 3 and 5, where external environmental conditions were constant, it was shown very clearly that variation in YP/grain was very much dependent on TGW. This relationship between TGW and YP/grain is not surprising given that these variables are not independent of one another. The previous section discussed how variation in YP/grain also occurs independently of TGW in response to environmental conditions external to the plant. Therefore, variation in YP/grain occurs in response to TGW, as shown in Experiments 3 and 5, most likely due to source/sink partitioning, whilst simultaneously occurring independently of TGW as shown in Experiments 1, 2 and 4 and in data presented in Chapter 2. Despite the relationship between YP/grain

and TGW, YP concentration was still significantly negatively associated with TGW; therefore the magnitude of the difference in YP/grain associated with variation in TGW, is less than the difference in TGW.

4.4.3 Relationship between Yellow Pigment and Grain Weight

Grain weight (TGW) was significantly ($P<0.001$) negatively correlated with YP concentration in every experiment for all genotypes. It is possible that this is due to starch dilution where the YP is diluted by starch in larger grains as suggested by Clarke et al. (2005). The YP data from the current study were generated from relatively small flour samples. However, if larger grain samples were available protein content could have been measured to provide a better estimate of the grain starch content. The conclusion of dilution of YP by starch is consistent with a report by Graham and Rosser (2000) that lutein content reaches a maximum 12-15 days post anthesis before concentrations decline as the grain size increases and the grain approaches maturity. In addition, Soriano (unpublished, 2007) found that between 20 and 45 days post anthesis, grain lutein concentrations declined as grain dry weight increased (Figures 4.10 and 4.11) and that between twenty days post anthesis until the end of grain fill there is generally no change in the amount of lutein per grain (Figure 4.13). Ramachandran *et al.* (2010) also found that the majority of the total grain lutein content was generally synthesised during early stages of grain development. Ramachandran *et al.* (2010) found that in some environments, genotypes that expressed high levels of YP/grain concentration could actually accumulate lutein throughout grain development. This does not necessarily suggest that starch dilution of YP was not still occurring under these conditions as the amount of YP being

synthesised may have been less than the amount of starch being deposited in the grain which would have resulted in lower YP concentration. In addition, data from Experiment 3 and 5 also support the role of starch dilution in the relationship between YP concentration and TGW since these experiments sought to eliminate external environmental variation from simultaneously and independently affecting both YP concentration and TGW.

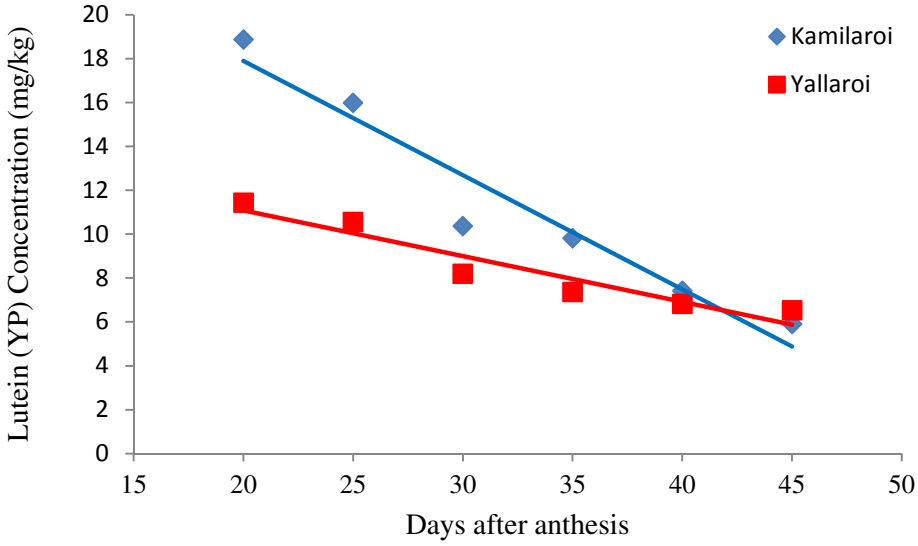

Figure 4.11: Average lutein (yellow pigment (YP)) concentration, measured with high pressure liquid chromatography (HPLC), at various stages of grain development of the durum wheat samples of genotypes Kamilaroi (blue line and diamond) and Yallaroi (red line and square). Adapted from Soriano (unpublished, 2007)

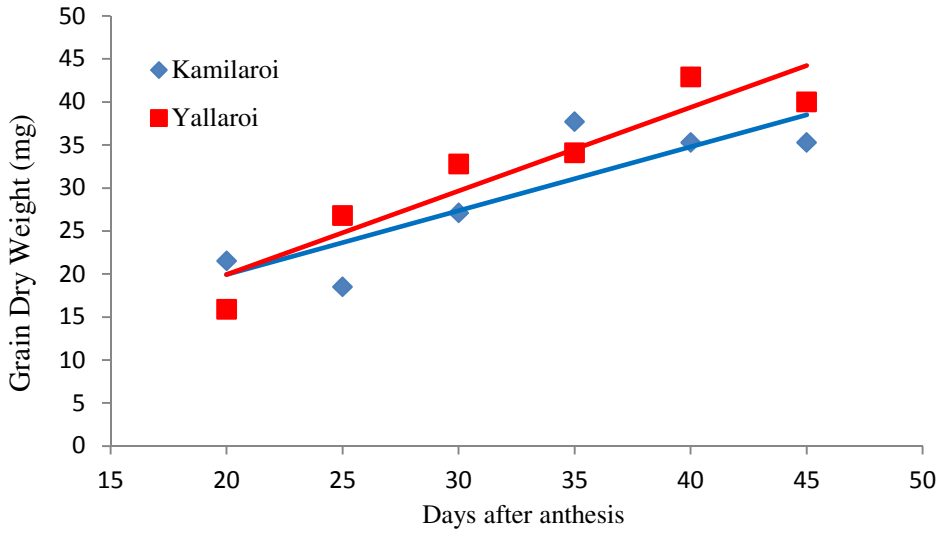

Figure 4.12: Average dry grain weight of durum wheat samples of genotypes Kamilaroi and Yallaroi at various stages of grain development. Adapted from Soriano (unpublished, 2007)

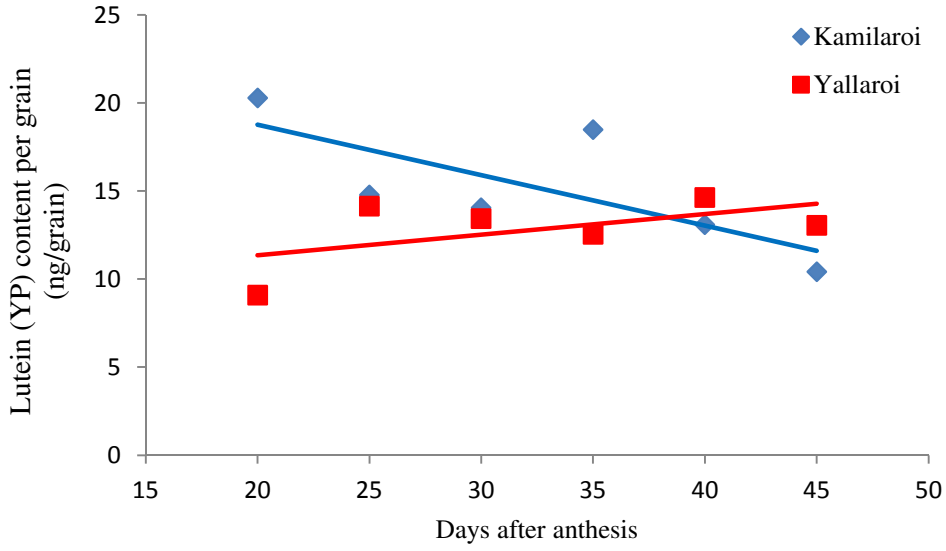

Figure 4.13: Average lutein (yellow pigment YP)) content per grain of durum wheat samples of genotypes Kamilaroi and Yallaroi at various stages of grain development. Adapted from Soriano (unpublished, 2007)

Conclusion

In summary non-genetic causes of variation in YP concentration and YP/grain has been shown to be influenced by both environmental factors and source/sink partitioning factors managed at the plant level. Variation in TGW that was not related to external environmental factors were found to be positively associated with YP/grain and negatively associated with YP concentration. Variation in temperature and humidity was shown to have no significant impact on YP/grain; however higher temperature and lower humidity were generally associated with higher YP concentration which was likely due to lower TGW also associated with higher temperature and lower humidity. Limiting water input during grain filling generally resulted in lower YP/grain, lower TGW and higher YP concentration. However, in some situations it appeared that the lower YP/grain response to water limitation was enough to offset the higher YP concentration usually associated with lower TGW response to water limitation.

The results from this study suggest that an environment with ample plant available water during grain filling is likely to produce high levels of YP/grain. However, any environmental conditions that lead to larger grains may result in lower YP concentrations through starch dilution. High temperatures and/or low humidity during grain filling are not likely to impact YP/grain but are likely to restrict TGW and therefore result in higher YP concentrations. Management practices and environments that produce smaller grains are likely to result in durum wheat with higher YP concentration. The combination of management strategies that do not result in very large grains and an environment providing ample water and high temperatures during grain filling are likely to produce durum wheat with high YP concentrations.

Chapter 5: General Discussion to Part 1

5.1 Endosperm Cell Number and Size and Proposed Hypothesis to explain the Relationship between Grain Weight and Yellow Pigment

5.1.1 Relationship between Grain Weight and both Yellow Pigment Concentration and Yellow Pigment per Grain

One of the key findings from this thesis is the relationships identified between thousand grain weight (TGW) and both yellow pigment (YP) concentration and yellow pigment content per grain (YP/grain). Grain weight was found to be negatively correlated with YP concentration, consistent with previous reports which suggested this may be due to starch dilution (Clarke et al. 2006; Hessler et al. 2002). Somewhat contradictory to the above was a positive relationship identified between the quantity of YP/grain and TGW. These observations imply that the final YP concentration of grain is a function of the synthesis of YP which is greater in larger grain, but partially offset by dilution effects as the rate and duration of grain filling is in turn affected by environmental conditions. Whilst it may not be surprising that greater grain mass was associated with greater total YP/grain, what was particularly interesting was the very strong correlation between these traits, particularly in the absence of, or at least limited, external environmental variation, such as in Experiments 3 and 5 described in Chapter 4. For such a close association to be identified between grain weight and YP/grain suggests that to some extent both these traits are being influenced by, or are a function of, a common factor.

5.1.2 Mechanisms Underlying Variation in Grain Weight

The majority of grain weight, approximately 84%, is made up of starchy endosperm (Kent and Evers 1994; Simmonds 1989). Variation in the number and size of cells that make up the endosperm has been reported (Singh 1982; Singh and Jenner 1981, 1982a, b, 1984). The endosperm has been shown to contain the bulk of the grain YP content (Law 2005). The amount of endosperm is obviously determined by the number and average weight of the endosperm cells. Singh (1992) reports that grain weight is mostly influenced by variation in endosperm cell number, which is fixed approximately 12 to 15 days post anthesis (Jenner et al. 1991). Environmental factors late in grain development, after the time endosperm cell number is fixed, have been shown to affect grain size (Stone and Nicolas 1995), indicating endosperm cell size must in turn be influenced by the environment. Due to source/sink relationships it is expected that compensatory mechanisms exist between final endosperm cell number and endosperm cell size. Similarly plants show a high degree of plasticity during pre-anthesis to early grain development in terms of grain number and grain size, and grain size and grain number have also been shown to be negatively correlated (Bennett et al. 2012; Harper et al. 1970) also indicating possible compensatory mechanisms between these traits. Therefore, if grain number affects grain size, then grain number must also affect final endosperm cell number and/or cell size. The size of the grain coat is also a critical mechanism controlling final grain size according to Fang et al. (2012). Therefore there appear to be a series of overlaying and interconnecting factors influencing the final endosperm cell number and size.

5.1.3 Possible Mechanisms Underlying Development of Yellow Pigment Content within the Wheat Grain

The majority of wheat grain lutein content, which is the predominant YP in wheat (Lepage and Sims 1968), is located in the endosperm (Law 2005) and more specifically in the amyloplasts (Fratianni et al. 2005; Hentschel et al. 2002; Panfili et al. 2004; Zandomeneghi et al. 2000; Zhou et al. 2004) which are specialised plastids for storage of starch granules (Kirk and Tilney-Bassett 1978). During cell division, division of the organelles ensures their inheritance and the maintenance of copy number within progeny cells (Eckardt 2003). Therefore it is expected that each endoperm cell forms the same number of organelles, one of which are amyloplasts. From this an assumption could be made that, at least at the time of cell division, each endosperm cell contains amyloplast and therefore also YP. It should be acknowledged that variation exists within the endosperm such as variation in types of starch granules and in protein content per unit area or unit mass. Variation within the endosperm could be explained by a number of factors, for example differential cell expansion or differential synthesis within, and/or loading into, the endosperm post cell development, and does not necessarily equate to variation between endosperm cells. In regard to differentiation in starch granules across the endosperm, both A and B type starch granules are contained within the amylosplast (Parker 1985) and so should not be considered evidence for differentiation between endosperm cells themselves. If each endosperm cell contains amyloplasts, complete with YP, then the number of endosperm cells influences the amount of YP/grain in addition to the weight of the grain. The absolute amount of YP/grain would then be determined by the amount of YP per endosperm cell and the number of endosperm cells. The quantity of YP per cell and total endosperm cell number is presumably under some genetic control; in

Chapter 3 significant differences in YP concentration were reported between genotypes of similar grain weight grown in the same environment.

It has been shown that YP content in the grain generally peaked at 12 to 15 days post anthesis, after which there was either no, or only limited, further synthesis of lutein. As starch synthesis continued beyond 15 days post anthesis the initial lutein content became diluted by the increased starch (Graham and Rosser 2000; Ramachandran et al. 2010; Soriano 2007). Therefore, the period of endosperm cell development aligns with the period of lutein development within the grain.

5.1.4 Hypothesis: Environmental Effects on Endosperm Cell Number Influence both Grain Weight and Flour Yellow Pigment Concentration

This hypothesis, explaining a possible mechanism for variation in YP across environments, fits with both observations reported in this thesis and published reports relating to formation of endosperm, the effect of temperature and water stress on the development of endosperm and thereby grain weight and YP concentration and YP/grain and with published reports of the development of YP within the grain. Earlier an assumption was made that individual endosperm cells within a genotype contain similar numbers of amyloplasts; if this assumption were in fact found to be incorrect then a slight variation on the above hypothesis could be considered, namely that: Environmental effects on number of endosperm cells (amyloplasts) influence both TGW and flour YP concentration. Since amyloplasts are starch storage organelles (Kirk and Tilney-Bassett 1978), and the majority of wheat grain weight is

starch (Kent and Evers 1994; Simmonds 1989), it is logical that number of amyloplasts could be related to TGW. Cell weight is dependent on the number of small and large starch granules per endosperm cell (Jenner et al. 1991). Starch synthesis does not reach its maximum rate until endosperm cell division and granule initiation have almost finished (Jenner et al. 1991) and cell weight continues to increase until endosperm volume stops expanding (Jenner 2009).

5.1.5 Formation of Endosperm Cell

The development of endosperm cells is unique compared to the development of most other biological cells. The first stage, called the syncytial stage, is characterised by the formation of a syncytium with many endosperm nuclei, followed by the cellularization stage in which cell walls form between the nuclei of the syncytium. During the third stage, the differentiation stage, the three endosperm cell types form, namely the aleurone, starchy endosperm and transfer cells (Becraft and Yi 2011; Mares et al. 1975; Mares et al. 1977; Olsen 2001). Division of the first endosperm nucleus, the product of fusion of one sperm nucleus with two polar nuclei, within the central cell begins within a few hours of fertilisation. After three days cell walls appear and cell division becomes much slower, about one-quarter of the rate in the preceding phase (Bennett et al. 1975; Gao et al. 1992). Cell number continues to rise but at a gradually diminishing rate for 10-15 days, after which few or no new cells are formed (Jenner et al. 1991).

5.1.6 Effect of Temperature and Water Stress on Endosperm Cell Number, Endosperm Size, Grain Weight, and Yellow Pigment per Grain

Grain weight components differ not just in their timing of development, but also in their response to environmental conditions. Variation in temperature for example does not significantly affect cell number, only the rate of cell division, that is, the time taken until maximum cell number has been reached (Hoshikawa 1962; Wardlaw 1970). High temperature has been shown to be related to low grain weight, thus cell weight is the attribute being affected by temperature (Jenner et al. 1991). Water stress, and particularly when associated with high temperature, has been shown to reduce both endosperm cell number and endosperm cell size (Nicolas et al. 1984, 1985). Mean temperature greater than 15-18ºC during grain development has been shown to result in reduced TGW in wheat (Wardlaw 1994). It is possible that the effects of extremely high temperature may be different from the effects of more modest increases in temperature but for the purposes of discussion here, no distinction has been made between the two and elevated temperatures that result in reduced TGW may be referred to as "temperature stress".

Different critical periods and response to environmental conditions of grain weight and YP concentration components may explain variation in the magnitude of effect of TGW on flour YP concentration across sites and seasons. In Chapter 4 it was shown that variation in temperature and humidity had little impact on YP/grain whereas water stress had a large and highly significant effect on YP/grain. This is not surprising given the effect of temperature is limited to affecting only endosperm cell size and that water stress reduces both endosperm cell number and size.

If the proposed hypothesis is correct, high YP concentrations would arise from a combination of genotype and environment factors that result in;

- a large number of endosperm cells,
- a high amount of YP synthesised per endosperm cell; and,
- a relatively small number of starch granules per endosperm cell

However, to prove this hypothesis, endosperm cell counts would be required and shown to be associated with variation in YP/grain across environments.

5.2 Implication of the Relationship between Grain Weight and Yellow Pigment Content for Breeding

Flour YP concentration is the result of the amount of YP/grain as a proportion of the total grain weight. Obviously breeders do not wish to increase YP concentration by reducing grain weight, and therefore increasing YP/grain is the breeding objective. If YP/grain is influenced by endosperm cell number it may also be influenced by genetic variation in YP/endosperm cell, as significant differences in YP concentration have been observed between genotypes of similar grain weight grown in the same environment. According to the proposed hypothesis the quantity of YP/grain is a function of the number of endosperm cells and the amount of YP per endosperm cell. By considering YP/grain the confounding effect of endosperm cell weight, which is heavily influenced by the environment, is removed. For this reason durum breeders may be wise to consider YP/grain when making selections. This should prevent selection for small grain and also reduce variation due to environmental effects on grain weight which may in turn improve the heritability and accuracy of selection.

5.3 Alternative explanations for variation in YP across environments

Cell number may not be the only mechanism responsible for variation in YP/grain. Clarke et. al. (2006) reported low YP associated with a killing frost just prior to maturity. Xanthophylls are strong antioxidants and are known to be involved in the protection of the photosynthetic apparatus. During a severe frost event, lipid cells would be under immense stress and so perhaps lutein is used to help protect these cells. In addition, lutein in flour has been found, in this study, to become severely degraded through the process of freezing and thawing (data not shown). In these cases the variation in pigment may not be due to changes in pigment development but rather to YP degradation. Alternatively, plant stress may result in a reduction in synthesis of lutein. Plants under stress have been shown to produce more abscisic acid (ABA) (Izanloo et al. 2008). ABA is synthesised in the plant from the same precursors as the carotenoids causing yellow pigments (Seo and Koshiba 2002) and metabolite feedback mechanisms exist between carotenoids and ABA in the carotenoid biosynthetic pathway (Cazzonelli 2011). If more carotenoid precursors are used for ABA synthesis in response to plant stress then perhaps this results in reduced synthesis of YP content per endosperm cell.

5.4 Implications of Effect of Temperature, Water input and Grain Weight on Yellow Pigment and General Ability to Explain Variation in Yellow Pigment across Environments

Non-genetic variation in YP/grain has, not surprisingly, been shown in this study to be dependent on final TGW. This study has also shown that variation in YP/grain also occurred independent of TGW. Variation in temperature (and humidity) as well as variation in plant available water both influenced TGW; however only variation in plant available water and was associated with variation in YP/grain. Variation in temperature and humidity was not associated with variation in YP/grain. Therefore, since variation in YP/grain was shown to be dependent on TGW, in addition to occurring independent of TGW, the final YP concentration will be influenced by the relative importance of temperature and plant available water in driving variation in TGW. In addition, the timing of heat and/or water stress events during grain development are also likely to be important. As has been discussed, temperature and water stress themselves may not be independent of one another as higher temperature may exacerbate water stress through increased transpiration and evaporation rates and water stress may exacerbate plant temperature through a reduction in transpiration rate. Temperature and precipitation were also shown to be significnalty related to one another in Chapter 2. In addition to the complexity due to the above reasons, positive and negative metabolite feedback mechanisms exist within the carotenoid pathway, and among the pathway products, carotenoids, ABA and also 2-C-methyl-D-erythritol 4-phosphate (MEP) (Cazzonelli 2011). In addition, production of ABA is strongly influenced by the environment and in particular by plant stress, and carotenoid biosynthesis requires substrates derived from the plastid-localised MEP pathway (Rodriguez-Concepcion 2010). Clearly the environmental control of YP is highly

complex and it is not surprising that no clear pattern in YP concentration was observed between a wide set of environments.

5.5 Future research – Non Genetic Control of YP Concentration

Further research is encouraged to test the hypothesis that endosperm cell number and cell size are implicated in YP concentration and YP/grain. This could be achieved most accurately by counting endosperm cells and relating this to the amount of YP/grain and the weight of the grain. A number of methods have successfully been used to count cell number in wheat including staining and counting with the aid of various microscopy techniques (Gao et al. 1992; Mares and Stone 1973) and even with the aid of a haemocytometer (Gao et al. 1992). Once the number of endosperm cells and the amount of endosperm is accurately estimated, the average size of cells can be calculated. The effect of other traits, in addition to water and temperature, could also be investigated for their effect on YP concentration and/or YP/grain. Light intensity or photon exposure may be an interesting trait to target. Carotenoids play a role in light harvesting (Howitt and Pogson 2006) and also have a critical role in limiting membrane damage by functioning as photoprotective compounds (Demmig-Adams and Adams 1996; Niyogi 1999). Light and circadian oscillations appear to regulate genes controlling key enzymes within the carotenoid biosynthetic pathway (Cazzonelli 2011) including *PSY* which controls Phytoene synthase which is a rate limiting regulatory enzyme in the lutein biosynthetic pathway (Cazzonelli and Pogson 2010). Although likely interesting, investigating the effect of light variation is unlikely to be straightforward as light also appears to regulate the expression of MEP genes (Cazzonelli 2011; Cordoba et al. 2009) which have been discussed earlier,

affects grain weight through an effect on endosperm cell number (Brocklehurst 1977; Singh and Jenner 1984; Wardlaw 1970), and is often correlated with temperature.

5.6 Summary

The environmental control of YP concentration has been found to be extremely complex; however significant progress has been made in understanding the effect of specific environmental variables which has led to the proposal of a hypothesis explaining environmental variation in YP concentration and YP/grain. In total, four separate supporting arguments for the hypothesis have been put forward that, in combination, suggest that the proposed hypothesis provides a viable explanation for the observed non-genetic variation in YP concentration. These arguments included;

- A logical argument describing the relationship between amyloplasts, YP, endosperm cells and TGW
- That the timing of YP synthesis within the grain coincides with the development of endosperm cells
- That the observed association between temperature and YP and between water stress and YP is in agreement with the model provided by the proposed hypothesis the context of published reports of the effects of temperature and water stress on endosperm cell number and size
- That the hypothesis provides an explanation for the observed relationship between TGW and both YP concentration and YP/grain in the context of the relationship between TGW and both endosperm cell number and cell size

Further research is suggested to better understand the environmental control of YP in durum wheat including characterising the effect of further environmental variables and characterising the relationship between endosperm cell number, grain weight and YP/grain.

Part II – Genetic Analysis of Variation in Yellow Pigment Concentration of Durum Wheat

Chapter 6: Genetic Control of Yellow Pigment in Durum

6.1 Introduction

A bright yellow colour which can be primarily attributed to endogenous yellow pigments, primarily lutein, is a trademark of good quality durum products and therefore is an important trait for durum wheat breeders. A clear understanding of the genetic control of yellow pigment (YP) concentration in durum wheat semolina is required to make improvements for this important quality trait through breeding.

Breeding for higher or lower levels of YP requires an understanding of the heritability of this trait and the level of genotype x environment interaction that can be expected. High broad sense heritability has been reported for YP in durum (Borrelli et al. 1999c; Braaten et al. 1962; Clarke et al. 2006; Elouafi et al. 2001; Johnston et al. 1983; Santra et al. 2005) and hexaploid bread wheat (Mares and Campbell 2001; Parker et al. 1998). However, Lee et al. (1976) and Patil et al. (2008) have reported a significant genotype x environment interaction effect on YP concentration of durum wheat. To date there has been no published research relating to genotype by environment interaction for YP concentration in Australian durum wheat.

Many breeding programs selecting for yellow pigment concentration do so by measuring the CIELAB b* value of the flour or semolina milled from grain as it is a cheap and quick method of estimating YP concentration. However, the colour of flour or semolina or flour (CIELAB b*) can be influenced by many confounding factors such as particle size (itself influenced by multiple genetic and environmental factors

such as protein, grain hardness and vitreousness) and brightness (CIELAB L*). It was concluded in Chapter 3 that estimating YP concentration indirectly by measuring the colour of flour (CIELAB b*) after the germ and bran had been removed is an effective tool for breeding; however, in early generations when grain sample sizes may be small, producing wholemeal flour may be a useful method of producing enough flour for flour colour (CIELAB b*) analysis.

Chapter 4 presented a strong phenotypic correlation between YP concentration and thousand grain weight (TGW) where TGW was negatively correlated with YP concentration both within and between environments. For selection purposes it needs to be established if there is also a genetic correlation between TGW and YP concentration. Clarke et al. (2006) found that negative genotypic correlations between YP and TGW were generally greater in magnitude than the phenotypic correlations and Hessler et al. (2002) found a QTL associated with both YP and TGW in opposing directions. Yellow pigment per grain (YP/grain) was also considered in the previous chapters. YP/grain is the product of both YP concentration and TGW and so considering YP/grain is an efficient method of considering both of these important quality traits simultaneously. YP concentration is of course the ultimate objective pertaining to end product quality and not YP/grain; however, if a negative genetic correlation exists between TGW and YP concentration then YP/grain may also need to be considered in breeding programs to prevent selection for small grains. The objectives of the research presented in this chapter are to;

1. Gain an improved understanding of the genetic control of YP concentration in Australian durum wheat germplasm

2. Determine effective selection methods/strategies to assist in improving YP concentration in durum wheat through plant breeding

These objectives will be achieved by;

1. Creating two bi-parental recombinant inbred line (RIL) populations expressing variation in YP concentration
2. Characterising the genetic relationship between YP concentration and TGW and between YP/grain and TGW
3. Assess the relationship between YP concentration and wholmeal flour colour (CIELAB b*), and;
4. Determine the extent of genotype by environment interaction for YP concentration

6.2 Materials and Methods

6.2.1 Field Trials and Germplasm

Field trials and germplasm were as described in Chapter 2 (Sections 2.2.6) for the Wollaroi/Tamaroi and WID22221/Tamaroi RIL populations.

6.2.2 Milling, Flour Colour Analysis and Analysis of Yellow Pigment Concentration

Thirty gram sub samples of grain from each field plot were milled in random order using a cyclone mill to produce wholemeal flour for flour colour analysis and flour YP concentration analysis. For some genotypes from some trials insufficient grain

was available to mill 30g which explains some variation in sample numbers between trials presented in the results. The genotypes that produced insufficient grain for analysis seemed random across trials and so their absence from specific data sets is not expected to have affected the overall trend of any of the results presented. The glasshouse trial in 2007/08 of WID22221/Tamaroi RILs provided less grain than the field trials. From the 222 WID22221/Tamaroi RILs with the most grain from this glasshouse trial, 8g samples were milled in random order using a cyclone mill to produce wholemeal flour. Flour colour analysis and analysis of YP concentration of all samples were as described in Chapter 2 (Section 2.2.2).

6.2.3 Statistical Analysis

Yellow pigment (YP) concentration data were statistically adjusted in a two stage analysis. In the first stage analysis, replicated laboratory data was analysed with a restricted maximum likelihood (ReML) analysis to calculate best linear unbiased estimates (BLUEs) of YP concentration of grain from each individual field plot. Field plot number was entered into the model as a fixed term. To account for laboratory error, batch number, day of analysis, and day of milling were entered into the model as random terms and remained in the model if the variance attributable to these terms was at least as large as the standard error.

The second stage analysis aimed to account for variation in YP concentration in the field due to spatial variation. For this a ReML analysis was again used to calculate BLUEs of YP concentration from grain samples of each genotype. Estimates of YP concentration of grain from each field plot from the first analysis were used in this

second analysis. An auto regressive model was used fitting genotype and spatial terms column and row as fixed terms and column x range as a random term. If either column or row were not significant ($p>0.05$) they were removed from the model.

Broad sense heritability (H^2) within trials was calculated following the methods of Reimer et al. (2008) which was as a ratio of the total genetic variance (σ^2_g) to the total phenotypic variance (σ^2_p) such that $H^2 = \sigma^2_g / \sigma^2_p$. The total phenotypic variance (σ^2_p) was estimated as the total genetic variance (σ^2_g) added to the residual variance (σ^2_e) divided by the number of replications (R) such that $(\sigma^2_p) = (\sigma^2_g) + (\sigma^2_e / R)$. The genetic and phenotypic variances were calculated using ReML. Genotypic correlation (r) values between YP and TGW were calculated using Microsoft Excel.

6.3 Results

6.3.1 Population Frequency Distributions

The distribution of YP concentration of Wollaroi/Tamaroi RILs was slightly skewed right (toward higher levels of YP concentration) at Turretfield in both 2007 and 2006 as well as the average of all the YP concentration data across all environments for this population (Table 6.1 and Figure 6.1). At Waite in 2006/2007 and in 2007 the distribution was significantly ($p<0.05$) normal (Table 6.1). Transgressive segregation was displayed for all traits assessed in the Wollaroi/Tamaroi and WID22221/Tamaroi populations (Table 6.2) but was more pronounced in the Wollaroi/Tamaroi population (Figures 6.1 and 6.2). There was a larger spread in YP concentration values among WID22221/Tamaroi RILs than among Wollaroi/Tamaroi RILs (Table 6.2).

Table 6.1: Significance (p value) of normality of the frequency distribution of yellow pigment concentration of Wollaroi/Tamaroi RILs according to an Anderson Darling test for normality. ns = Not significant (p>0.1)

Environment	p value
Turretfield 2007	ns
Waite 2007	0.045
Waite 2006/2007	0.016
Turretfield 2006	ns
Average all sites	ns

Table 6.2: Parental, mean, median, minimum, maximum and standard deviation of average yellow pigment concentration (YP conc.), yellow flour colour (CIELAB b* value), thousand grain weight (TGW) and yellow pigment content per grain (YP(ng)/grain) of Wollaroi/Tamaroi RILs and of yellow pigment concentration of WID22221/Tamaroi RILs

	Wollaroi/Tamaroi RILs				WID22221/Tamaroi RILs
	YP Conc. (mg/kg)	b*	TGW(g)	YP(ng)/grain	YP Conc. (mg/kg)
Mean	7.15	16.9	42.4	303.6	8.54
Median	7.09	16.9	42.2	299.1	8.53
Minimum	5.01	14.9	23.3	187.6	6.02
Maximum	9.95	19.5	58.3	486.2	11.43
Population size	290	290	290	290	339
Tamaroi	5.92	16	43.3	256.4	6.46
Other Parent	7.18	17.4	40.4	290	10.09
Standard deviation	0.92	0.80	5.34	52.26	1.10

Average yellow flour colour (CIELAB b*), average TGW and average YP/grain were all significantly ($p<0.01$) normally distributed according to an Anderson-Darling test for normality (Figures 6.3, 6.4 and 6.5).

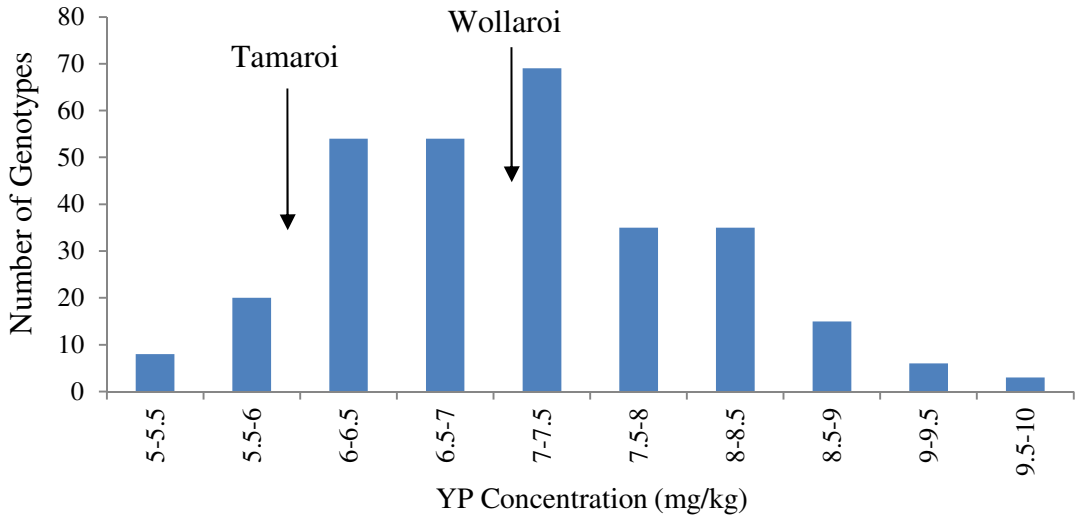

Figure 6.1: Frequency distribution of average yellow pigment (YP) concentration of Wollaroi/Tamaroi RILs across all environments. YP concentration of Tamaroi (5.92 mg/kg) and Wollaroi (7.18 mg/kg) are marked with arrows

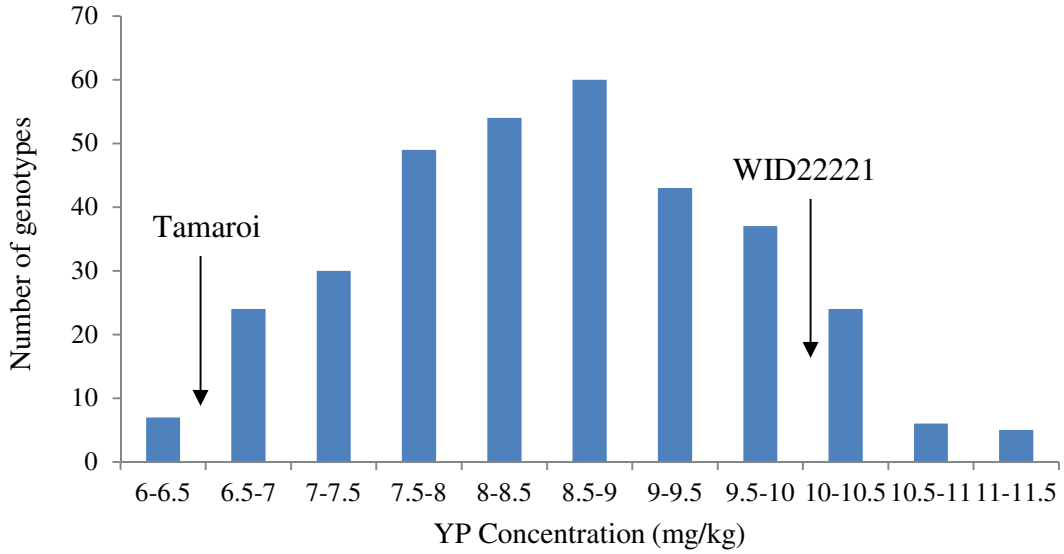

Figure 6.2: Frequency distribution of average yellow pigment (YP) concentration of WID22221/Tamaroi RILs at Waite, SA in 2008. YP concentration of Tamaroi (6.46 mg/kg) and WID22221 (10.09 mg/kg) are marked with arrows

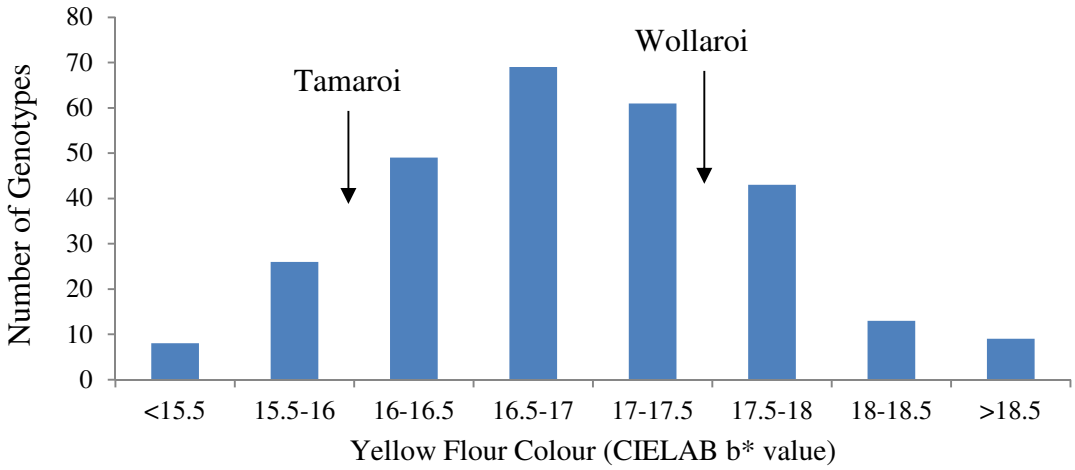

Figure 6.3: Frequency distribution, significantly normally distributed (p<0.01), of average yellow flour colour (CIELAB b* value) of Wollaroi/Tamaroi RILs across all environments. Average CIELAB b* value of Tamaroi (16.0) and Wollaroi (17.4) are shown

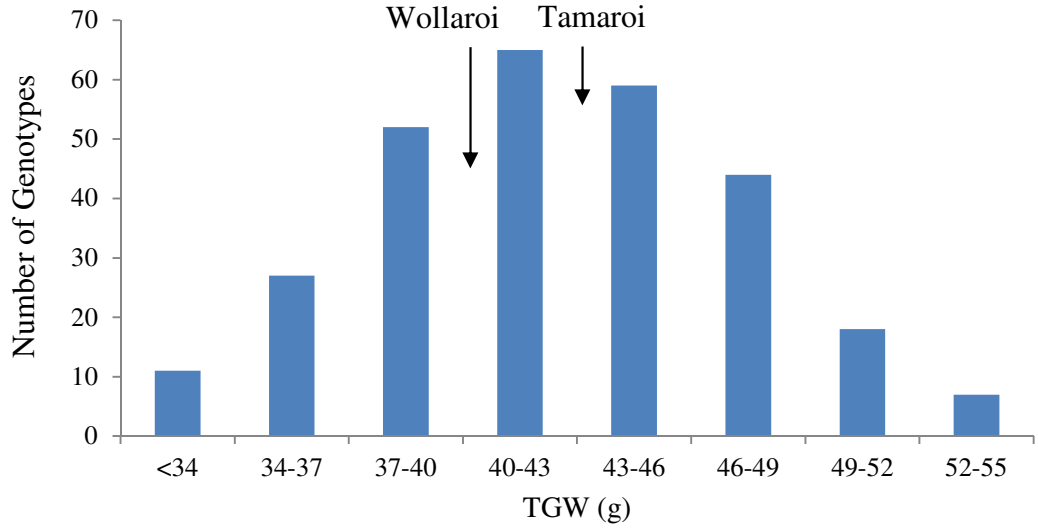

Figure 6.4: Frequency distribution, normally distributed (p<0.001), of thousand grain weight (TGW) of Wollaroi/Tamaroi RILs across all environments. Average TGW of Tamaroi (43.3g) and Wollaroi (40.4g) are shown

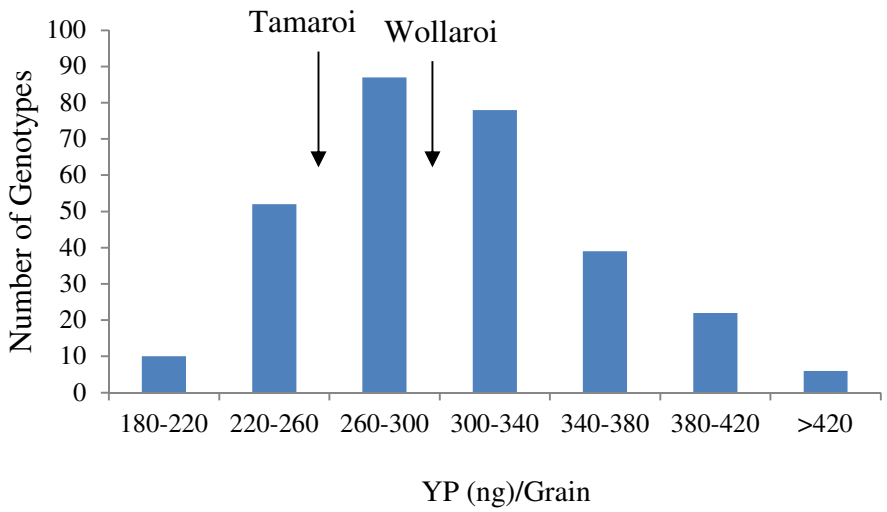

Figure 6.5: Frequency distribution, significantly ($p<0.05$) normally distributed, of average yellow pigment content per grain (YP(ng)/Grain) of Wollaroi/Tamaroi RILs across all environments. Average YP content per grain of Tamaroi (256.4 ng/grain) and Wollaroi (290.0 ng/grain) are presented

6.3.2 Genetic relationship between Yellow Pigment Concentration and Grain Weight

There was a weak genetic correlation between YP concentration and TGW of Wollaroi/Tamaroi RILs which was significant ($p<0.01$) at two of the four environments (Table 6.3). This relationship was strongest at Turretfield in 2007 (Table 6.3). In this field trial the correlation between YP concentration and TGW appeared to be leveraged by four genotypes which had the largest average grain weight and low YP concentration relative to the rest of the population (Figure 6.6); however, the negative correlation remained significant even after removal of these four data points (data not shown). There was also a weak negative correlation between YP concentration and TGW of WID22221/Tamaroi RILs in 2008; which was not significant (data not shown).

Table 6.3: Genetic correlation (r) values between flour YP concentration and thousand grain weight (TGW) of Wollaroi/Tamaroi RILs at all four environments individually and the average across the four environments and the significance (p) of each correlation. ns = Not significant

Environment	r	p
Waite 2007	-0.05	ns
Turretfield 2007	-0.23	<0.005
Waite 2006/2007	-0.16	<0.01
Turretfield 2006	-0.06	ns
Average all sites	-0.05	ns

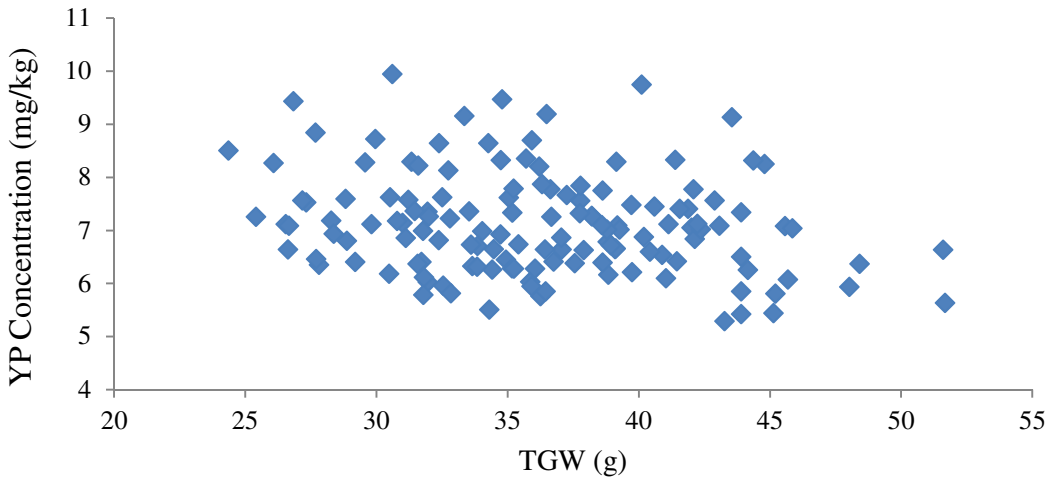

Figure 6.6: Genetic relationship between flour yellow pigment (YP) concentration and one thousand grain weight (TGW) at Turretfield in 2007 for Wollaroi/Tamaroi RILs; r= -0.23, n=144, p<0.01

6.3.3 Genetic Relationship between Yellow Pigment per Grain and Grain Weight

There was a significant (P<0.001) positive correlation between YP/grain and TGW for WID22221/Tamaroi RILs at Turretfield in 2008 and of Wollaroi/Tamaroi RILs at each of the four environments (Table 6.7). The average YP/grain and TGW values across all four environments of Wollaroi/Tamaroi RILs were also significantly (p<0.001) correlated (Table 6.4 and Figure 6.7).

Table 6.4: Significant (P<0.001) correlation (r) values between yellow pigment per grain (YP/grain) and thousand grain weight (TGW) of Wollaroi/Tamaroi RILs at four environments individually; between the mean TGW and mean YP concentration across the four environments; and between TGW and YP/grain of WID22221/Tamaroi RILs at Waite in 2008. The sample number for each correlation is presented (n)

Population	Environment	r	n
Wollaroi/Tam	Turretfield 2007	0.70	145
	Waite 2007	0.77	199
	Turretfield 2006	0.71	239
	Waite 2006/2007	0.72	265
Wollaroi/Tam	Average all sites	0.58	290
WID22221/Tam	Waite 2008	0.50	339

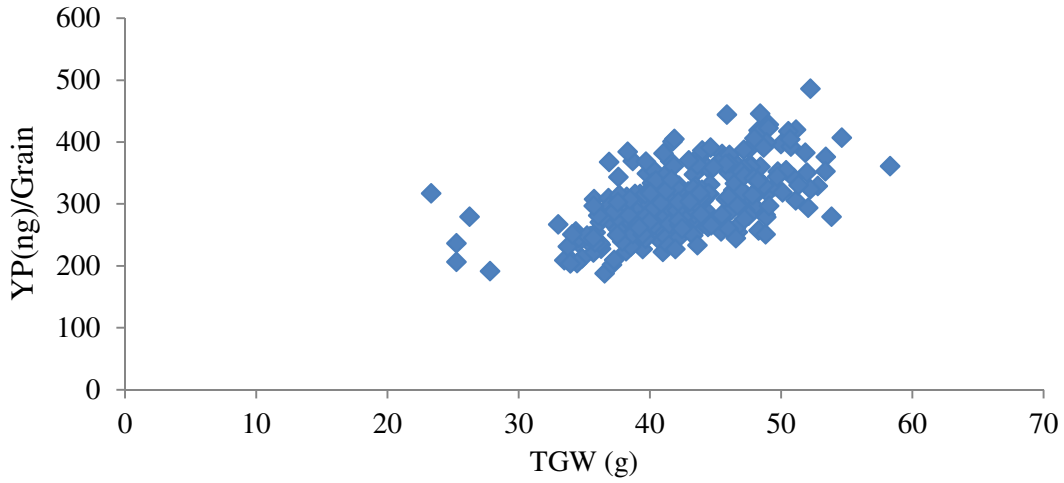

Figure 6.7: Relationship between average one thousand grain weight (TGW) and average yellow pigment content per grain (YP(ng)/grain) across all four environments of Wollaroi/Tamaroi RILs (r = 0.58, n=282, p<0.001))

6.3.4 Relationship between Yellow Pigmnet concentration and Wholemeal Flour Colour (CIELAB b)*

For both the Wollaroi/Tamaroi and WID22221/Tamaroi populations at each individual trial, and for all data averaged across environments, there was a significant ($p<0.001$) correlation between YP concentration and wholemeal flour colour (Figure 6.8 and Table 6.5).

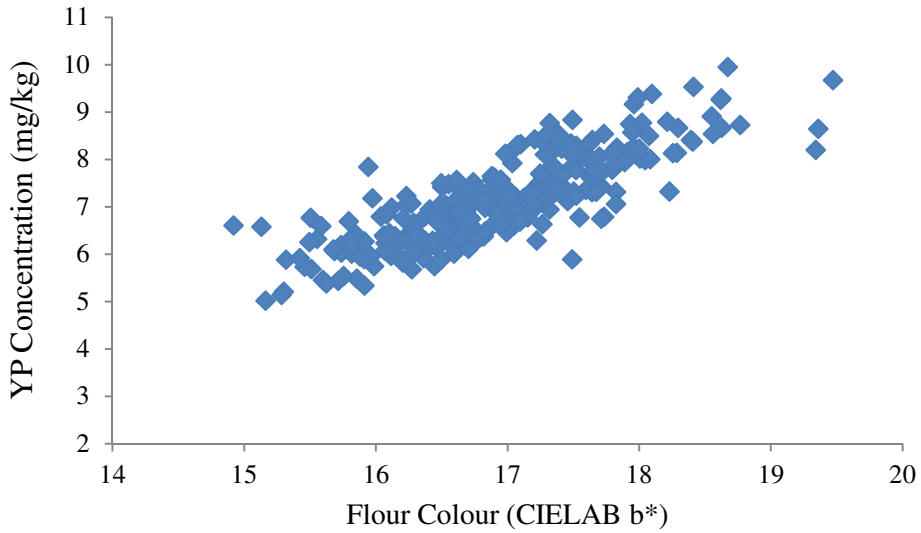

Figure 6.8: Relationship between average wholemeal flour yellow pigment (YP) concentration and average wholemeal flour colour across all four environments of Wollaroi/Tamaroi RILs (r=0.83, n=296, p<0.001)

Table 6.5: Correlation (r) between yellow pigment concentration and wholemeal flour colour (CIELAB b*) of Wollaroi/Tamaroi RILs at each of the four environments and of WID22221/Tamaroi RILs at Turretfield in 2008 and number of data points (n) for each trial

Population	Environment	r	n
Wollaroi/Tam	Turretfield 2007	0.67	257
	Waite 2007	0.84	281
	Turretfield 2006	0.80	249
	Waite 2006/2007	0.78	268
Wollaroi/Tam	Average all sites	0.83	296
WID22221/Tam	Waite 2008	0.69	339

6.3.5 Heritability and GxE

The broad sense heritability estimates for YP concentration within each of the four trials were very high and ranged from H^2=0.86 at Waite in 2007 to H^2=0.97 at Turretfield in 2006 (Table 6.6). Comparing the mean squares from the ANOVA revealed that the environment (mean square = 0.012) contributed substantially more to the total observed variance across trials than genotype (mean square = 0.003) (Table 6.7). There was a strong and highly significant (P<0.001) correlation between each of the four environments for YP concentration (Table 6.8).

Table 6.6: Broad sense heritability (H^2) of yellow pigment concentration for each environment the Wollaroi/Tamaroi trial was grown

Environment	Heritability (H^2)
Turretfield 2006	0.97
Turretfield 2007	0.91
Waite 2006/2007	0.94
Waite 2007	0.86

Table 6.7: Results from analysis of variance of average yellow pigment concentration of Wollaroi/Tamaroi RILs across four environments. Sum of squares, means squares and significance (p value) of effect of Environment and Genotype are presented

Source	Sum of Squares	Means Squares	p
Environment	0.037	0.012	<.001
Genotype	0.929	0.003	<.001

Table 6.8: Correlation (r) values for yellow pigment concentration between each of the four environments and the average across all environments. Number of data points presented in parentheses. All correlation values are significant (p<0.001) and calculated with a simple linear regression analysis

	Waite 2007	Turretfield 2007	Waite 2006/2007	Turretfield 2006
Average all sites	0.92 (280)	0.89 (252)	0.88 (270)	0.88 (239)
Turretfield 2006	0.76 (239)	0.71 (239)	0.61 (239)	*
Waite 2006/2007	0.74 (270)	0.73 (252)	*	
Turretfield 2007	0.74 (257)	*		

6.1 Discussion

6.4.1 Relationship between Yellow Pigment Concentration and Wholemeal Flour colour

The correlation between wholemeal YP concentration and wholemeal flour colour was very strong ranging from r=0.67 at Turretfield in 2007 to r=0.84 at Waite in 2007. This is generally a lower correlation that reported in Chapter 3 between YP concentration and flour colour based on flour which had the germ and bran removed. This suggests that there may be additional confounding factors involved in estimating YP concentration from wholemeal flour indirectly by measuring flour colour (CIELAB b*). Alternatively, the slightly lower correlation value between these traits when using wholemeal may be a function of a reduced range in YP concentrations and flour colour (CIELAB b*) values. Either way, with such high correlation (r) values, estimating YP concentration indirectly from the colour of wholemeal flour appears to be an effective tool for breeding.

6.4.2 Heritability and GxE

For all environments the broad sense heritability of YP concentration was high and sites were highly significantly correlated with one another, indicating that there was little genotype by environment (G x E) interaction for YP concentration. Due to limited seed and limited resources only the Wollaroi/Tamaroi population at Waite, SA in 2007 was replicated and so the precise extent of genotype by environment interaction across trials could not be calculated. The limited G x E interaction indicated by these results are in agreement with results reported in Chapter 3 and with other authors (Clarke et al. 2006; Elouafi et al. 2001; Mares and Campbell 2001; Parker et al. 1998; Patil et al. 2008), who all reported high heritability and low genotype by environment interaction. Together, the high heritability, lack of (or at least limited) GxE interaction, and close correlation between environments should give Australian breeders confidence in selecting for YP in just one or two environments and so expensive testing across multiple locations and/or years should not be required.

6.4.3 Population Frequency Distributions

There was generally a normal population frequency distribution for all traits of the Wollaroi/Tamaroi population and for YP concentration of the WID22221/Tamaroi population. For all three traits in both populations transgressive segregation was observed. Assessment of the frequency distributions, and, the extent of transgressive segregation, suggests that YP concentration, YP/grain, and TGW are likely to be under polygenic control. Transgressive segregation for YP concentration appeared to

be more pronounced in the Wollaroi/Tamaroi population than in the WID22221/Tamaroi population. This indicates that Wollaroi and Tamaroi are likely to share fewer alleles influencing YP concentration than WID22221 and Tamaroi. The larger range in YP concentrations among the WID22221/Tamaroi RILs compared to the Wollaroi/Tamaroi RILs is likely due to a function of differences in population size.

6.4.4 Genetic Relationship between Grain Weight and Yellow Pigment Concentration

There was a significant ($P<0.01$) negative correlation observed between TGW and YP concentration of Wollaroi/Tamaroi RILs at Turretfield in 2007 ($r=-0.23$) and at Waite in 2006/2007 ($r=-0.16$). The correlation was generally not strong and could easily go unnoticed within a breeding program; however, due to this relationship, selection for increased YP concentration could result in selection of genotypes with small grains. In terms of selection within a breeding program, genetic relationships are very different and much more important than phenotypic relationships. The genotypic correlation between TGW and YP concentration observed here within environments was much weaker than the phenotypic correlation observed between environments in Chapters 3 and 4. This was not due to a lack of variation in either TGW or YP concentration as the range in TGW and YP concentration reported here are similar to that reported in Chapters 3 and 4.

6.4.5 Genetic Relationship between Grain Weight and Yellow Pigment Content per Grain

There was a strong and significant genetic correlation observed between TGW and YP/grain of Wollaroi/Tamaroi RILs at each of the four environments; which might be expected since YP/grain and TGW are not independent. This is consistent with results reported in Chapters 3 and 4 in which significant environmental and physiological correlations between TGW and YP/grain were identified. Unlike YP concentration, which is affected mostly by environmentally induced variation in TGW, YP/grain seems to be affected by both environmentally and genetically induced variation in TGW. As there is a positive genetic correlation between TGW and YP/grain, then selecting for higher levels of YP/grain will obviously result in selection of genotypes with large grain size. As discussed previously, selection for high YP concentration on its own may lead to selection for small grain. Genotypes with small grain size are not desirable as they will tend to produce high screenings and lower milling yield (Marshall et al. 1986; Wiersma et al. 2001). Therefore the best approach is to select for YP concentration, but only amongst genotypes which have suitably large grain size thus avoiding selecting genotypes with a propensity for screenings and/or low milling yield.

6.4.6 Summary

YP concentration is significantly influenced by genotype and appears to be under complex polygenic control. YP concentration was found to be a highly heritable trait with limited interaction between genotype and the environment. The high heritability and limited GxE interaction make selection for this trait efficient and the close correlation between wholemeal flour colour and wholemeal flour YP concentration

allows for cheap and fast selection. In some environments a negative correlation between TGW and YP concentration was observed and so it is recommended that a selection strategy be adopted that integrates simultaneous selection for high YP concentration and large grain size.

Chapter 7: Analysis of QTL Associated with Yellow Pigment

7.1 Introduction

The previous chapter described the nature of the genetic control of variation in yellow pigment (YP) concentration and YP content per grain (YP/grain) for the Wollaroi/Tamaroi and WID22221/Tamaroi populations at a number of environments. Previous chapters reported a strong and significant ($P<0.01$) effect of genotype on both YP concentration and YP/grain and very limited genotype by environment interaction for these traits. It was concluded from the frequency distributions of Wollaroi/Tamaroi RILs and WID22221/Tamaroi RILs presented in Chapter 5 that genetic variation for YP concentration and YP/grain are under complex polygenic control. Reports in the literature have identified multiple QTL associated with variation in flour colour and/or yellow pigment (Atienza et al. 2007; Carrera et al. 2007; Elouafi et al. 2001; He et al. 2009b; He et al. 2008; Hessler et al. 2002; Howitt et al. 2009; Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998; Patil et al. 2008; Pozniak et al. 2007; Reimer et al. 2008; Singh et al. 2009; Zhang and Dubcovsky 2008). Furthermore, candidate genes coding for key enzymes of the carotenoid (including lutein) biosynthetic pathway, specifically phytoene synthase and ε-cyclase, have been mapped to group 7 (He et al. 2009b; He et al. 2008; Patil et al. 2008; Pozniak et al. 2007; Reimer et al. 2008; Singh et al. 2009) and 3 (Howitt et al. 2009) chromosomes respectively and have been reported to be associated with variation in YP concentration. However, there have been no studies identifying or validating quantitative trait loci (QTL) associated with YP in exclusively Australian durum wheat germplasm. Identification and/or validation of QTL associated with YP in Australian germplasm will assist breeding efforts to select for this important quality trait in Australia. Results from Chapter 3 illustrated that the two popular South

Australian durum wheat cultivars Tamaroi and Kalka were among the poorest of the germplasm tested for YP concentration. Therefore there is an urgent need within the South Australian durum breeding program to efficiently and effectively select for genotypes with improved YP concentration. Marker assisted selection (MAS) offers one method of improving selection efficiency for complex quantitative traits such as YP concentration. The successful application of MAS within breeding programs has been well documented (Jefferies et al. 2003; Kuchel et al. 2006b; Yousef and Juvik 2001; Yu et al. 2000). For MAS to be used sensibly by Australian durum breeders molecular markers associated with high levels of YP need to be identified, preferably in Australian adapted germplasm. The objective of this chapter is to identify QTL associated with flour YP concentration, flour colour (CIELAB b*), and YP/grain in Australian durum wheat germplasm. The importance of the relationship between thousand grain weight (TGW) and YP has been explained previously and therefore association between QTL and TGW will also be investigated. Furthermore, due to extensive literature reporting the effects of the phytoene synthase genes, this chapter aims to characterise presence or absence of polymorphism at these genes on chromosomes 7AL and 7BL and the effect of allelic differences at these genes on YP concentration and YP/grain.

7.2 Materials and Methods

7.2.1 Field and Glasshouse Trials, Germplasm and Development of Phenotypic data

Phenotypic data were derived from the field trials of Wollaroi/Tamaroi RILs and the field trial and glasshouse trial of WID22221/Tamaroi RILs described in Chapter 2

(section 2.2.7). Phenotypic data of Wollaroi/Tamaroi RILs and WID22221/Tamaroi RILs presented in Chapter 5 were used for QTL analysis.

7.2.2 Molecular Marker Assays

Wollaroi/Tamaroi population

Ten Wollaroi/Tamaroi RILs and their parents Wollaroi and Tamaroi, were assayed with 37 simple sequence repeat (SSR) markers on chromosomes 2A (*gwm425*, *gwm95*, *wmc296*), 3A (*wmc664*, *barc67*, *gwm32*, *gwm674*), 3B (*gwm376*, *gwm285*, *barc73*), 4B (*gwm495*, *gwm113*, *gwm368*), 5B (*gwm371*, *gwm499*, *wmc75*), 6B (*gwm193*, *barc134*, *barc14*), 7A (*gwm276*, *cfa2293*, *barc49*, *barc174*, *wmc116* and *gwm344*) and 7B (*cfa2257*, *barc340*, *barc1073*, *wmc526* and *gwm146*). These 37 markers were chosen as they have been mapped within the vicinity of chromosome regions which have previously been reported to be associated with variation in YP concentration or flour colour in durum or hexaploid wheat (Elouafi et al. 2001; Hessler et al. 2002; Kuchel et al. 2006b; Mares and Campbell 2001; Pozniak et al. 2007). The durum wheat genotypes AC Avonlea, AC Navigator, Renville, and Senatore Capelli were genotyped (for comparison purposes) for the phytoene synthase gene, *Psy1-A1*, on chromosome 7A using markers *YP7A* (He et al. 2009b), *YP7A-2* (He et al. 2009b) and *Psy1-A1_STS* (Singh et al. 2009); for *Psy1-B1* on chromosome 7B using primer pair *Psy1F5/R5* (Pozniak et al. 2007); and for *Psy2-B2* on chromosome 5B using *Psy2-2F/Psy2R2* (Pozniak et al. 2007). The ten Wollaroi/Tamaroi RILs were chosen such that there were five RILs with the highest and five RILs with lowest YP concentration based on phenotypic data from the trial conducted at Turretfield in 2006. This approach was used to improve the chances of polymorphisms being identified for any markers that may be associated with YP.

Polymorphisms at *Psy1-A1* (using marker YP7A) and *Psy1-B1* (using marker *Psy1-1F5/R5*) were identified and so the remaining 280 Wollaroi/Tamaroi RILs were genotyped with these markers.

An additional 295 SSR markers were assayed, using methods and technology (multiplex ready technology (MRT)) developed by Hayden et al. (2008), across four separate bulks of Wollaroi/Tamaroi RILs in a modified bulk segregant analysis (BSA) approach. The 295 markers were part of a whole genome scan kit (Hayden et al. 2008) and were chosen on the basis that they were of good quality, easy to score and collectively spanned the entire genome by aiming to cover every 20 cM with at least one marker. The markers were scored according to their clear amplification, and, following separation by capillary electrophoresis, were detected by fluorescence (ABI3730, BioSystems). Each of the four bulks were made up of six Wollaroi/Tamaroi RILs. The four bulks were developed such that the RILs making up each bulk satisfied the following criteria based on phenotypic data from the Turretfield 2006 and Waite 2006/2007 trials and the genotypic data generated for the *YP7A* and *Psy1F5/R5* markers;

- Bulk 1: High YP and fixed for Wollaroi alleles at *Psy1-A1* (*YP7A*) and *Psy1-B1* (*Psy1-1F5/R5*)
- Bulk 2: Low YP and fixed for Wollaroi alleles at *Psy1-A1* (*YP7A*) *and Psy1-1B* (*Psy1-1F5/R5*)
- Bulk 3: High YP and fixed for Tamaroi alleles at *Psy1-A1* (*YP7A*) and *Psy1-B1* (*Psy1-1F5/R5*)
- Bulk 4: Low YP and fixed for Tamaroi alleles at *Psy1-A1* (*YP7A*) and *Psy1-B1* (*Psy1-1F5/R5*)

Bulks were developed in this way to improve the chance of finding markers associated with YP concentration independent of any potential effect of either *Psy1-A1* (*YP7A*) or *Psy1-B1* (*Psy1-1F5/R5*). This method also allowed for identification of markers associated with YP concentration and also linked to either *Psy1-A1* (*YP7A*) or *Psy1-B1* (*Psy1-1F5/R5*) by comparing bulk 1 to bulks 3 and 4 and bulk 2 to bulks 3 and 4; thereby increasing map resolution in these chromosome regions which have previously been reported to be associated with variation in YP concentration in durum or bread wheat (Elouafi et al. 2001; Hessler et al. 2002; Kuchel et al. 2006b; Mares and Campbell 2001; Parker and Langridge 2000; Patil et al. 2008; Pozniak et al. 2007; Reimer et al. 2008; Zhang et al. 2008; Zhang and Dubcovsky 2008; Zhang et al. 2009).

The preliminary results of the modified BSA identified eight loci putatively associated with either YP concentration or flour colour (CIELAB B*). Marker density at these loci was low and so a further 280 MRT SSR markers where chosen which according to a range of sources (Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998; Pozniak et al. 2007; Qi et al. 2004; Reimer et al. 2008; Somers et al. 2004; Zhang et al. 2008; Zhang and Dubcovsky 2008), map at, or near to, one of the eight putative loci. These 280 MRT SSR markers were assayed across 10 randomly chosen Wollaroi/Tamaroi RILs to identify those markers polymorphic in this population which were then assayed across all individuals of the population.

As a result of the above assays, a total of 75 markers polymorphic in the Wollaroi/Tamaroi population were successfully assayed and scored across all

individuals of the population. These 75 markers either map to regions reported in the literature to be associated with YP or flour colour in durum or hexaploid wheat, or, were polymorphic between the bulks described earlier; thus indicating they may be associated with variation in YP concentration, or, that they may be linked to either *YP7A* or *Psy1-1F5/R5* and thereby would increase resolution at these loci.

WID22221/Tamaroi population

A total of 73 molecular markers from each of the 14 chromosomes were assayed across 10 WID22221/Tamaroi RILs. The 10 RILs were chosen such that five expressed high YP and 5 low YP concentration to improve the chance of finding polymorphic markers associated with variation in YP concentration, similar to the methods applied to the Wollaroi/Tamaroi population. The 73 markers represented all the markers that were significantly associated with YP concentration or flour colour in the Wollaroi/Tamaroi population, or have been reported in the literature to be associated with YP concentration or flour colour in durum or bread wheat, or, have been reported as linked to these regions according to maps published by a range of authors (Elouafi et al. 2001; Hessler et al. 2002; Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998; Patil et al. 2008; Pozniak et al. 2007; Qi et al. 2004; Reimer et al. 2008; Somers et al. 2004; Zhang et al. 2008; Zhang and Dubcovsky 2008). Included in these markers were *Psy1-A1_STS* (Singh et al. 2009) *YP7A* (He et al. 2008) and *Psy1F5/R5* (Pozniak et al. 2007). From these 73 markers, 21 were found to be polymorphic and were assayed across every individual in the population. Markers that did not form linkage groups were assigned to their chromosome according to the consensus map (Somers et al. 2004) and/or the physical map (Qi et al. 2004).

7.2.3 Map Construction and QTL Analysis

For the Wollaroi/Tamaroi population, map construction, QTL analysis, calculation of logarithm of odds (LOD) score, percentage variation accounted for and effect of marker were performed using interval mapping in Map Manager QTX software (Manly et al. 2001) using the Kosambi mapping function (Kosambi 1943). Associations between molecular markers and phenotypic traits were tested using interval analysis (Lander and Botstein 1989). Graphical representations of QTL associations were generated using QGENE 4.0 software (Joehanes and Nelson 2008). QTL exhibiting LOD $\geq$ 3.0 were regarded as statistically significant.

As only 21 markers were assayed across the WID22221/Tamaroi population QTL analysis was not performed on this population as many markers were unlinked or formed only very small linkage groups. Regression analysis between polymorphic markers in WID22221/Tamaroi population and the response variate YP concentration, yellow flour colour, TGW and YP/grain was performed using Genstat 11 software. Chromosome location of the makers assayed across the WID22221/Tamaroi population were predicted based on the mapped location of these markers from a range of publications (Elouafi et al. 2001; Hessler et al. 2002; Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998; Patil et al. 2008; Pozniak et al. 2007; Qi et al. 2004; Reimer et al. 2008; Somers et al. 2004; Zhang et al. 2008; Zhang and Dubcovsky 2008).

7.2.4 Statistical Analysis

A significance threshold LOD score of 3 was used for determining significance of QTL and putative QTL with a LOD score above 2 were also reported. Interactions between QTL were tested with ANOVA fitting either YP concentration or YP/grain as the response variate and individual markers (linked to QTL) as factors. This analysis calculated a predicted mean for each genotype combination for each of the fitted QTL which is presented. Percentage of phenotypic variation accounted for by combinations of QTL were calculated by multiple linear regression by fitting YP concentration or YP/grain as the response variate and marker score at markers linked to QTL as the explanatory variates. Significant differences in YP concentration between individuals with either Wollaroi or Tamaroi alleles at *cfa2293* and *YP7A* were tested with a Tukey test. Mean YP concentration and YP/grain for each genotypic class at *cfa2293* and *YP7A* were also calculated with ANOVA which enabled QTL at other loci to be fitted as blocking (nuisance) factors. The model fitted YP concentration as the response variate and marker score at *cfa2293* and *YP7A* as factors and marker score at *gwm46*, *PSY1-1F5/R5*, *wmc84*, *gwm193*, *barc164* on chromosomes 7BS, 7BL, 1A, 6B and 3B respectively as blocking (nuisance) factors to remove the effect of the association between these QTL and YP.

7.3 Results

7.3.1 Wollaroi/Tamaroi Population

Sixty markers were mapped to fourteen separate linkage groups on eleven chromosomes. There were no linkage groups mapped to chromosomes 2B and 5B while chromosomes 3A, 7A and 7B each had two separate linkage groups. There were also 15 markers which were polymorphic in the Wollaroi/Tamaroi population identified as unlinked. One of these, *barc164*, was assigned to chromosome 3B based on the mapped location of this marker reported in a number of publications (Maccaferri et al. 2008; McCartney et al. 2005; Qi et al. 2004; Reimer et al. 2008; Somers et al. 2004; Zhang et al. 2008; Zhang et al. 2009).

Markers for both *Psy1-A1* and *Psy1-B1* on chromosomes 7A and 7B respectively were polymorphic in this population. Amplification of *Psy1-A1* with *Psy1-A1_STS* produced an 897 base pair fragment from Wollaroi suggesting that Wollaroi has the *Psy1-A1o* allele (Singh et al. 2009). No product was obtained for Tamaroi with this primer pair. Amplification of *Psy1-A1* with *YP7A-2F/2R* produced a 1001 base pair fragment from Tamaroi suggesting that Tamaroi has the *Psy1-A1c* allele reported by He et al. (2009b). AC Avonlea, AC Navigator, Renville and Senatore Capelli were also shown to carry the *Psy1-A1c* allele.

Six QTL were identified to be associated with flour YP concentration, three of these were significant ($p<0.001$) across all four environments while the remaining three were of lesser effect and not significant in every environment (Table 7.1). The most significant of these QTL was on chromosome 7AL (Figure 7.1). In the first instance

YP concentration was mapped for each environment on chromosome 7AL but each map was very similar (data not shown) therefore a single map of the average data across all environments is presented (Figure 7.1). This QTL explained 16, 17 and 18 per cent of the phenotypic variation in average YP concentration, average yellow flour colour and average YP/grain across the four environments of Wollaroi/Tamaroi RILs respectively (Tables 7.1, 7.2 and 7.3). At this QTL, marker *cfa2293* was most tightly linked to the peak of the QTL. Wollaroi contributed the favourable allele(s) for high YP concentration. The functional marker for the phytoene synthase gene, *YP7A* (He et al. 2008), located distal to this QTL on chromosome 7AL and was polymorphic within this population but was not significantly associated with any of the YP or flour colour traits in any of the environments tested. *YP7A* mapped approximately 28cM distal to marker *cfa2293*.

A comparison of individuals sharing the same allele at *cfa2293* with a Tukey test revealed that there was no significant association between *YP7A* and either YP concentration (Tables 7.4 and 7.5) or YP/grain (Table 7.6). In all environments, except at Waite in 2006/2007, RILs carrying Tamaroi alleles at *YP7A* were associated with higher YP concentration independent of the alleles present at *cfa2293* but the effect was not significant ($p>0.05$) (Table 7.4). This result held even when the effect of the other five QTL associated with YP concentration were accounted for by including these QTL as blocking terms in the analysis of variance (Table 7.5).

Table 7.1: QTL associated with flour yellow pigment (YP) concentration in the Wollaroi/Tamaroi population across four environments and the average YP across all four environments. T.field=Turretfield, the 2006 trial was also at Turretfield and the 2006/07 trial was at Waite. Chromosome arm, most significant marker, flanking markers, parent contributing positive (+ve) allele(s) for higher YP concentration (W=Wollaroi, T=Tamaroi), LOD score for the association and percentage of phenotypic variation accounted for by QTL (%V.A.) are shown. N/A=Not applicable due to no linked markers. ns = Not significant (LOD<2)

				T.field 2007		Waite 2007		2006/07		2006		Average 4 trials	
Chromosome	Marker	Flanking Markers	+ve allele	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.
7AL	*cfa2293*	*wmc346, stm0415tcac*	W	6.9	13	10.4	16	8.5	14	6.9	13	10.7	16
7BL	*Psy1-1F5/R5*	*wmc276, wmc526*	W	3.4	7	5.1	10	4.7	8	3.2	9	6.2	12
7BS	*gwm46*	*barc267*	W	6.4	12	8.7	14	5.9	10	6.2	12	8.7	14
6B	*wmc397*	*gwm193*	T	2.6	5	ns		4.2	7	ns		3.3	5
1A	*wmc84*	*gwm164, barc83*	T	3.0	6	2.1	4	ns		3.0	6	3.0	5
3B	*barc164*	N/A	T	ns		2.6	4	ns	ns	2.4	5	2.6	4
2A	*stm0552tgag*	*gwm515, wmc522*	T	ns		ns		3.4	6	ns		ns	

Table 7.2: QTL associated with yellow flour colour (CIELAB b*) in the Wollaroi/Tamaroi population across four environments and the average flour across all four environments. T.field=Turretfield, the 2006 trial was also at Turretfield and the 2006/07 trial was at Waite. Chromosome arm, most significant marker, flanking markers, parent contributing positive (+ve) allele(s) for higher levels of yellow flour colour (W=Wollaroi, T=Tamaroi), LOD score for the association and percentage of phenotypic variation accounted for by QTL (%V.A.) are shown. N/A=Not applicable due to no linked markers. ns = Not significant (LOD<2)

				T.field 2007		Waite 2007		2006/07		Waite2006		Average 4 trials	
Chromosome	Marker	Flanking Markers	+ve allele	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.
7AL	*cfa2293*	*wmc346 stm0415tcac*	W	6.6	12	10.8	17	12.2	19	3.4	7	11.7	17
7BL	*Psy1-1F5/R5*	*gwm304 wmc526*	W	3.1	7	2.1	4	4.7	8	3.2	11	4.6	9
7BS	*gwm46*	*barc267*	W	5.1	9	6.0	10	3.8	7	4.9	10	6.4	10
6B	*wmc397*	*gwm193*	T	3.6	7	2.4	4	4.8	8	2.6	5	4.6	7
1A	*wmc84*	*gwm164 barc83*	T	2.2	4	ns	ns	2.1	4	ns	ns	3.0	5
3B	*barc164*	N/A	T	ns	ns	ns	ns	2.2	4	ns	ns	ns	ns

Table 7.3: QTL associated with yellow pigment (YP) per grain in the Wollaroi/Tamaroi population across four environments and the average YP per grain across all four environments. T.field=Turretfield, the 2006 trial was also at Turretfield and the 2006/07 trial was at Waite. Chromosome arm, most significant marker, flanking markers, parent contributing positive (+ve) allele(s) for high YP/grain (W=Wollaroi, T=Tamaroi), LOD score for the association and percentage of phenotypic variation accounted for by QTL (%V.A.) are shown. ns = Not significant (LOD<2)

				T.field 2007		Waite 2007		2006/07		2006		Average 4 trials	
Chromosome	Marker	Flanking Markers	+ve allele	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.
2A	*stm0552tgag*	*gwm515, wmc522*	W	7.3	21	4.3	10	1.6	3	3.21	7	4.9	8
7AL	*cfa2293*	*cfa2019, stm0415tcac*	W	3.4	12	9.1	19	11.5	19	4.97	9	12.3	18
7BL	*Psy1-1F5/R5*	*gwm304, wmc526,*	W	3.1	9	3.1	6	3.2	6	3.3	8	4.3	9
7BS	*gwm46*	*barc267*	W	2.8	5	2.5	6	4.2	8	3.90	8	4.5	7
3B	*barc164*		T	ns		2.8	6	ns		2.04	4	2.6	4
1B	*barc240*	*barc302*	W	ns		ns		ns		4.27	8	3.8	6
1A	*wmc611*	*wmc469, wmc333*	T	ns		ns		2.1	4	2.54	5	2.5	4

Table 7.4: Average yellow pigment concentration (YP) (mg/kg) for individuals in each genotype class for each of the four possible combinations of Tamaroi (T) and Wollaroi (W) alleles at markers *cfa2293* and *YP7A* (*Psy1-A1* gene) at each of the four environments separately and for the average across all four environments. Number of individuals of the population within each genotype class tested (N) is shown. YP values not connected by the same letter are significantly (P<0.05) different from one another according to a Tukey test (^{A,B,C})

Alleles at *cfa2293* and *YP7A*	Average YP all sites	N	Waite 2007 YP	N	Turretfield 2007 YP	N	Waite 2006/2007 YP	N	Turretfield 2006 YP	N
TT	6.84^{A}	93	6.96^{A}	90	6.96^{A}	87	6.39^{A}	89	6.93^{A}	73
TW	6.75^{A}	58	6.87^{A}	55	6.75^{B}	46	6.60^{AB}	53	6.90^{A}	46
WT	7.71^{B}	22	7.98^{B}	19	7.77^{C}	15	7.20^{BC}	20	7.83^{B}	17
WW	7.47^{B}	106	7.65^{B}	102	7.44^{C}	89	7.29^{C}	95	7.47^{B}	88

Table 7.5: Average yellow pigment concentration (YP) (mg/kg) for individuals in each genotype class for each of the four possible combinations of Tamaroi (T) and Wollaroi (W) alleles at markers *cfa2293* and *YP7A* (*Psy1-A1* gene) at each of the four environments separately and for the average across all four environments. Number of individuals of the population within each genotype class tested (N) is shown. QTL influencing YP at 7BS (*gwm46*), 7BL (*PSY1-1F5/R5*), 1A (*wmc84*), 6B (*gwm193*) and 3B (*barc164*) were included in the model as blocking terms to account for the effect of any possible unequal segregation of these QTL between groups shown. YP values not connected by the same letter differ by more than the sum of the standard error associated with each pair of means calculated by analysis of variance (^{A,B,C})

Alleles at *cfa2293* and *YP7A*	Average YP all sites	N	Waite 2007 YP	N	Turretfield 2007 YP	N	Waite 2006/2007 YP	N	Turretfield 2006 YP	N
TT	6.90^{A}	93	7.02^{A}	90	7.05^{A}	87	6.42^{A}	89	6.96^{A}	73
TW	6.63^{A}	58	6.72^{A}	55	6.60^{A}	46	6.60^{B}	53	6.66^{A}	46
WT	7.35^{B}	22	7.65^{B}	19	7.53^{B}	15	6.90^{BC}	20	7.38^{B}	17
WW	7.68^{B}	106	7.68^{B}	102	7.44^{B}	89	7.29^{C}	95	7.44^{B}	88

Table 7.6: Average yellow pigment content per grain (YP(ng)/grain) for individuals of each genotype class for each of the four possible combinations of Tamaroi (T) and Wollaroi (W) alleles at markers *cfa2293* and *YP7A* (*Psy1-A1* gene) at all four environments separately and the average YP/grain across all four environments. The number of individuals (N) tested in each genotype class for each environment are shown. YP values not connected by the same letter differ by more than the sum of the standard error associated with each pair of means calculated by analysis of variance ([A,B,])

Alleles at *cfa2293* and *YP7A*	Average YP(ng/grain) all sites	N	Waite 2007 YP(ng/grain)	N	Turretfield 2007 YP(ng/grain)	N	Waite 2006/2007 YP(ng/grain)	N	Turretfield 2006 YP(ng/grain)	N
TT	281[A]	93	299[A]	62	242[A]	48	270[A]	88	302[A]	73
TW	284[A]	56	300[A]	42	240[A]	27	282[A]	53	298[A]	46
WT	333[B]	22	353[B]	13	273[AB]	6	321[B]	20	359[B]	17
WW	325[B]	104	358[B]	72	273[B]	58	324[B]	93	335[B]	88

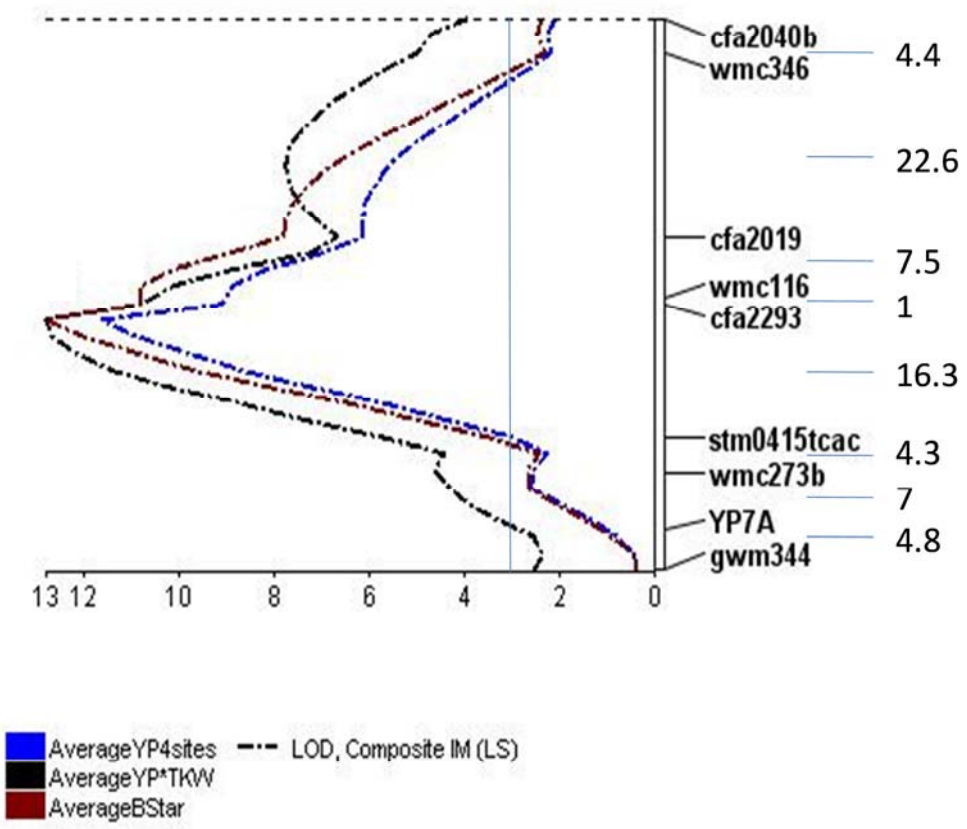

Figure 7.1: Location on chromosome 7AL of a region associated with average yellow pigment concentration across four environments (AverageYP4sites), average yellow pigment content per grain across four environments (AverageYP*TKW) and average flour colour (CIELAB b* value) across four environments (AverageBStar). The x axis displays the LOD score, the blue line marks the LOD score 3 significance threshold and map distances in centimorgans between markers are displayed on the right hand side

The second most significant QTL associated with YP concentration, yellow flour colour and YP/grain in all environments was on the distal arm of chromosome 7BS (Figure 7.2). This QTL explained 14, 10 and 7 per cent of the total phenotypic variation in average YP concentration, yellow flour colour and YP/grain across all four environments respectively (Tables 7.1, 7.2 and 7.3 respectively). Wollaroi contributed the favourable alleles for higher YP concentration.

The third QTL associated with YP concentration at all environments was located on chromosome 7BL (Figure 7.3). The peak of this QTL was between markers *gwm304* and *Psy1-1F5/R5* (Figure 7.3) and the marker most closely linked to the peak of the QTL and most significantly associated with YP concentration using single marker regression was *Psy1-1F5/R5*. This QTL explained 12, 9, and 9 per cent of the total phenotypic variation in average YP concentration, yellow flour colour and YP/grain across all four environments respectively (Tables 7.1, 7.2 and 7.3 respectively). Wollaroi contributed the favourable allele(s) for higher YP concentration.

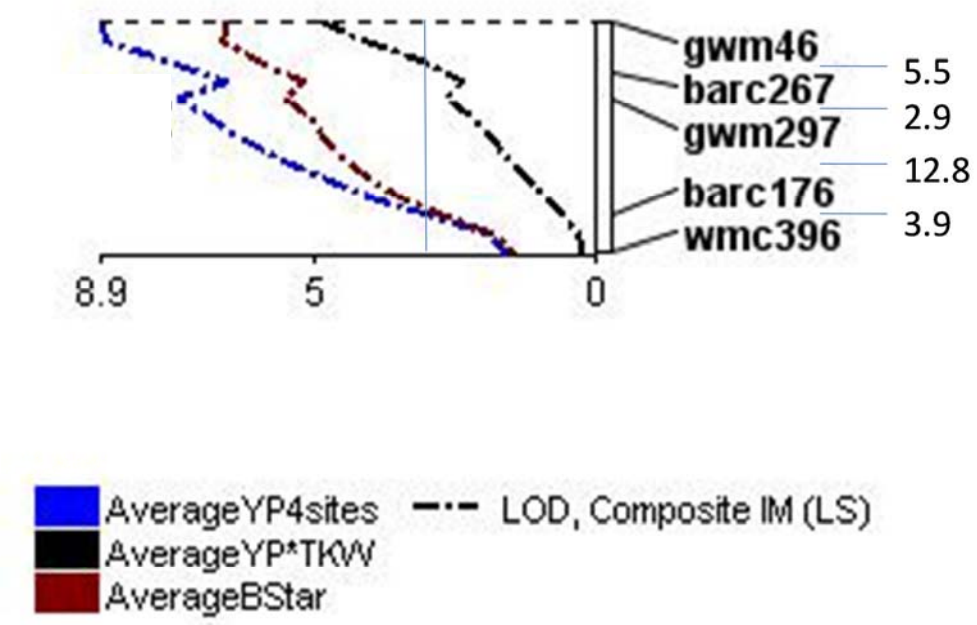

Figure 7.2: Location on chromosome 7BS of a region associated with average yellow pigment concentration across four environments (AverageYP4sites), average yellow pigment content per grain across four environments (AverageYP*TKW) and average flour colour (CIELAB b* value) across four environments (AverageBStar). The x axis displays the LOD score and the blue line marks the LOD 3 significance threshold and map distances in centimorgans are displayed between markers on the right hand side

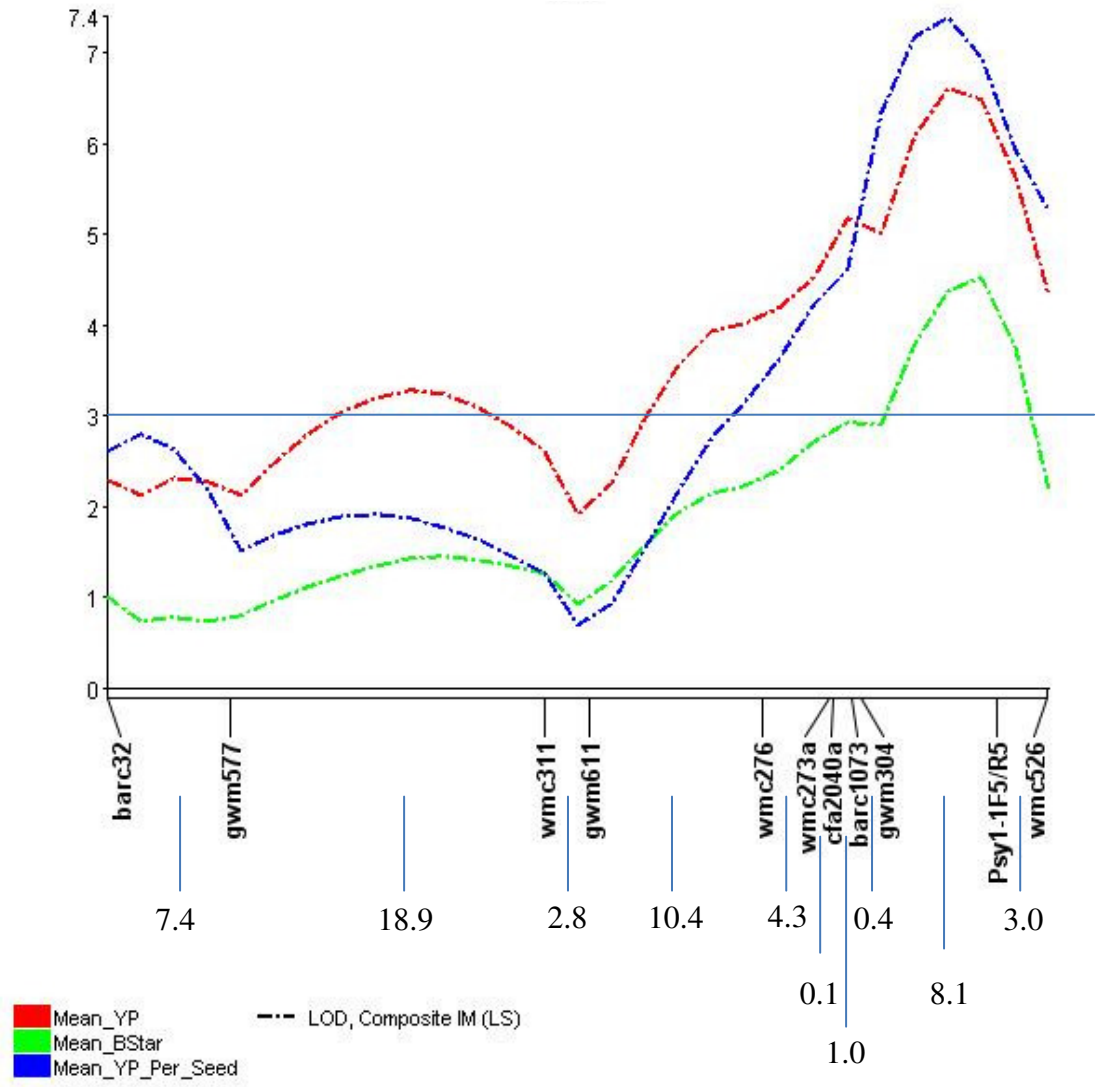

Figure 7.3: Location on chromosome 7BL of a region associated with mean yellow pigment concentration across all four environments (Mean_YP), mean yellow pigment content per grain (seed) across four environments (Mean_YP_Per_Seed) and average flour colour (CIELAB b* value) across four environments (Mean_BStar). The y axis displays the LOD score, the blue line marks the LOD 3 significance threshold. Map distances, in centimorgans, between markers are pointed out by a blue line along the x axis

There were three QTL on chromosomes 6B (Figure 7.4), 1A (Figure 7.5), and 3B which were associated with variation in flour YP concentration in some but not all environments with Tamaroi contributing the favourable alleles for higher YP concentration at each of these QTL (Table 7.1). Each of these QTL explained between 4 to 5 per cent of the variance in average YP concentration of Wollaroi/Tamaroi RILs across all four environments.

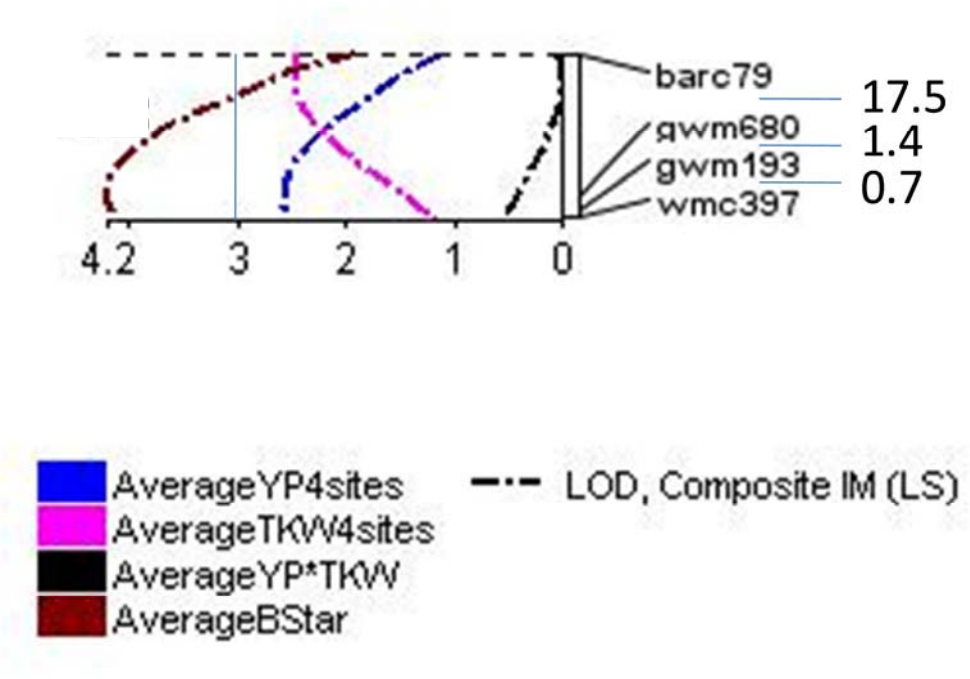

Figure 7.4: Location on chromosome 6B of a region associated with average flour colour (CIELAB b* value) across four environments (AverageBStar). The x axis displays the LOD score and the blue line marks the LOD 3 significance threshold. The association of average yellow pigment concentration across four environments (AverageYP4sites), average yellow pigment content per grain across four environments (AverageYP*TKW), and average thousand grain (kernel) weight (TKW) across four environments (AverageTGW4sites) all below the LOD 3 significance threshold are also shown. Map distances in centimorgans between markers are displayed on the right hand side

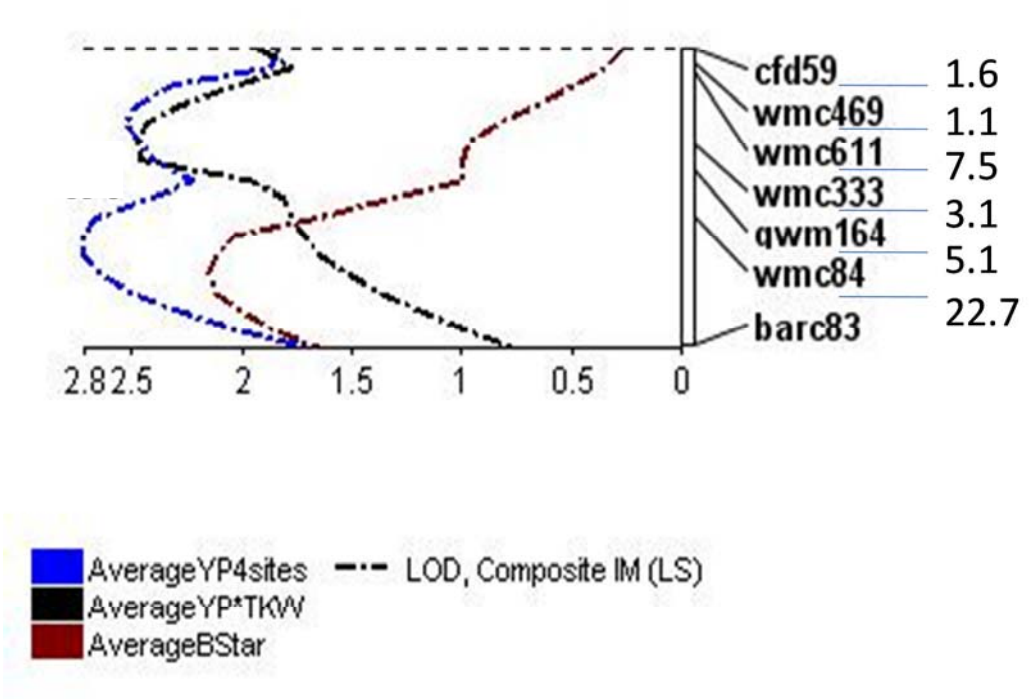

Figure 7.5: Location on chromosome 1A of a region associated with average yellow pigment concentration across four environments (AverageYP4sites), average yellow pigment content per grain across four environments (AverageYP*TKW) and average flour colour (CIELAB b* value) across four environments (AverageBStar). The x axis displays the LOD score and map distances in centimorgans are displayed between markers on the right hand side

All six QTL found to be associated with YP concentration (Tables 7.7, 7.10 and 7.11) and seven QTL associated with YP/grain (Tables 7.8 and 7.13) behaved in an additive manner and an analysis of variance revealed that there were no significant interactions between any of the QTL. The association of the 3B QTL with YP concentration was consistently lower among RILs with favourable (higher YP) alleles at any of the other QTL associated with YP concentration (Table 7.5); however, these differences were not significant.

Table 7.7: Average yellow pigment (YP) concentration (mg/kg) across all four environments for individuals with either Tamaroi (T) or Wollaroi (W) alleles at each of the identified six QTL showing the additive nature of the QTL. The following markers were used to represent the various QTL and the favourable (higher YP) alleles are shown in parentheses: 7A=*cfa2293*(W), 7BL=*barc1073*, *Psy1-1F5/R5*(W), 7BS=*gwm46*(W), 6B=*gwm193*(T), 1A=*wmc84*(T) and 3BL=*barc164*(T). Calculated by analysis of variance, for each pair of QTL, favourable alleles at both QTL resulted in higher YP concentration than at either one QTL alone, which in turn resulted in significantly higher YP concentration than unfavourable alleles at both QTL. The YP concentrations of the favourable combination of alleles for each pair of QTL are presented in bold

		Yellow Pigment Concentration (mg/kg)									
Chromosome		7BL		7BS		6B		1A		3BL	
	Allele	T	W	T	W	T	W	T	W	T	W
7A	T	6.54	6.99	6.48	7.17	6.96	6.66	6.99	6.69	7.02	6.51
	W	7.17	**7.89**	7.20	**7.86**	**7.68**	7.29	**7.74**	7.32	**7.59**	7.35
7BL	T	*	*	6.57	7.20	6.90	6.81	7.05	6.75	6.99	6.63
	W	*	*	7.05	**7.74**	**7.68**	7.05	**7.59**	7.20	**7.44**	7.29
7BS	T	*	*	*	*	6.99	6.66	6.99	6.66	7.05	6.57
	W	*	*	*	*	**7.62**	7.32	**7.80**	7.26	**7.5**	7.44
6B	T	*	*	*	*	*	*	**7.68**	7.08	**7.47**	7.20
	W	*	*	*	*	*	*	7.05	6.84	7.08	6.66
1A	T	*	*	*	*	*	*	*	*	**7.44**	7.23
	W	*	*	*	*	*	*	*	*	7.14	6.75

Table 7.8: Average yellow pigment content per grain (YP/grain) across all four environments for individuals with either Tamaroi (T) or Wollaroi (W) alleles at each of the identified seven QTL showing the additive nature of the QTL. The following markers were used to represent the various QTL and the favourable (higher yellow pigment) alleles are shown in parentheses: 7A=*cfa2293*(W), 2A=*stm0552tgag*(W), 7BL=*Psy1-1F5/R5*(W), 7BS=*gwm46*(W), 3B=*barc164*(T), 1A=*wmc333*(T) and 1B=*barc302*(T). Calculated by analysis of variance, for each pair of QTL, favourable alleles at both QTL resulted in higher YP/grain than at either one QTL alone, which in turn resulted in significantly higher YP/grain than unfavourable alleles at both QTL. The YP/grain of the favourable combination of alleles for each pair of QTL are presented in bold

		Yellow Pigment content per grain (ng/grain)											
Chromosome		2A		7BL		7BS		3B		1A		1B	
	Allele	T	W	T	W	T	W	T	W	T	W	T	W
7A	T	266	300	268	292	272	293	295	266	293	273	274	293
	W	318	**335**	304	**350**	308	**346**	**335**	314	**339**	319	310	**341**
2A	T	*	*	279	296	278	299	294	281	294	284	279	299
	W	*	*	296	**331**	299	**339**	**331**	301	**335**	304	302	**334**
7BL	T	*	*	*	*	271	305	296	275	291	283	279	296
	W	*	*	*	*	303	**331**	**325**	303	**328**	305	301	**329**
7BS	T	*	*	*	*	*	*	295	283	295	283	280	302
	W	*	*	*	*	*	*	**336**	306	**336**	305	302	**330**
3B	T	*	*	*	*	*	*	*	*	**325**	303	305	**323**
	W	*	*	*	*	*	*	*	*	303	282	277	305
1A	T	*	*	*	*	*	*	*	*	*	*	300	**329**
	W	*	*	*	*	*	*	*	*	*	*	282	308

Table 7.9 Results from multiple linear regression analysis presenting significance levels (p) of association of each marker with average yellow pigment (YP) concentration across four environments of Wollaroi/Tamaroi RILs in a model including all five markers simultaneously. Amount of increase in YP concentration (Effect) associated with the Wollaroi allele at each marker and the standard error (S.E.) are also presented. The chromosome that each marker was mapped to is presented in parentheses

Marker	Effect (mg/kg)	S.E.	p
cfa2293 (7AL)	0.8312	0.097	<.001
Psy1-1F5/R5 (7BL)	0.6295	0.0974	<.001
gwm46 (7BS)	0.6342	0.0967	<.001
wmc84 (1A)	-0.4353	0.0976	<.001
gwm193 (6B)	-0.3702	0.0957	<.001
barc164 (3B)	-0.3017	0.0996	0.003

Table 7.10: Average yellow pigment concentration across four environments for individuals within each combination of genotype classes at the three most significant QTL at 7AL (*cfa2293*), 7BL (*Psy1-1F5/R5*) and 7BS (*gwm46*). T=allele inherited from Tamaroi, W=allele inherited from Wollaroi

		Yellow Pigment concentration (mg/kg)	
		7BS	
7AL	7BL	T	W
T	T	6.15	6.93
T	W	6.72	7.35
W	T	6.87	7.50
W	W	7.47	8.40

Multiple linear regression showed that combining all six QTL that were found to be associated with YP concentration explained 51 per cent of the total phenotypic variance in average YP concentration across all four environments (Tables 7.9 and 7.11) and that the effect of all six QTL remained significant even when they are all included in the model simultaneously (Table 7.9). Similarly for YP/grain, combining seven QTL associated with YP/grain explained 52.9 per cent of the average YP/grain across all four environments (Tables 7.12 and 7.13). As much as 30.4 per cent of the variation in average YP concentration was explained just by the two QTL on chromosome 7AL and 7BL (Table 7.11). Favourable alleles at one, two or three of the QTL with the most significant association with YP concentration increasingly shifts the proportion of the mapping population with high YP concentration (Figure 7.6) and yellow flour colour (Figure 7.7).

Table 7.11: Percentage of total phenotypic variation in average yellow pigment concentration (YP conc.) of Wollaroi/Tamaroi RILs across all four environments accounted for by a range of QTL allele combinations calculated with multiple regression analysis. The following markers were used to represent the various QTL and the favourable (higher YP) alleles are shown in parentheses: 7AL=*cfa2293*(W), 7BL=*Psy1-1F5/R5*(W), 7BS=*gwm46*(W), 6B=*gwm193*(T), 1A=*wmc84*(T) and 3B=*barc164*(T)

QTL	% Variation in average YP conc. accounted for
7AL, 7BL, 7BS, 1A, 6B, and 3B	51.0
7AL, 7BL, 7BS, 1A, and 6B	48.0
7AL, 7BL, 7BS, and 1A	44.7
7AL, 7BL, and 7BS	40.9
7AL and 7BL	30.4
7AL	16.0

Table 7.12 Results from multiple linear regression analysis presenting significance levels (p) of association of each marker with average yellow pigment content per grain of Wollaroi/Tamaroi RILs across four environments in a model including all five markers simultaneously. Amount of increase in YP concentration (Effect) associated with the Wollaroi allele at each marker and the standard error (S.E.) are also presented. The chromosome that each marker was mapped to is presented in parentheses

Marker	Effect (ng/grain)	S.E.	p
cfa2293 (7AL)	50.89	5.35	<.001
Psy1-1F5/R5 (7BL)	23.91	5.44	<.001
gwm46 (7BS)	28.62	5.28	<.001
barc240 (1B)	23.12	5.37	<.001
barc164 (1A)	-20.75	5.5	<.001
gwm164 (3B)	-19.74	5.44	<.001
stm0552tgag (2A)	23.68	5.36	<.001

Table 7.13: Percentage of total phenotypic variation in average yellow pigment content per grain (YP(ng)/grain) of Wollaroi/Tamaroi RILs across all four environments accounted for by a range of QTL combinations calculated with multiple regression analysis. The following markers were used to represent the various QTL with donor of the favourable (higher yellow pigment) alleles shown in parentheses: 7A=*cfa2293*(W), 2A=*stm0552tgag*(W), 7BS=*gwm46*(W), 7BL=*Psy1-1F5/R5* (W), 1B=*barc240*(W), 3B=*barc164*(T) and 1A=*wmc333*(T)

QTL	% Variation in YP(ng)/grain account for
7AL, 7BL, 7BS, 7BL, 1B, 3B and 2A	52.9
7AL, 7BL, 7BS, 1B, 1A and 3B	48.2
7AL, 7BL, 7BS, 1B and 1A	44.3
7AL, 7BL, 7BS and 1B	40.8
7AL, 7BL and 7BS	36.6
7AL and 7BL	30.3
7AL	18.9

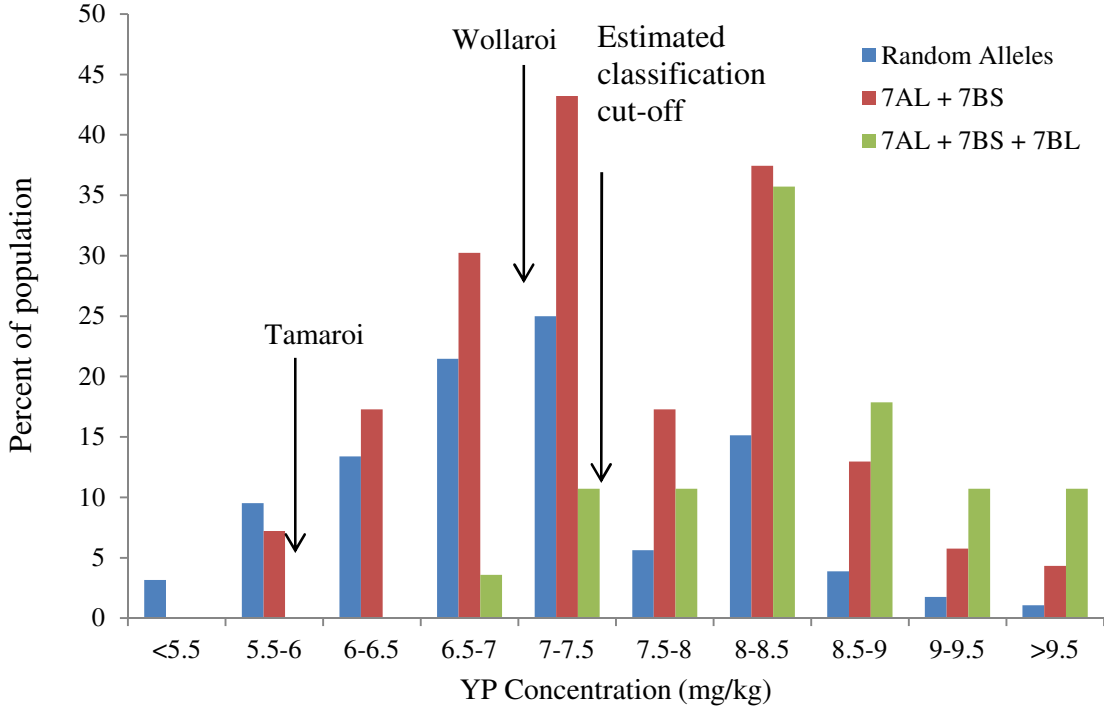

Figure 7.6: Frequency distribution of Wollaroi/Tamaroi RILs within each class of average yellow pigment (YP) concentration (mg/kg) from four environments with either; random alleles (blue bar on the left), favourable alleles at *cfa2293* on chromosome 7AL and *gwm46* on chromosome 7BS (red bar in the middle) or favourable alleles at 7AL, 7BS and *Psy1-B1* on chromosome 7BL (green bar on the right). YP concentration of Tamaroi (5.92 mg/kg), Wollaroi (7.18 mg/kg) and the estimated minimum YP concentration required at this environment to achieve Australian Durum classification are marked with arrows

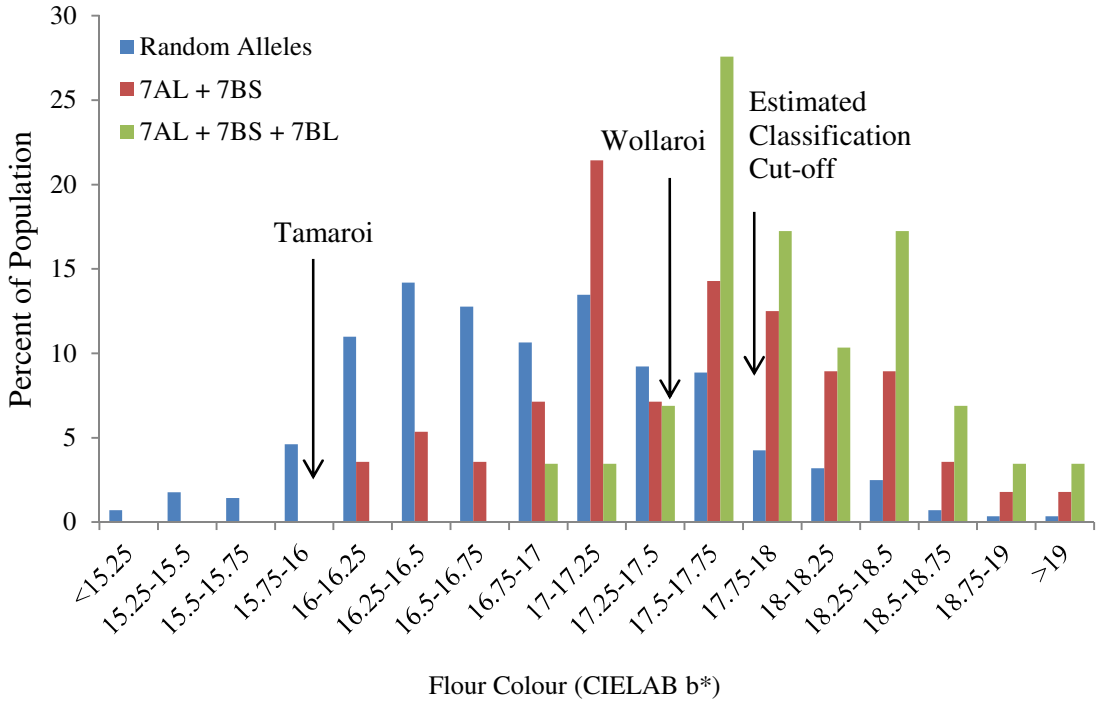

Figure 7.7: Frequency distribution of Wollaroi/Tamaroi RILs within each class of average flour colour (CIE b*) from four environments with either; random alleles (blue bar on the left), favourable alleles at *cfa2293* on chromosome 7AL and *gwm46* on chromosome 7BS (red bar in the middle) or favourable alleles at 7AL, 7BS and *Psy1-B1* on chromosome 7BL (green bar on the right). Average yellow flour colour (CIELAB b* value) of Tamaroi (16.0), Wollaroi (17.4) and the estimated minimum yellow flour colour required at this environment to achieve Australian Durum classification are marked with arrows

TGW was significantly (LOD>3) associated with a major QTL on chromosome 2A at all environments. At this locus marker *stm0552tgag* was most significantly associated with TGW explaining up to 43 per cent of the variance in TGW at Turretfield in 2007 (Table 7.13). Wollaroi contributed the favourable alleles for higher TGW at this locus. This QTL was also significantly associated with YP/grain explaining up to 21 per cent of the variance in YP/grain at Turretfield in 2007. This QTL was generally not associated with YP concentration or flour colour.

A second QTL on chromosome 1B was significantly (LOD score 3.77) associated with TGW (Table 7.13) and YP/grain (Table 7.3) at Turretfield in 2006 and with the average TGW across all four environments, but not at the other three sites individually. Wollaroi contributed the favourable alleles for higher YP/grain and TGW at this locus.

Table 7.13: QTL associated with one thousand grain weight (TGW) in the Wollaroi/Tamaroi population across four environments and the average TGW across all four environments. T.field=Turretfield, the 2006 trial was also at Turretfield and the 2006/07 trial was at Waite. Chromosome arm, most significant marker, flanking markers, parent contributing positive (+ve) allele(s) for larger TGW (W=Wollaroi, T=Tamaroi), LOD score for the association and percentage of phenotypic variation accounted for by QTL (%V.A.) are shown

				T.field 2007		Waite 2007		2006/07		2006		Average 4 trials	
Chromosome	Marker	Flanking Markers	+ve allele	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.	LOD	% V.A.
2A	*stm0552tgag*	*gwm515, wmc522*	W	17.3	43	12.6	27	18.9	27	10.3	17	24.5	33
1B	*barc240*	*barc302*	W	ns		2.4	6	ns		3.8	7	3.7	6
3B	*gwm181*	*gwm547*	W	2.4	8	ns		ns		ns		ns	
6B	*barc79*	*gwm680*	W	ns		ns		ns		ns		2.5	4
7A	*gwm344*	*YP7A*	W	ns		ns		ns		ns		2.3	4

7.3.2 WID22221/Tamaroi Population

Single marker regression analysis of WID22221/Tamaroi RILs grown at Waite in 2008 indicated that the regions on chromosomes 7AL, 7BL and 7BS identified in the Wollaroi/Tamaroi RILs to be associated with YP concentration, flour colour and YP/grain were also associated with these traits in the WID22221/Tamaroi population (Table 7.14). Similarly, the previously described chromosome 6B region associated with YP concentration and flour colour in the Wollaroi/Tamaroi population was also found to be associated with flour colour in the WID22221/Tamaroi population.

The marker *YP7A* for the *Psy1-A1* gene on chromosome 7A was polymorphic between WID22221/Tamaroi RILs and was linked to marker *cfa2293*. DNA amplification and digestion with *Psy1-A1_STS* (Singh et al. 2009) resulted in a fragment length of 897 base pairs from WID22221, indicating that WID22221 is likely to have the same *Psy1-A1* allele as Wollaroi. Comparing the predicted means from the regression model of an ANOVA of individuals sharing the same allele at *cfa2293* revealed that as for the Wollaroi/Tamaroi population, there was no significant association between *YP7A* and either YP concentration, flour colour or YP/grain of WID22221/Tamaroi RILs (Table 7.15).

Table 7.14: Marker trait associations in the WID22221/Tamaroi population analysed with single marker regression analysis. Phenotypic data used was from two separate environments, the glasshouse in the summer of 2007/08 (Glasshouse) and the field at the Waite Institute, Adelaide, SA during the spring season in 2008 (Field). Percentage of the total phenotypic variation for the relevant trait and donor parent of the favourable allele (high parent) are shown. The predicted chromosome location of each marker is shown in parenthesis. YPConc.= Yellow pigment concentration, YP/grain= Yellow pigment content per grain and TGW= Thousand grain weight

Marker	Trait	Environment	p value	% Variation accounted for	High parent
cfa2293 (7AL)	YPConc.	Field	<.001	27.8	WID22221
cfa2293 (7AL)	Flour colour	Field	<.001	25.2	WID22221
cfa2293 (7AL)	Flour colour	Glasshouse	<.001	10.3	WID22221
cfa2293 (7AL)	YP/grain	Field	<.001	22.8	WID22221
gwm334 (6AS)	YPConc.	Field	<.001	6.5	WID22221
gwm334 (6AS)	Flour colour	Field	0.004	2.3	WID22221
gwm334 (6AS)	Flour colour	Glasshouse	0.027	2	WID22221
gwm334 (6AS)	YP/grain	Field	<.001	5.9	WID22221
gwm46 (7BS)	YPConc.	Field	<.001	5.7	WID22221
gwm46 (7BS)	Flour colour	Field	<.001	4.7	WID22221
gwm46 (7BS)	Flour colour	Glasshouse	0.034	1.7	WID22221
gwm46 (7BS)	YP/grain	Field	<.001	4	WID22221
gwm344 (7BL)	YPConc.	Field	<.001	5.7	WID22221
gwm344 (7BL)	Flour colour	Field	<.001	4	WID22221
gwm344 (7BL)	Flour colour	Glasshouse	0.049	1.4	WID22221
gwm344 (7BL)	YP/grain	Field	<.001	5.9	WID22221
gwm0570 (6AL)	YPConc.	Field	0.004	2.8	WID22221
gwm0570 (6AL)	YP/grain	Field	<.001	4.4	WID22221
gwm0570 (6AL)	TGW	Field	0.067	0.9	WID22221
WMC418 (3B)	YPConc.	Field	0.011	1.7	Tamaroi
WMC418 (3B)	Flour colour	Field	0.043	1	Tamaroi
WMC418 (3B)	YP/grain	Field	<.001	4.3	Tamaroi
WMC418 (3B)	TGW	Field	0.005	2.2	Tamaroi
barc77 (3BL)	YPConc.	Field	0.035	1.1	WID22221
barc77 (3BL)	Flour colour	Glasshouse	0.069	1.1	WID22221
gwm680 (6B)	Flour colour	Field	0.01	1.8	Tamaroi
gwm193 (6BS)	Flour colour	Field	0.021	1.4	Tamaroi

Table 7.15: Predicted mean yellow pigment (YP) concentration (mg/kg), flour colour (CIELAB b* value) and YP/grain (ng/grain) from ANOVA of WID22221/Tamaroi RILs at Waite, SA in 2008 with different allelic combinations at *cfa2293* and *YP7A* on chromosome 7A. T=allele inherited from Tamaroi, W=allele inherited from WID22221, N=Number of RILs with each combination. Approximate effective standard errors from ANOVA are displayed. Values within a column for a trait which are not connected by the same letter are significantly (p<0.05) different from one another according to a Tukey test (^{AB})

Alleles at *cfa2293* & *YP7A*	N	YP conc. (mg/kg) 2008	Flour colour (CIELAB b*)	YP(ng) /grain
TT	99	7.896^{A}	16.54^{A}	236.9^{A}
TW	31	8.113^{A}	16.50^{A}	243.4^{A}
WT	33	9.136^{B}	17.12^{B}	274.1^{B}
WW	111	9.145^{B}	17.10^{B}	274.3^{B}

The marker *Psy1-1F5/R5* for the *Psy1-B1* gene on chromosome 7B was not polymorphic among WID22221/Tamaroi RILs. Despite this, variation in YP concentration was found to be associated with marker *gwm344* on chromosome 7BL. Marker *gwm344* maps 3 cM distal to *wmc526* according to the consensus map of Sommers et al. (2004). Marker *Psy1-1F5/R5* also mapped 3.0 cM distal to *wmc526* on the linkage map for the Wollaroi/Tamaroi population indicating that the same region is associated with YP concentration in both populations.

QTL associated with YP concentration in WID22221/Tamaroi RILs generally behaved in an additive manner and independent of alleles at other loci (Table 7.16). The main exception was the 3BL QTL which was not significantly associated with YP concentration when the effects of the 7BS, 6AS, or 3B QTL were included in the ANOVA model. Similarly, the 7BL QTL was not significantly (p>0.1) associated with YP concentration when the 7AL QTL was included in the ANOVA model, as was the case for the 3B QTL when including the 6AL QTL in the ANOVA model.

Interaction effects between QTL were limited but there were significant interaction effects between QTL involving the 3BL QTL; the association of the 3BL QTL with YP concentration was dependent on the genotype of RILs at either the 7BL or the 6AL QTL (Table 7.16).

Collectively, markers *cfa2293*, *gwm334*, *gwm46*, *gwm570* and *wmc418* linked to QTL on 7AL, 6AS, 7BS, 6AL and 3B respectively, explained 43.6 per cent of the total observed phenotypic variance in YP concentration of WID22221/Tamaroi RILs at Waite in 2008 (Table 7.18). All marker affects were significant in the multiple linear regression model which included all marker effects simultaneously (Table 7.17). Markers linked to just three key QTL on 7AL, 6AS and 7BS accounted for 40.9 per cent of the total observed phenotypic variation in YP concentration (Table 7.18). WID22221/Tamaroi RILs with favourable alleles at these markers had on average a 33 per cent, or 2.42 mg/kg, higher mean YP concentration than RILs with unfavourable alleles at these loci (Table 7.19). As was observed for Wollaroi/Tamaroi RILs, WID22221/Tamaroi RILs with favourable alleles at one, two or three of the QTL with the most significant association with YP concentration increasingly shifted the proportion of the mapping population with high YP concentration (Figure 7.8) and high levels of yellow flour colour (Figure 7.9).

Table 7.16: Mean yellow pigment (YP) concentration (mg/kg) for each combination of QTL identified in the WID22221/Tamaroi population at Waite, SA in 2008. The following markers were used to represent their relevant QTL 7AL=*cfa2293*, 6AS=*gwm334*, 7BS=*gwm46*, 6AL=*gwm570*, 3B=*wmc418*, 7BL=*gwm344* and 3BL=*barc77*. T=Tamaroi allele at the relevant marker and W=WID22221 allele at the relevant marker. Calculated by analysis of variance, for each pair of QTL, each individual QTL was significantly associated with variance in YP concentration, except for the values presented in *red italics* where one of the two QTL in the model was not significantly associated with YP concentration when modelled with its partner QTL in the pair. No interaction effects between QTL were significant except for combinations where values are in bold (p<0.05)

		Yellow Pigment Concentration (mg/kg)											
Chromosome		6AS		7BS		6AL		3B		7BL		3BL	
	Allele	T	W	T	W	T	W	T	W	T	W	T	W
7AL	T	7.71	8.17	7.51	8.32	7.76	8.30	8.00	7.84	*7.86*	*8.04*	7.81	8.04
	W	8.73	9.43	8.87	9.39	9.01	9.19	9.27	8.95	*9.14*	*9.11*	8.91	9.31
6AS	T	*	*	8.01	8.54	8.05	8.40	8.39	8.03	8.00	8.48	*8.21*	*8.22*
	W	*	*	8.57	9.05	8.67	9.03	9.00	8.64	8.43	9.08	*8.60*	*8.96*
7BS	T	*	*	*	*	7.99	8.65	8.52	7.99	7.82	8.53	*8.26*	*8.29*
	W	*	*	*	*	8.74	8.88	8.88	8.76	8.65	9.04	*8.60*	*8.97*
6AL	T	*	*	*	*	*	*	*8.49*	*8.24*	8.03	8.64	**8.12**	**8.59**
	W	*	*	*	*	*	*	*8.78*	*8.62*	8.51	8.90	**8.76**	**8.69**
3B	T	*	*	*	*	*	*	*	*	8.34	8.92	*8.61*	*8.69*
	W	*	*	*	*	*	*	*	*	8.09	8.60	*8.17*	*8.56*
7BL	T	*	*	*		*	*	*	*	*	*	**7.85**	**8.54**
	W	*	*	*	*	*	*	*	*	*	*	**8.74**	**8.82**

Table 7.17: Results from multiple linear regression analysis presenting significance levels (p) of association of each marker with average yellow pigment (YP) concentration of WID22221/Tamaroi RILs in a model including all five markers simultaneously. Amount of increase in YP concentration (Effect) associated with the Wollaroi allele at each marker and the standard error (S.E.) are also presented

Marker	Effect (mg/kg)	S.E.	p
cfa2293 (7AL)	1.097	0.111	<.001
gwm46 (7BS)	0.623	0.111	<.001
gwm334 (6AS)	0.643	0.111	<.001
gwm570 (6AL)	0.238	0.112	0.036
wmc418 (3B)	-0.279	0.112	0.014

Table 7.18: Total percentage phenotypic variance in yellow pigment concentration of WID22221/Tamaroi RILs at Waite in 2008 accounted for by various combinations of markers *cfa2293*, *gwm334*, *gwm46, gwm570* and *wmc418* linked to QTL on chromosomes 7AL, 6AS, 7BS, 6AL and 3B respectively

QTL fitted to regression model	% Variation accounted for
7AL, 6AS, 7BS, 6AL, 3B	43.6
7AL, 6AS, 7BS, 6AL	41.8
7AL, 6AS, 7BS	40.9
7AL, 6AS	33.5
7AL, 7BS	35.6
7AL	27.8

Table 7.19: Mean yellow pigment concentration (YP conc.) of WID22221/Tamaroi RILs with either favourable alleles from WID22221 (W) or unfavourable alleles from Tamaroi (T) at each of *cfa2293*, *gwm334* and *gwm046* linked to QTL on chromosomes 7AL, 6AS and 7BS respectively. For comparison, mean yellow pigment concentration from the same trial for each parent and two relevant check cultivars, Wollaroi and Bellaroi are presented

		7BS		Control	YP conc.
7AL	6AS	T	W	Genotype	(mg/kg)
T	T	7.3	8.11	Tamaroi	6.46
T	W	7.77	8.49	WID22221	10.09
W	T	8.55	8.96	Wollaroi	8.27
W	W	9.12	9.72	Bellaroi	9.35

Although marker *gwm344* on chromosome arm 7BL alone accounted for 5.7 per cent of the phenotypic variation in YP concentration at Waite in 2008 (Table 7.14), this marker was not associated with a significantly higher YP concentration when combined with favourable alleles at the most important loci on chromosome arm 7AL (Table 7.16). Therefore this marker, and for similar reasons *barc77* on chromosome arm 3BL, were not included in a multiple linear regression analysis to determine the proportion of phenotypic variance in YP concentration that can explained collectively by all the markers associated with YP concentration of WID22221/Tamaroi RILs at Waite, SA in 2008.

In total across both populations, eight QTL on seven of the fourteen chromosomes were associated with at least one of the YP or flour colour traits measured in Wollaroi/Tamaroi and/or WID22221/Tamaroi RILs. Five of these eight QTL associated with YP or flour colour traits appear to be coincident in the two populations (Figures 6.10 and 6.11).

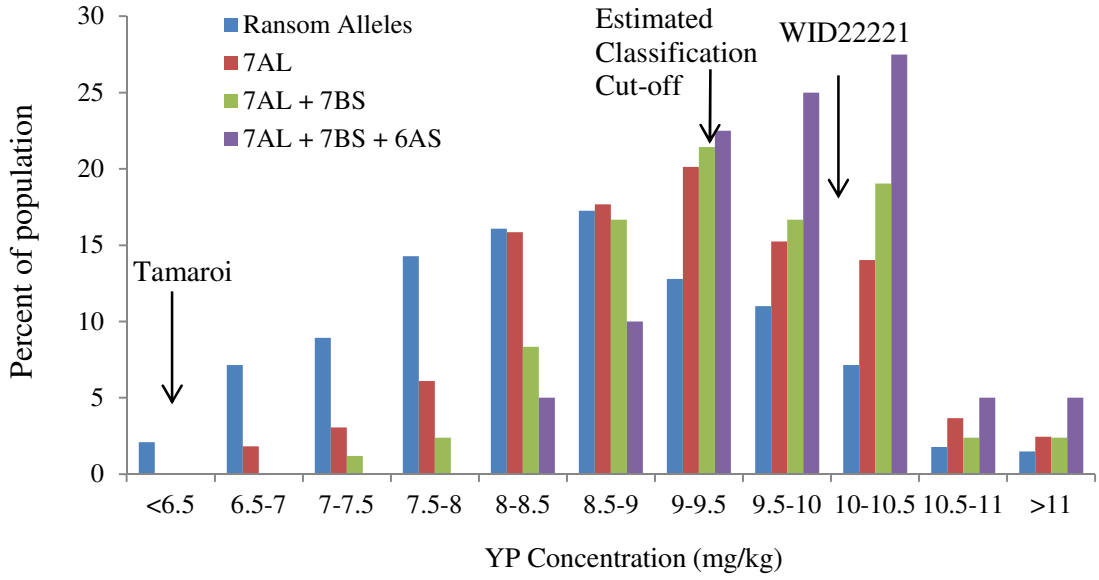

Figure 7.8: Frequency distribution of WID22221/Tamaroi RILs within each genotype class of yellow pigment (YP) concentration at Waite in 2008 with either; random alleles (blue bar on the left), favourable allele (for high YP concentration) at *cfa2293* on chromosome arm 7AL (red bar second from left), favourable alleles at 7AL and *gwm46* on chromosome arm 7BS (green bar second from right), favourable alleles at 7AL, 7BS and *gwm334* on chromosome arm 6AS (purple bar on the right). YP concentration of Tamaroi (6.46 mg/kg), WID22221 (10.09 mg/kg) and the estimated minimum YP concentration required to achieve classification in Australia are marked with arrows

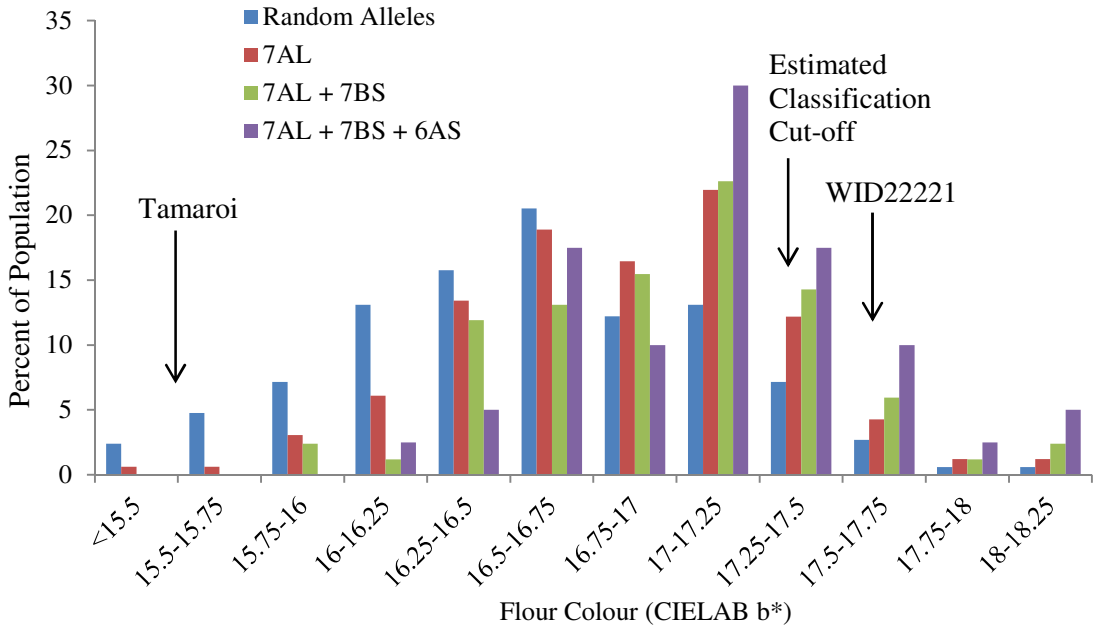

Figure 7.9: Frequency distribution of WID22221/Tamaroi RILs within each class of flour colour (CIELAB b*) at Waite in 2008 with either; random alleles (blue bar on the left), favourable allele (for high YP concentration) at *cfa2293* on chromosome arm 7AL (red bar second from left), favourable alleles at 7AL and *gwm46* on chromosome arm 7BS (green bar second from right), favourable alleles at 7AL, 7BS and *gwm334* on chromosome arm 6AS (purple bar on the right). Flour colour (CIELAB b* value) of Tamaroi (15.52), WID22221 (17.66) and the estimated yellow flour colour required at this environment to achieve Australian Durum classification in Australia are marked with arrows

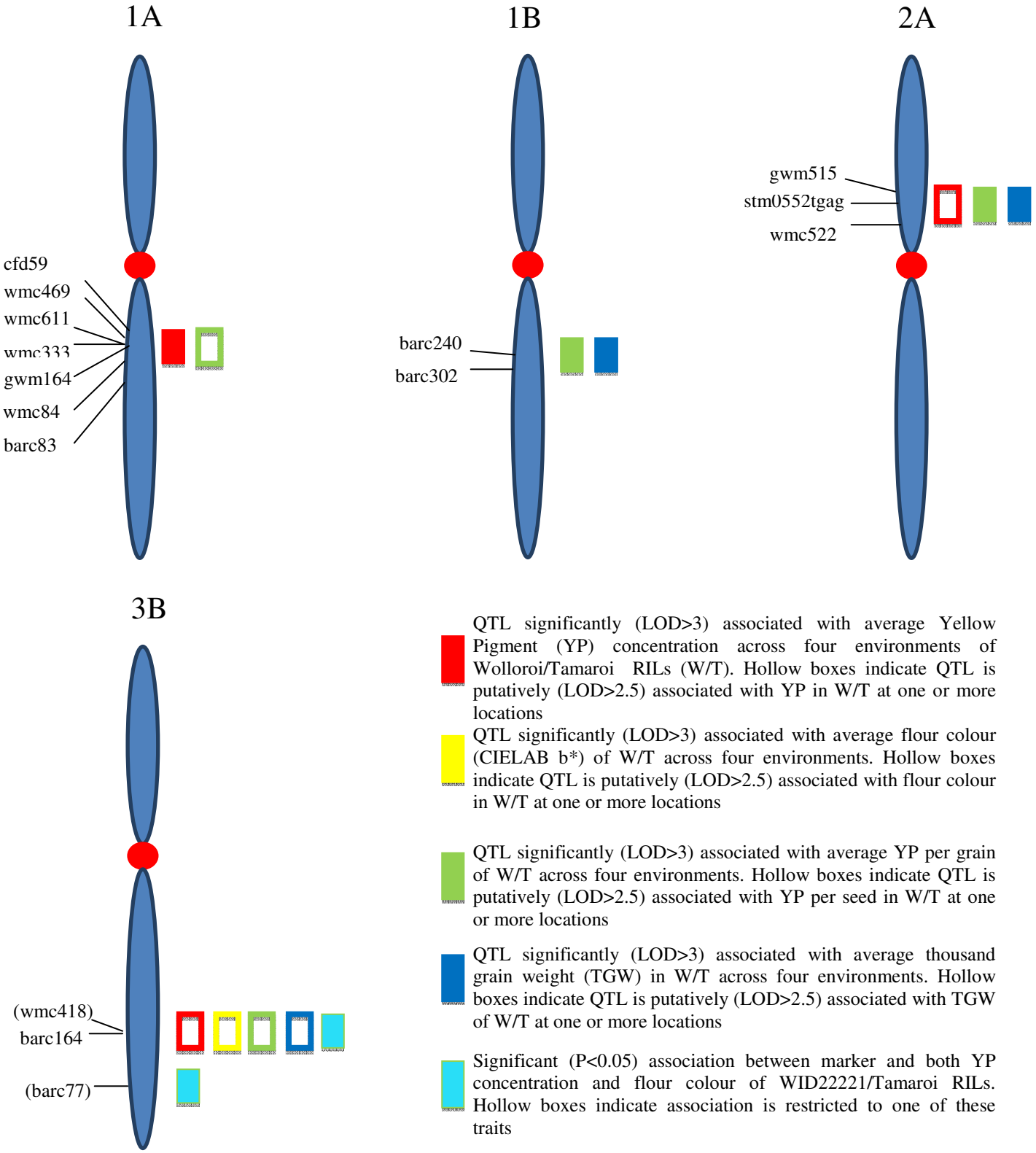

Figure 7.10: QTL associated with flour yellow pigment (YP) concentration, flour colour (CIELAB b*), YP per grain or thousand grain weight of Wollaroi/Tamaroi or WID22221/Tamaroi RILs on chromosomes 1A, 1B, 2A and 3B. Marker and QTL locations are approximate and not to scale. Markers in parentheses were polymporhpic only in WID22221/Tamaroi and so location has been estimated according to consensus map (Somers et al. 2004) and/or physical map (Qi et al. 2004)

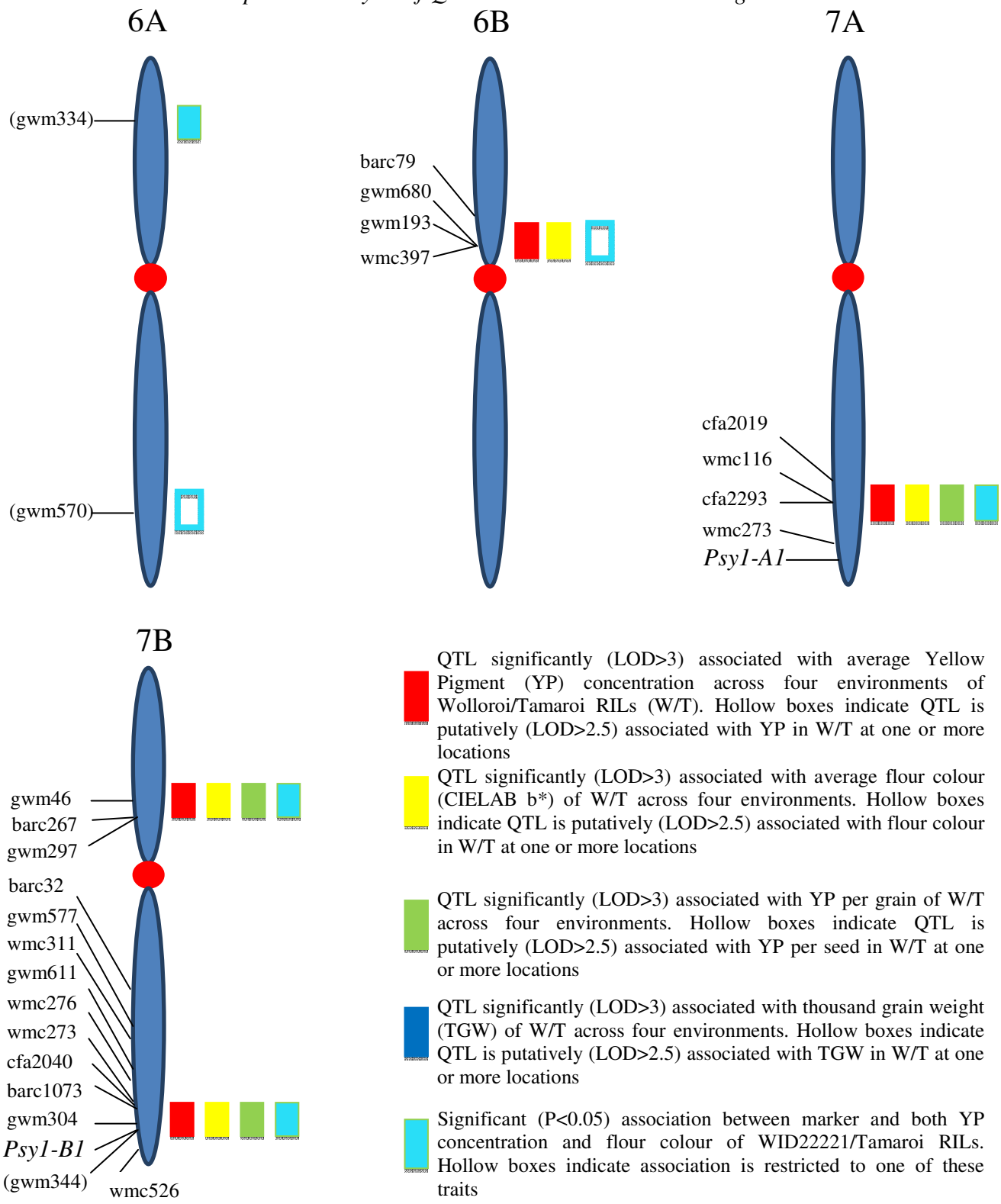

Figure 7.11: QTL associated with flour yellow pigment (YP) concentration, flour colour (CIELAB b*), YP per grain or thousand grain weight of Wollaroi/Tamaroi or WID22221/Tamaroi RILs on chromosomes 6A, 6B, 7A and 7B. Marker and QTL locations are approximate and not to scale. Markers in parentheses were polymporhpic only in WID22221/Tamaroi and so location has been estimated according to consensus map (Somers et al. 2004) and/or physical map (Qi et al. 2004)

7.4 Discussion

7.4.1 Complexity of Genetic Control of Yellow Pigment

YP concentration, YP/grain, and flour colour are highly complex traits under the control of multiple loci. Greater than 40 per cent of the total phenotypic variance in YP concentration and YP/grain was explained by the QTL identified in this study in both populations. The additive effect of more than 5 loci was required to explain this high proportion of phenotypic variance. Given that the phenotyping experiments had very high heritability, that is, most of the variation was genetic and not environmental, and the fact that approximately half of the phenotypic variance remained unexplained, implies that there are likely to be further loci involved in the control of these traits than those identified in this study. These unidentified loci are quite possibly of very small effect, and therefore many in number, as they were not identified in the whole genome scan which was shown to be effective in identifying a number of QTL of relatively small effect. The frequency distributions presented in Chapter 5, which were generally normally distributed for YP/grain, flour colour and for YP concentration and displayed transgressive segregation, support this conclusion. This conclusion is also supported by Clarke et al. (2006) who calculated that the number of effective factors differing between parents for pigment concentration in Canada ranged from 3 to 27, with the range being variable depending on the parental combination and the environment.

7.4.2 Psy1-A1, Chromosome Arm 7AL and Yellow Pigment Concentration

7.4.2.1 Characterisation of RIL parental Genotypes Tamaroi, Wollaroi and WID22221 at *Psy1-A1* and Comparisons with Previous Studies

The Tamaroi allele at *Psy1-A1* appears to be the same allele carried by Canadian durum wheat cultivars AC Avonlea and AC Navigator based on the *YP7A*, *YP7A-2* and *Psy-A1_STS* marker data (data not shown) and also according to Singh et al. (2009). This allele has been designated *Psy1-A1l* (Singh et al. 2009). Comparing the gene bank accessions (http://www.ncbi.nlm.nih.gov) for the allele designated *Psy1-A1c* (He et al. 2009b) identified in CIMMYT spring bread wheat lines and *Psy1-A1l* (Singh et al. 2009) suggests that these are likely to be the same allele. The Wollaroi allele at *Psy1-A1* appears to be the same allele carried by Canadian durum wheat cultivar Commander and has been designated *Psy1-A1o* (Singh et al. 2009). Based on the length of the genomic sequence at *Psy1-A1* from marker data using *YP7A*, WID22221 has the same allele, *Psy1-A1o*, as Wollaroi.

7.4.2.2 Association between *Psy1-A1* Alleles and Yellow Pigment

7.4.2.1 Classification of Psy1-A1 Alleles and Association with Yellow Pigment

In the present study, YP concentration of RILs with the Wollaroi or WID22221 allele at *Psy1-A1*, designated *Psy1-A1o* (Singh et al. 2009), was not significantly different to RILs with the Tamaroi allele, designated *Psy1-A1l* (Singh et al. 2009), once linkage to the QTL at *cfa2293* had been accounted for. This is in contrast to results reported by Singh et al. (2009) who found that the *Psy1-A1o* allele was associated with

significantly higher YP concentration than the *Psy1-A1l* allele in two separate mapping populations and also among a diverse set of 93 durum wheat cultivars in an association mapping study. Among Wollaroi/Tamaroi RILs the reverse was actually observed, although the difference was not significant. Other authors have also reported allelic variation at *Psy1-A1* to be associated with variation in YP concentration (He et al. 2009a; He et al. 2009b; He et al. 2008; Howitt et al. 2009; Zhang and Dubcovsky 2008; Zhang et al. 2009). Zhang and Dubcovsky (2008) found an association between variation in YP concentration and *Psy-E1* in tetraploid 7AL/7EL and 7DL/7EL recombinant lines. There have been no previously published reports of allelic variation at *Psy1-A1* allele which is not significantly associated with differences in YP concentration, except for one population (Stongfield x Commander (D05.58)) of 106 lines tested in a single environment (Singh et al. 2009). Singh et al. (2009) suggested that the lack of significant association of *Psy1-A1* with YP concentration in this population may have been due to it only being tested in a single location, citing that the association between YP concentration and *Psy1-B1* is environmentally dependent (Pozniak et al. 2007) and that this may also be the case for *Psy1-A1*. The lack of agreement between this study and other published studies regarding association between YP concentration and allelic variation at *Psy1-A1* could be due to;

1. Potential misclassification of alleles at *Psy1-A1*
2. Gene by environment interaction at *Psy1-A1*
3. Alternative gene(s) to *Psy1-A1* on chromosome 7AL being associated with YP concentration

7.4.2.2 Potential Misclassification of Alleles at Psy1-A1

As previously mentioned, the Wollaroi and Tamaroi alleles at *Psy1-A1* are likely to be the same as the *Psy1-A1l* and *Psy1-A1o* alleles designated by Singh et al. (2009) based on the length of genomic sequences of the *Psy1-A1* gene. This would mean that the Wollaroi and Tamaroi alleles at *Psy1-A1* are identical in state to the *Psy1-A1l* and *Psy1-A1o* alleles respectively; however, they are not necessarily identical by decent. This means that the Wollaroi and Tamaroi alleles at *Psy1-A1* may not necessarily differ for the functional mutation in amino acid sequence in the exon that may be responsible for variation in YP concentration, or flour colour, that has been reported in other populations (He et al. 2009a; He et al. 2009b; He et al. 2008; Howitt et al. 2009; Singh et al. 2009; Zhang and Dubcovsky 2008; Zhang et al. 2009). If Wollaroi and Tamaroi don't differ for that functional mutation then allelic variation at *Psy1-A1* would not be associated with variation in YP concentration in the Wollaroi/Tamaroi population. This is a possible explanation for the lack of association observed between YP concentration and allelic variation at *Psy1-A1* in both the Wollaroi/Tamaroi and WID22221/Tamaroi populations. The hypothesis that Wollaroi and Tamaroi do not differ for the functional mutation in *Psy1-A1* could be tested by one of three experiments.

1) The *Psy1-A1* genes from Wollaroi and Tamaroi and parents from any population where allelic differences at *Psy1-A1* have been found to be associated with variation in YP concentration, for example Strongfield and Commander (Singh et al. 2009), could be sequenced and the sequences compared.
2) The ancestors of the above set of genotypes could all be assayed for allelic variants at *Psy1-A1* to determine if the donors of the Wollaroi and Tamaroi

allelic variants are the same or different from germplasm with allelic variants that have been shown to be associated with variation in YP variation. This process may not necessarily be conclusive and depending on how divergent these genotypes are in their lineage may involve a large amount of research.

3) Create a population between Wollaroi and another line carrying *Psy1-A1o*, such as Commander (Singh et al. 2009), phenotype the population for YP concentration, genotype the population with markers linked to *Psy1-A1*, and determine if variation in YP concentration maps to the *Psy1-A1* region. An alternative to this third method might be to create a population between Tamaroi and a genotype which has been confirmed to carry *Psy1-A1o*, such as Commander (Singh et al. 2009), genotype the population with a *Psy1-A1* gene based marker, and from this determine if variation in YP concentration is associated with allelic variation at *Psy1-A1*. Wollaroi/Tamaroi RILs should also be tested in the same experiment to compare the association of allelic variation at *Psy1-A1* with YP concentration between the populations. In addition, linkage to the QTL identified in this study on chromosome arm 7AL peaking at *cfa2293* should also be considered.

7.4.2.3 Gene by Environment Interaction at Psy1-A1

An alternative explanation for not observing an association between allelic variation at *Psy1-A1* and YP concentration of Wollaroi/Tamaroi and WID22221/Tamaroi RILs might be that the association is environmentally dependent. Although possible this conclusion in unlikely as both in this study and in published literature, QTL associated with YP concentration have been shown to generally be stable across environments (Clarke et al. 2006; Elouafi et al. 2001; Mares and Campbell 2001;

Parker et al. 1998; Patil et al. 2008). The one exception to this is the study reported by Pozniak et al. (2007) mentioned previously. Phytoene synthase is potentially very complex and can be transcriptionally responsive to abscisic acid, salt, drought, temperature, photoperiod, development cues, and post-transcriptional feedback regulation (Cazzonelli and Pogson 2010). To test the *Psy1-A1* by environment interaction theory Wollaroi/Tamaroi and/or WID22221/Tamaroi RILs could be trialled alongside a population in which variation between the *Psy-A1o* allele and the *Psy-A1l* allele has been found to be associated with variation in YP concentration, such as W9262-260D3/Kofa (Pozniak et al. 2007) or D03.77 (Singh et al. 2009). If the reason for not observing an association between allelic variation at *Psy1-A1* and YP concentration in all five typically Australian spring season environments in this study is a *Psy1-A1* by environment interaction, then it could be concluded that selection for allelic variants at *Psy1-A1* is not worthwhile in Australian durum breeding programs.

7.4.2.4 Alternative Gene to Psy1-A1 on 7AL

Another explanation for the contrast in results regarding the association between *Psy1-A1* and YP concentration between this study and previous studies is that allelic variation at *Psy1-A1* is actually not associated with variation in YP concentration but rather published studies have identified a gene or QTL associated with YP concentration which is tightly linked to *Psy1-A1*, and that is not segregating in Wollaroi/Tamaroi or WID22221/Tamaroi RILs. The possibility of this has been acknowledged by Singh et al. (2009) and fits the "alternative gene hypothesis" presented by Zhang and Dubcovsky (2008). Zhang and Dubcovsky (2008) also proposed the hypotheses that *Psy1-A1* is associated with variation in YP

concentration, either exclusively or in addition to at least one other gene on the distal region of the long arm of homeologous group 7 chromosomes and called these the "*Psy-1* hypothesis" and the "more-than-one-gene-on-7L hypothesis" respectively. Zhang and Dubcovsky (2008) concluded that the most likely scenario is the "more-than-one-gene-on-7L hypothesis". The technical difficulties of proving the causal effect of allelic variation at *Psy1-A1* on YP concentration are discussed by Howitt et al. (2009) where they pointed out that that this would require either genetic complementation, or demonstration that PSY activity in vivo is reduced as a result of allelic variation at *Psy1-A1*.

7.4.2.3 QTL on Chromosome Arm 7AL Associated with Yellow Pigment Concentration in this Study

Of all the QTL identified in this study, the QTL on chromosome arm 7AL was associated with the largest variation in YP concentration and flour colour in both mapping populations. The association of this QTL, linked to *cfa2293* and *wmc116*, with YP concentration has also been identified in other populations (Singh et al. 2009; Zhang and Dubcovsky 2008) and supports the "more-than-one-gene-on-7L hypothesis" proposed by Zhang and Dubcovsky (2008). Patil et al. (2008) also found a major QTL on the distal end of chromosome arm 7AL which was not associated with *Psy1-A1*. Elouafi et al. (2001) reported two QTL on the long arm of chromosome 7A associated with YP concentration in durum, one which appeared to locate near the centromere and another at the distal end of 7AL. The more proximal of the two QTL reported by Elouafi et al. (2001) appears to locate closer to the centromere than the QTL identified in the Wollaroi/Tamaroi and WID22221/Tamaroi populations; the markers linked to this QTL in the Wollaroi/Tamaroi and WID22221/Tamaroi

populations have generally been reported to map to the distal end of the chromosome (Qi et al. 2004; Reimer et al. 2008; Singh et al. 2009; Somers et al. 2004; Zhang et al. 2008; Zhang and Dubcovsky 2008; Zhang et al. 2009). Singh et al. (2009) hypothesised that the variation at *Psy1-A1* was associated with the most distal of the two QTL associated with YP on chromosome 7AL reported by Elouafi et al. (2001). However, it is also possible that the distal QTL on 7AL reported by Elouafi et al. (2001) is in fact the same as identified in the Wollaroi/Tamaroi population centred at *cfa2293*.

7.4.3 Psy1-B1, Chromosome Arm 7BL and Yellow Pigment Concentration

7.4.3.1 Association between *Psy1-B1* and Yellow Pigment in this Study

The QTL on the long arm of chromosome 7B was significantly associated with YP concentration of Wollaroi/Tamaroi RILs and flour colour at all four environments. This chromosomal region has also been reported in the literature to be associated with YP concentration or flour colour in durum (Elouafi et al. 2001; Pozniak et al. 2007; Reimer et al. 2008; Zhang et al. 2008; Zhang and Dubcovsky 2008) and bread wheat (Howitt et al. 2009; Kuchel et al. 2006b; Mares and Campbell 2001; Zhang et al. 2009). It has been suggested that the phytoene synthase gene, *Psy1-B1* is a candidate gene for the 7BL QTL and that allelic variants of this gene may be responsible for variance in YP concentration and/or flour colour (He et al. 2009a; Howitt et al. 2009; Pozniak et al. 2007). He et al. (2009b) have suggested that markers for this gene could be regarded as functional markers for flour colour. The Wollaroi allele at *Psy1-B1* has been designated the *Psy1-B1b* allele (Reimer et al. 2008) and appears to be equivalent

to *Psy1-B1e* allele in bread wheat (He et al. 2009b). The *Psy1-B1b* allele has been reported to be associated with significantly higher YP concentrations than the *Psy1-B1a* allele (He et al. 2009b; Reimer et al. 2008), which is the allele carried by Tamaroi (Reimer et al. 2008). Since the *Psy1-B1* gene is very tightly linked to the QTL associated with variation in YP concentration in the Wollaroi/Tamaroi population and that the *Psy1-B1b* allele has previously been reported to be associated with higher concentrations of YP than the *Psy1-B1a* allele (He et al. 2009a; He et al. 2009b; Pozniak et al. 2007; Reimer et al. 2008; Zhang and Dubcovsky 2008), it is likely that variation in YP concentration associated with this 7BL QTL in the current study is due to allelic variation at *Psy1-B1*. In addition, Zhang and Dubcovsky (2008) concluded from the YP concentration of 7BL/7EL translocation lines that the *Psy1-1* gene most likely plays an important role in determining YP concentration. The difference in YP concentration in mg/kg associated with this 7BL QTL in the Wolaroi/Tamaroi population is similar to the variation reported to be associated with allelic variation in *Psy1-B1* in durum wheat in several studies (He et al. 2009a; Pozniak et al. 2007; Zhang et al. 2008) and with group 7 chromosomes in bread wheat (He et al. 2008; Howitt et al. 2009; Kuchel et al. 2006b; Mares and Campbell 2001; Parker et al. 1998; Zhang et al. 2009).

7.4.3.2 Potential for Other Genes Additional to *Psy1-B1* on Chromosome Arm 7BL

The peak of the QTL on chromosome 7BL identified in the Wollaroi/Tamaroi population did not map directly at *Psy1-B1* but slightly distal to *Psy1-B1*, between markers *Psy1-1F5/R5* and *gwm304*, which concurs with results from the UC1113/Kofa (Zhang et al. 2008) and the W9262-260D3/Kofa (Pozniak et al. 2007) populations. This could simply be the result of random variation or could be due to

the presence of other gene(s) associated with variance in YP concentration tightly linked to *Psy1-B1* on 7BL.

On chromosome 7BL, marker *gwm344*, which was significantly associated with YP concentration of WID22221/Tamaroi RILs, was not part of a linkage group, but according to the consensus map constructed by Somers et al. (2004) and the physical map constructed by Qi et al. (2004), *gwm344* maps to a similar region as *Psy1-B1*. There was no allelic variation at *Psy1-B1* between WID22221/Tamaroi RILs that could be detected with *Psy1-1F5/R5*. This suggests that there is an additional gene, or genes, to *Psy1-B1* associated with variation in YP concentration on the distal end of chromosome 7BL. Alternatively, it is possible that there is allelic variation at *Psy1-B1* between WID22221/Tamaroi RILs but the polymorphism could not be detected with the gene based marker *Psy1-1F5/R5*. Comparing the chromosome 7BL QTL between populations, the 7BL QTL identified in the Wollaroi/Tamaroi population behaved in a much more additive manner than the 7BL QTL in WID22221/Tamaroi, particularly in combination with the QTL on chromosome 7AL. The 7BL QTL identified in the Wollaroi/Tamaroi population was associated with much greater variation in YP concentration than the 7BL QTL identified in the WID22221/Tamaroi population. These data suggest that the gene(s) responsible for these QTL on chromosome 7BL may be different between the two populations.

7.4.3.3 QTL for Yellow Pigment Concentration on Chromosome Arm 7BS

The QTL on the short arm of chromosome 7B accounted for the second greatest proportion of variance in YP concentration and yellow flour colour of Wollaroi/Tamaroi RILs and WID22221/Tamaroi RILs. A QTL in this region

associated with YP concentration has previously been reported in two environments for one population (Patil et al. 2008) and across four environments in an association mapping panel of 93 diverse durum accessions that included Wollaroi and Tamaroi (Reimer et al. 2008). The linkage group at this QTL in the Wollaroi/Tamaroi population was relatively small and the marker most significantly associated with YP concentration and YP/grain was located at the end of the linkage group. Additional markers are required to better characterise this QTL and identify the most appropriate makers to use for marker assisted selection. Patil et al. (2008) mapped marker *gwm46* to the edge of this QTL associated with YP concentration for one environment and in another environment *gwm46* was mapped just proximal to this QTL associated with YP concentration. Therefore, *gwm46* was found to be significantly associated with YP concentration in this study, polymorphic markers distal to *gwm46* may have revealed a stronger relationship.

7.4.4 QTL on Chromosome Arm 2AS and Association with Both Yellow Pigment Content per Grain and Grain Weight

7.4.4.1 Relationship between Chromosome Arm 2AS and Grain Weight and Yellow Pigment Content per Grain

The QTL on chromosome 2AS was found to be highly significantly (LOD>10) associated with TGW and YP/grain in all environments. This QTL generally was not related to flour YP concentration or yellow flour colour and therefore selection for Wollaroi alleles at this QTL could be expected to increase TGW without diluting or reducing YP concentration. This QTL may be especially useful for selection for

higher TGW and because of the concomitant association with YP/grain, the increase in TGW should be associated with lower YP concentration.

7.4.4.2 Potential Effect of Phenology on Grain Weight and Yellow Pigment

It is possible that the association between this QTL and TGW may be an indirect result of an association between this QTL and some other trait, such as phenology for example. It is sensible to expect that in the South Australian Mediterranean type environment later genotypes are more likely to be exposed to terminal drought and heat stress which could affect TGW. Flowering times were unfortunately not recorded; however, Wollaroi and Tamaroi have been genotyped for the major phenology genes *Vrn-A1, Vrn-B1,* and, *Ppd-A1* (Eagles 2012; Kane 2012). Wollaroi and Tamaroi carried the same allele at *Vrn-A1* and *Vrn-B1* but were polymorphic at *Ppd-A1* (Eagles 2012; Kane 2012). The major photoperiod insensitivity gene (*Ppd-A1*) has been mapped to chromosome 2A in durum wheat (Wilhelm et al. 2009); also Maccaferri et al. (2008) mapped an association between ear emergence and this chromosome region in durum wheat. Kuchel et al. (2006a) reported a QTL associated with the control of vernalisation requirement also on chromosome 2A linked to *barc220*. It is difficult to accurately place the ear emergence and vernalisation requirement QTL relative to the QTL reported here for TGW; however, comparison with a range of published maps (Kuchel et al. 2006a; Maccaferri et al. 2008; Qi et al. 2004; Somers et al. 2004; Wilhelm et al. 2009) suggests that *Ppd-A1* is distal to the 2AS QTL reported in the current study. Nevertheless, this QTL appears sufficiently close to *Ppd-A1* that the association of this QTL with TGW could be due to *Ppd-A1*. This QTL was most significant in the data set from the summer season of 2006/2007 at Waite. During the long days of summer, variation in phenology between genotypes

due to photoperiod response is reduced relative to the short days of winter. Therefore if the association between TGW and the 2A QTL is due to linkage to the *Ppd-A1* gene then it might be expected this effect would be less significant during a summer season and not more significant as was observed. This question could be resolved by assaying Wollaroi/Tamaroi RILs with a *Ppd-A1* gene based marker and mapping *Ppd-A1* relative to the 2A QTL. Alternatively Wollaroi/Tamaroi RILs could be phenotyped for photoperiod response to determine if photoperiod response maps to this region on chromosome 2A identified in the Wollaroi/Tamaroi population. Marker *barc220*, found to be associated with vernalisation requirement in hexaploid wheat (Kuchel et al. 2006a), maps to the long arm of chromosome 2A and therefore is unlikely to be associated with TGW in this population. There have been no published reports of variation in TGW mapping to this region that have not also been associated with another trait which is related in some way to TGW. McCartney et al. (2005) identified a QTL associated with grain yield just proximal to *wmc522* and *gwm515* and a similar region on chromosome 2A has been found to be associated with harvest index (Peleg et al. 2009) and water soluble carbohydrate (Yang et al. 2007), which in turn are both implicated in variation in grain weight (Rebetzke et al. 2008). There was only limited variation in phenology among Wollaroi/Tamaroi RILs (data not shown) which was likely due to variation at *Ppd-A1*. With the exception of variation at *Ppd-A1* which may have contributed to the 2A QTL as just discussed, limited variation in phenology and evidence of both Wollaroi and Tamaroi carrying the same allele at *Vrn-A1* and variation at *Vrn-A1* and *Vrn-B1* indicates that, at least to some extent, the effect of phenology on YP data in this study can be discounted.

7.4.4.3 Possible Genetic Effect of 2AS on Both Grain Weight and Yellow Pigment Content per Grain

The 2AS QTL for TGW was also found to be associated with variation in YP/grain suggesting that this QTL, either directly or indirectly through association with another trait, may be having an effect on the developing grain at the cellular level, thus affecting both grain size and total amount of YP produced per grain. The linkage group on chromosome 2A is very small with only three markers and spanning only 13.8 cM. It is possible that greater marker density in this region could assist in better characterising the relationship between this QTL, TGW, and YP/grain.

7.4.5 QTL on Chromosome 6B Associated with Grain Weight

The QTL on chromosome 6B did not account for a large proportion of the variance in YP concentration but importantly, this QTL did have a significant association with TGW where TGW were negatively correlated with YP concentration. Consequently this QTL had no effect on YP/grain. It is likely that this QTL influences YP concentration indirectly through its effect on grain size, possibly as a result of starch dilution. This is an example of a genetic correlation between TGW and YP concentration and therefore potentially limiting application of this QTL in breeding. Hessler et al. (2002) found a QTL on chromosome 5A associated with variation in TGW which in turn affected YP concentration. Pozniak et al. (2007) and Reimer et al. (2008) reported a significant association between yellow endosperm colour and *gwm193* on chromosome 6B. Grain size or weights were not reported in these studies but Pozniak et al. (2007) acknowledged that this QTL may have an effect on endosperm colour due to variation in starch content similar to the effect of the 5A

QTL reported by Hessler et al. (2002). A large and major QTL on chromosome 6B, and linked to marker *gwm193*, associated with TGW was identified by Elouafi and Nachit (2004) which may support the association between TGW and the 6B QTL identified in the current study. It is difficult to determine the precise location of the QTL reported by Elouafi and Nachit (2004) since it spans such a large chromosome region (approximately 50 cM). Since phenology data was not recorded on Wollaroi/Tamaroi RILs it is not known if the relationship between the 6B QTL and TGW is due to plant phenology as discussed for the 2A QTL.

7.4.6 QTL on Chromosome 6AS Associated with Yellow Pigment Concentration, Flour Colour and Yellow Pigment per Grain

Molecular marker *gwm334* was significantly associated with YP concentration, yellow flour colour and YP/grain of WID22221/Tamaroi RILs. Variation at this marker explained 6.5 per cent of the observed phenotypic variance in YP concentration at Waite, SA in 2008. Importantly, the association of this QTL with YP concentration was additive with other QTL identified in the WID22221/Tamaroi population. According to the consensus map (Somers et al. 2004), *gwm334* is on the distal end of chromosome 6AS while the 6B QTL linked to marker *gwm193* identified in Wollaroi/Tamaroi RILs is near the centromere. This suggests that the QTL on 6B and 6AS are not homoeologous. In addition, *gwm334* was not associated with TGW whereas the QTL on chromosome 6B in Wollaroi/Tamaroi RILs was. This appears to be the first report of an association between this chromosomal region and YP concentration in a durum RIL population. A QTL associated with YP and pasta colour has previously been mapped to chromosome 6A (Zhang et al. 2008); however, cross

referencing with consensus and physical maps (Qi et al. 2004; Somers et al. 2004) indicates that this QTL is proximal to both *gwm334* and also to *gwm570*, which was also found to be significantly associated with a relatively small level of variance in YP concentration and YP per grain of WID22221/Tamaroi RILs. Mares and Campbell (2001) identified a QTL associated with yellow flour colour (CIELAB b*) on chromosome 6A in a bread wheat population and Reimer et al. (2008) identified a marker trait association between *gwm334* and YP in an association mapping panel which supports the association identified in this study. Lycopene-β-cyclase is involved in the conversion of lycopene to lutein (Cunningham et al. 1996) and has been reported to map to the group 6 chromosomes (Singh et al. 2009). It is possible that the group 6 QTL identified in this study are associated with Lycopene-β-cyclase expression.

7.4.7 QTL on Chromosome 1A Associated with Yellow Pigment Concentration

The QTL on chromosome 1A has not previously been reported in any mapping population. However, in an association mapping study Reimer et al. (2008) identified chromosome regions on all 14 durum chromosomes, including chromosome 1A around *barc83*, associated with YP concentration in a panel of 93 diverse durum lines. Inclusion of this QTL in the regression analysis increased the total percentage of variation in average YP concentration accounted for from 40.9 to 44.7 per cent. However, this QTL was of relatively small effect and was not significantly associated with YP concentration in all environments. This QTL is therefore unlikely to be an economically viable target for MAS in a commercial breeding program.

7.4.8 QTL on Chromosome 1B associated with TGW

A QTL on chromosome 1B was found to be associated with TGW at two of the four environments tested. This QTL was not found to be associated with YP concentration or yellow flour colour in any of the environments, that is, this QTL seems to influence TGW independent of YP concentration in the grain. The significant association of this QTL with YP/grain at Turretfield in 2006 supports this conclusion. As the effect of this QTL on TGW was environmentally dependent and not of large effect, it is unlikely that it would be economically viable to target it in a MAS strategy aimed at increasing TGW. In addition, as for many QTL found to be associated with TGW, it is possible this QTL is simply associated with TGW indirectly through phenology or some other developmental trait.

7.4.9 QTL on Chromosome 3B Associated with Yellow Pigment Concentration

The putative association between the 3B QTL and YP concentration was relatively weak. Markers *wmc418* and *barc164*, which were found to be associated with YP concentration of Wollaroi/Tamaroi and WID22221/Tamaroi RILs respectively, map to the same location on chromosome 3B according to both the consensus map (Somers et al. 2004) and a durum map constructed by Reimer et al. (2008). Therefore, the interesting thing about this QTL is that it seems to have been identified in both populations despite its small effect. Since these markers were not part of any linkage group, the weak association between this QTL and YP concentration may be due to poor marker coverage of this chromosome region. A QTL has previously been reported on chromosome 3B accounting for 20 per cent of the variance in yellow flour

colour (CIELAB b*) of bread wheat (Mares and Campbell 2001) and has since been found to co-locate to the ε-cyclase (*ε-LCY*) gene (Howitt et al. 2009). ε-LCY is the first committed step in the β- ε pathway that leads to the production of lutein (Cunningham and Gantt 1998). Harjes et al. (2008) found that in maize, four natural polymorphisms in ε-LCY explained 58 per cent of the variation in flux between alpha carotene and beta carotene branches down the carotenoid pathway and a threefold difference in endosperm provitamin A compounds. It has been proposed that allelic variation at the *ε-LCY* gene in wheat results in higher ε-LCY activity relative to β-cyclase which would explain a higher lutein content (Howitt et al. 2009). Wollaroi/Tamaroi and WID22221/Tamaroi RILs could be assayed for allelic variation at the *ε-LCY* gene to determine if this explains variation in YP concentration in these populations.

7.4.10 Interaction between QTL

All the QTL found to be associated with YP concentration and YP/grain of Wollaroi/Tamaroi RILs and most QTL identified in WID22221/Tamaroi RILs behaved in an additive manner and were not influenced by significant epistatic interactions with any of the other QTL identified. This is consistent with reports by Elouafi et al. (2001) that YP is mainly affected by genotypic effect in an additive manner; and Lee et al. (1976) and Johnston et al. (1983) who found that no epistatic effects influenced YP concentration. Similarly, Singh et al. (2009) found that there was no statistically significant interaction between *Psy1-B1* and *Psy1-A1*.

7.4.11 Mechanisms Potentially Responsible for Variation in Yellow Pigment Associated with Identified QTL

All these QTL associated with YP synthesis are possibly linked to genes associated with regulation of enzymes involved in the synthesis of xanthophylls or other yellow pigments. Alternatively, these QTL could be linked to gene(s) coding for transcription factors which affect regulation of carotenoid biosynthetic genes (Corona et al. 1996). More specifically, any enzymes or transcription factors involved with YP synthesis in durum are likely to be involved with lutein synthesis since lutein is the predominant YP in durum wheat (Lepage and Sims 1968). The potential for ε-cyclase, Lycopene-β-cyclase and phytoene synthase to be associated with QTL on the group 3, 6 and 7 chromosomes respectively has already been discussed. Zeta-carotene desaturase has been mapped to the group 2 and group 4 chromosomes (Cenci et al. 2004) and so are unlikely to be associated with any of the QTL identified in this study. Multiple paralogues of the *Psy* gene family have been identified in the *Proaceae*; a second *Psy* gene has been mapped to the group 5 chromosomes (Pozniak et al. 2007) and a third has been identified in maize (Li et al. 2008). It is therefore possible that further *Psy* genes exist in wheat and that this is responsible for the variation in YP associated with QTL identified in this study.

7.4.12 Selection for Improved YP Concentration in a Breeding Program

7.4.12.2 GxE Interactions

The lack of significant GxE described for these populations in Chapter 5 was generally also observed at an individual QTL level. Generally, the association of QTL

with YP concentration identified in this study were stable across environments, which provides confidence in applying these QTL in a MAS strategy. Previously reported QTL associated with YP concentration have also generally been found to be stable across environments (Clarke et al. 2006; Elouafi et al. 2001; Mares and Campbell 2001; Parker et al. 1998; Patil et al. 2008). Pozniak et al. (2007) reported that the association between the QTL at *Psy1-B1* and YP concentration was environmentally dependent. This contrasts with the results of the current study as the association of the 7BL QTL with YP of Wollaroi/Tamaroi RILs was stable across all four environments.

7.4.12.1 *Psy1-A1*

It has been suggested that the lack of observed association between *Psy1-A1* and YP concentration in this study may be due to the lack of a functional mutation between polymorphism at this gene. If this is found to be correct, then an opportunity may exist to introgress the *Psy1-A1* allele from a genotype that has been shown to be associated with higher YP concentrations, such as Canadian variety Commander (Singh et al. 2009) for example, into Australian germplasm. Selecting for functional genes rather than for QTL using linked markers has many benefits. Because linkage phase between the QTL and markers used for selection is unknown, MAS using linked markers can only be used with confidence in populations derived from a validated source, in this case, Wollaroi. Given this limitation, the opportunities for applying MAS targeting linked QTL within a breeding program are likely to be very few. In contrast, when targeting a characterised functional gene with a gene based marker, the source is usually not important. In addition, when selecting for markers linked to a QTL often flanking markers are also used. Utilisation of flanking markers results in greater truncation of population size which may reduce useful genetic

variation, increased cost of MAS due to having to assay more markers, and increases the potential for linkage drag which may be deleterious. Furthermore, even with the use of flanking markers the potential for recombination between the target gene(s) and the flanking markers still exists.

Genotype WID22221, and RILs derived from this parent, were found to express very high concentrations of YP concentrations despite not having favourable alleles at *Psy1-B1*; assuming the 7BL QTL in the WID22221/Tamaroi population was not due to undetected polymorphism at *Psy1-B1* as discussed. Favourable alleles at this gene could easily be introgressed from Wollaroi (and potentially other, better adapted Australian lines) into recurrent parents carrying the *Psy1-B1a* (Tamaroi) allele such as WID22221. This should improve the YP concentration of the recurrent parent thereby improving its breeding value for subsequent hybridisations as well as improving its chance of making Australian Durum classification. During selection for *Psy1-B1b* it should be considered that due to linkage repulsion with the QTL identified on chromosome 7BL in the WID22221/Tamaroi population, it may be difficult to simultaneously select for both this QTL and *Psy1-B1b*.

7.4.12.3 MAS for QTL Associated with Yellow Pigment Concentration

The benefits of selecting for functional genes has been discussed and so, to this end, the *Psy1-1* genes are obvious targets for utilisation within a breeding program selecting for improved YP concentration with MAS. Due to their relatively strong association with YP concentration, the 7AL QTL and the 7BS QTL are also obvious targets for a MAS strategy. Together, these QTL explained 28.3 and 33.5 per cent of the total observed variance in YP concentration across four environments of

Wollaroi/Tamaroi RILs and WID22221/Tamaroi RILs respectively. The association of the 6AS QTL identified in the WID22221/Tamaroi population with YP concentration was additive with the other QTL and so may also provide a viable MAS target, particularly within populations without variation at the other more important loci.

7.4.12.4 Selection Strategy and Putting MAS into Context

The relatively low cost and accuracy of phenotyping for YP concentration using methods such as NIR (McCaig et al. 1992), and the high heritability of this trait, means selection for these QTL is likely to be most economically beneficial only in early and very early generations, for example in F_2 seeds or plants and F_1 seeds or plants derived from complex crosses (backcrosses and/or topcrosses). During these early stages it is not practical to phenotype for YP and so MAS offers a very useful method for identifying selections likely to have high YP concentration. During later generations phenotyping for YP is likely to not only be cheaper than MAS, but given the highly complex nature of the genetic inheritance of this trait, is likely to be the most effective way of identifying and discarding individuals that have not combined the multiple genes required to have sufficiently high concentrations of YP to achieve the classification standards. Populations that have been enriched for favourable alleles in early generations should have fewer individuals that require culling in later generations due to insufficient YP concentration, thus maintaining large enough population sizes for effective selection for other economically important traits such as yield, disease resistance, other quality traits and agronomic traits. The effect of fixing favourable alleles at key loci has a large impact on the proportion of the remaining population with sufficient YP concentration and is shown graphically in Figures 7.6,

7.7, 7.8 and 7.9. Therefore combining very early generation MAS with later generation phenotyping is likely to be the most effective and efficient way of making progress in a breeding program.

7.4.12.5 Consideration of TGW When Selecting for Yellow Pigment Concentration

When selecting for improved YP based on phenotype, TGW should also be considered to avoid selection for smaller grain size in populations where these traits have been found to be negatively correlated. When large grain size is also an important target breeding objective, or in populations where grain size could be a problem, selecting for the 2A QTL associated with TGW could also be useful and should simultaneously improve YP/grain. Selecting for the QTL on chromosome 6B is not advisable as this QTL confers high YP concentration through producing small grain size. The value of selecting for the QTL found on chromosome 1A the QTL linked to marker *barc164* on chromosome 3B is limited as the association of these loci with YP was either not significant or environmentally dependent and their effect on YP was small relative to other QTL.

7.4.12.6 Selection for Yellow Pigment Concentration Using Flour Colour Data

In Chapter 5 a very strong correlation was reported between YP concentration measured spectrophotometrically and flour colour (CIELAB b*). This was further supported by the similarities demonstrated between QTL associated with both YP concentration and flour colour. Often the percentage of variation accounted for, and significance of the QTL, were higher for YP concentration than for flour colour; possibly a function of YP concentration data being less confounded by other factors

such as protein and flour particle size. Although the correlation between YP concentration and flour colour is likely to be strong enough for breeders to use for selection, the higher levels of significance and the greater percentage of variation accounted for by QTL associated with YP concentration relative to flour colour indicates it would be preferable to measure YP concentration directly in research.

7.4.13 Summary

In summary, allelic variation at the *Psy1-A1* gene amongst Australian germplasm did not appear to be associated with variation in YP concentration. However, allelic variation at *Psy1-B1* exists within Australian germplasm and this variation is associated with variance in YP concentration. An opportunity exists to utilise allelic variation at *Psy1-B1* to improve YP concentration in Australian durum wheat germplasm. Allelic variation at *Psy1-A1* may also be useful but only if utilising a source from which the association between variation in YP concentration and *Psy1-A1* has been verified. In addition to *Psy1-B1*, two QTL explaining a relatively large proportion of phenotypic variance in YP concentration were identified on chromosomes 7AL and 7BS and two further QTL explaining a moderate proportion of variance were identified on chromosomes 6AS and 7BL. MAS for the functional genes *Psy1-A1* and *Psy1-B1* as well as the QTL identified in this study could assist breeders in raising the frequency of alleles positive for YP concentration in early generations and in turn contribute to greater rates of genetic gain for YP concentration and other important traits in Australian durum wheat breeding programs.

Chapter 8: General Discussion to Part 2

8.1 Implications of Research Outcomes for Wheat Breeding Programs

8.1.1 New Selection Methodologies - Phenotyping

A selection strategy has been recommended that involves enriching populations for key QTL and/or genes in the F_2 generation (and F_1 generation of complex populations) followed by phenotyping as soon as sufficient grain is available in later generations. Although developing phenotypic data of YP concentration is fast and cheap and selection is effective due to high heritability and limited genotype by environment interaction, one of the arguments for leaving phenotypic selection for later generations (later than F_2) is that to date it has not been possible to assay YP content of single grains non-destructively. High throughput NIR reflectance/ transmittance spectroscopy has been reported to phenotype, and even sort, single wheat grains with high throughput, for a range of traits including test weight, wet gluten content, sedimentation volume, water absorption, falling number, gelatinisation temperature, hardness index (Tonning et al. 2009) protein, oil content, moisture, starch content, grain weight, grain colour and fungal infection in wheat and/or maize (Bramble et al. 2006; Peiris and Dowell 2011; Spielbauer et al. 2009; Tallada et al. 2011; Weinstock et al. 2006) and for carotenoid content of bananas (Davey et al. 2009). It is feasible that in the near future this technology could be extended to YP content of individual wheat grains making an ideal method for enriching large, bulk populations with individuals that express high concentrations of YP; particularly

given the high heritability of YP concentration. A method such as this may allow selection pressure to be applied on very large populations very quickly and cost effectively.

Results from this study and other studies indicate that the genetic control of YP concentration is under additive gene action (Elouafi et al. 2001; Johnston et al. 1983; Lee et al. 1976; Mares and Campbell 2001; Santra et al. 2005). This facilitates early generation selection based on phenotype because the genetic control of the phenotype of a single grain is less complex and it is not necessary to wait until later generations for specific epistatic combinations have become fixed. Therefore, selection could begin on very early generation seed, perhaps as early as on F_2 seed.

8.1.2 Complexities of Early Generation Selection and Future Research Required

Dominant genetic control of high YP concentration could result in selections having a lower YP concentration phenotype in subsequent generations. Recessive control of YP concentration will result in the inability to distinguish between individuals that have become homozygous for unfavourable alleles and individuals that are heterozygous as they will all express low YP concentration. This may result in culling more individuals than necessary and reduce population size and in turn genetic variation, thereby affecting effective selection pressure for other traits. Breeders would need to be aware of this when selecting based on phenotype in early generations otherwise population sizes may become unnecessarily truncated. In addition, the complexity that results from the double fertilisation phenomenon (Gale

1989) that affects the phenotype of the endosperm would also need to be considered during early generation selection based on phenotypic data. The best approach may be to not select too aggressively and simply enrich populations at each generation, removing only genotypes with the lowest YP concentration. This would maintain a sufficiently large population in later generations to make genetic gain for yield and other economically important traits.

Due to the correlation between thousand grain weight (TGW) and YP concentration, it is important that grain weight is considered in selection strategies. If selection is based on the phenotype of individual grains, as suggested earlier with NIR, then it is even more important to consider the impact of grain weight. This is because when assessing the phenotype of an individual grain, both the genetic association as well as the physiological association, reported in Chapter 4 to be very strong, between grain weight and YP concentration will be influencing the phenotype of the individual grain. Ideally, as grains are being phenotyped for YP content by NIR they would also be phenotyped for grain size or weight and selected on the basis of both traits. Grain weight is highly heritable (Bhatt 1972; Johnson et al. 1966) suggesting that selection as early as the F_2 generation could be effective (Johnson et al. 1966). There is of course variation in TGW due to phenology as well as non-genetic variation in the weight of single grains due to spatial variation in the field and intra-spike hierarchy; therefore selection intensities for large grain should not be high.

8.1.3 Selection Methodologies – Marker Assisted Selection

Data from Wollaroi/Tamaroi RILs suggests that to achieve sufficient concentration of YP for commercial release (YP concentrations similar to Wollaroi) it was necessary to have favourable alleles at a minimum of two to three of the identified loci. In a complex cross (top-cross or back-cross) if only one minor parent (contributing on average ≤ 25% genome to future generations) has favourable alleles at a given loci then this can be targeted in the TC or BC F_1 such that only F_1 plants carrying the required allele(s) would contribute to the following generation; resulting in, on average, 75% of the F_2 seed carrying the favourable allele instead of 37.5% without marker assisted selection (MAS). If two or more parents have favourable alleles at a given loci this loci could be fixed in the F_1 with the use of co-dominant markers so that all progeny in subsequent generations will be fixed (homozygous) for favourable alleles at this locus. The benefits of enriching populations at the F_1 stage become exponentially greater as the number of loci or genes being selected increase. Table 8.1 shows the frequency (percentage) of individuals of a population derived from a complex cross where target loci are contributed by the minor parent at each generation (from F_2 to F_7) which is either homozygous or heterozygous for 1, 2, 3, 4 or 5 target QTL/genes using 4 different selection methods. In Table 8.1 it can be seen from some simple quantitative genetics calculations that in a complex (3 or 4 way) cross where the donor parent of positive loci for YP concentration is the minor parent, MAS can double the probability of retaining a single targeted loci and increases the chance of retaining five independent (unlinked) loci by 32 fold. The calculations presented in Table 8.1 have been made for the purpose of discussion and are based on a simplistic model assuming 100% accuracy of phenotypic data and 100% heritability of YP concentration.

The trade-off accompanying early generation selection with MAS is of course that the number of unique F_1 plants contributing to future generations decreases by 50% for every (unlinked) loci being targeted; so a larger number of initial F_1 seeds may be required in order to maintain genetic variation for other loci. However, this is not necessarily a limitation of the selection strategy but rather a function of the breeding objective; if the targeted loci really are necessary for commercial release then even without MAS, a similarly large number of F_1 seeds will be required in order to ensure all the targeted loci can be recombined in a homozygous line.

Table 8.1: Example of percentage of population at each generation from F_2 to F_7 which is either homozygous or heterozygous for 1, 2, 3, 4 or 5 target QTL/genes using 4 different selection methods. Model is based on a population derived from a complex cross (back-cross or top-cross) where the target QTL/genes are being conferred by the minor parent. Selection methods include; no selection (No $sel^{n.}$), MAS at F1 (+ MAS), retention of seed in each generation from F_2 from 75% of individuals with highest YP concentration based on phenotypic data generated on single grains by NIR (+ NIR) and combining the MAS approach with the phenotyping approach with NIR (MAS + NIR). Calculations for no selection method and MAS are based on simple quantitative genetics. Phenotypic data using NIR is based on unselected population percentages and adjusted to reflect selection pressure of 75% (discarding 25% of grains with lowest YP concentration) by dividing the unselected percentage with target loci at a given generation by 0.75^N where N=the generation number to reflect the number of selection cycles; ie % + NIR = % No selection / 0.75^N. Selection method combining MAS with phenotypic selection is calculated similarly but based on percentages of population with target loci after selection with MAS in F1 and then adjusted to reflect selection pressure of 75%; ie % MAS + NIR= % + MAS / 0.75^N where N = generation (F) number. A simplistic model is used assuming 100% accuracy of phenotypic data and 100% heritability

	1 QTL/gene				2 QTL/genes				3 QTL/genes				4 QTL/genes				5 QTL/genes			
	% No seln.	% + MAS	% + NIR	% MAS + NIR	% No seln.	% + MAS	% + NIR	% MAS + NIR	% No seln.	% + MAS	% + NIR	% MAS + NIR	% No seln.	% + MAS	% + NIR	% MAS + NIR	% No seln.	% + MAS	% + NIR	% MAS + NIR
F_2	37.5	75.0	50.0	100.0	10.9	43.8	14.6	58.3	2.9	23.4	3.9	31.3	0.8	12.1	1.0	16.1	0.0	6.2	0.3	8.2
F_3	31.3	62.5	55.6	100.0	9.0	35.9	16.0	63.9	2.5	19.7	4.4	35.1	0.7	10.5	1.2	18.7	0.0	5.5	0.3	9.8
F_4	28.1	56.3	66.7	100.0	7.7	30.9	18.3	73.1	2.1	16.6	4.9	39.4	0.6	8.8	1.3	20.9	0.1	4.6	0.3	11.0
F_5	26.6	53.1	84.0	100.0	7.0	28.0	22.1	88.6	1.8	14.7	5.8	46.5	0.5	7.7	1.5	24.2	0.1	4.0	0.4	12.6
F_6	25.8	51.6	100.0	100.0	6.6	26.5	28.0	100.0	1.7	13.6	7.2	57.5	0.4	7.0	1.8	29.5	0.1	3.6	0.5	15.1
F_7	25.4	50.8	100.0	100.0	6.4	25.8	36.2	100.0	1.6	13.1	9.2	73.5	0.4	6.6	2.3	37.3	0.1	3.4	0.6	18.9
F_∞	25.0	50.0			6.3	25.0			1.6	12.5			0.4	6.3			0.1	3.1		

The benefits of early generation selection using MAS and phenotyping become even more obvious when they are used in combination, particularly as the number of target loci increase. For example, if targeting just five loci from a minor parent, by F_7, without any prior selection, only 0.1% of the population will retain all five loci. Even enriching with MAS at the F_1 stage or culling the bottom 25% of the population (based on phenotypic data) at each generation starting from the F_2 only increases the expected percentage to 3.1% by F_7. However, combining these methods would theoretically result in (on average) almost 19% of the population retaining all 5 loci in a single genotype by F_7. The example presented in Table 8.1 models up to five segregating loci. To put this into context there were five and six loci associated with YP concentration of Wollaroi/Tamaroi and WID22221/Tamaroi RILs respectively accounting for approximately half the genetic variance for YP concentration, and wheat breeders select for many more traits than just YP concentration. Within a practical breeding program there are obviously much higher numbers of loci needing to be managed. For a successful selection strategy that involves F_1 enrichment with MAS followed by phenotyping it is important that the target trait is highly heritable and that the genetic control of the trait is mostly additive. Data from this thesis indicate that YP concentration satisfies these criteria.

Another strategy that could be considered is whole genome selection taking advantage of the high density SNP arrays that are coming on stream and continually becoming cheaper. Whole genome selection is particularly effective for complex polygenic traits (Jannink et al. 2010) and can be used to simultaneously select for a number of traits.

8.2 Suggested Further Research to Identify Additional Candidate Genes Associated with Yellow Pigment

The benefit of having markers derived from functional genes rather than linked markers have been discussed in Chapter 7 and is an obvious priority for further research. There are a number of approaches that can be taken to identify or develop 'perfect markers' which include; fine mapping of QTL and identification of candidate genes using genomic resources already available for other species; and, more recently for wheat, followed by validation using mapping, gene expression in relation to trait development, genetic modification, or TILLING. One of these methods could be used to identify perfect markers for QTL identified in the current study.

8.3 Summary

Due to the polygenic, highly heritable and additive nature of the genetic control of YP concentration, strategies based around MAS in very early generations combined with phenotyping are recommended. Further research in the area of identifying candidate genes and subsequently developing gene-specific markers associated with variation in YP concentration is recommended.

Chapter 9: Final Conclusions

This thesis has made significant contributions to the understanding of environmental and genetic control of yellow pigment (YP) concentration in durum wheat. Specifically, this study has found that;

- Allelic variation at *Psy1-A1* in Australian germplasm was found not to be associated with variation in YP concentration, flour colour, or yellow pigment content per grain (YP/grain);
- A major QTL linked to, and proximal to *Psy1-A1*, was found to be associated with variation in YP concentration explaining up to 27.8 per cent of the phenotypic variation in YP concentration;
- The previously reported association between allelic variation at *Psy1-B1* and YP concentration has been confirmed for the first time in Australian durum germplasm;
- A QTL associated with YP concentration on chromosome 7BL was identified which did not appear to be associated with allelic variation at *Psy1-B1*, but is expected to be linked to *Psy1-B1;*
- Six and Five QTL were identified to be associated with YP concentration of Wollaroi/Tamaroi and WID22221/Tamaroi RILs respectively explaining 51.0 and 43.6 per cent of the phenotypic variance in YP concentration respectively;
- The highly heritable, additive and polygenic control of YP concentration was confirmed as was the limited interaction between genotype and environment for this trait;
- Flour colour (CIELAB b*) was confirmed to be significantly and closely correlated with YP concentration and in turn confirming it as a useful tool for making breeding selections;

- The environmental influences on YP concentration were found to be very complex. Despite a very large data set of 46 site-year combinations across five years, no discernible pattern was observed between YP concentration and a wide range of environmental variables;
- A strong environmental effect on YP concentration was confirmed with up to a 2.5 fold difference in concentration observed from one environment to another. Despite this, neither specific sites nor regions within Australia were found to consistently produce grain with significantly higher or lower YP concentration from one season to another;
- A significant genotype effect on YP concentration was also confirmed and all popular Australian durum wheat varieties were characterised for their expression of YP content; Hyperno, Bellaroi and Wollaroi produced the highest concentrations of YP while Arrivato, Kalka and Tamaroi the lowest;
- Genotypes performed consistently relative to one another across environments for YP concentration indicating limited genotype by environment interaction for this trait;
- A highly significant ($p<0.001$) **negative** phenotypic correlation between thousand grain weight (TGW) and YP concentration was observed both between environments and, within environments, where variation in TGW is expected to be due to processes and resource allocation within the plant;
- A strong and highly significant ($p<0.001$) **positive** phenotypic correlation between TGW and YP/grain was observed both between environments and, within environments, where variation in TGW is expected to be due to processes and resource allocation within the plant;
- A weak negative genetic correlation was observed between TGW and YP concentration and was significant in some environments. This was supported by QTL

analysis where QTL were identified to simultaneously affect TGW and YP concentration in opposite directions;

- Water stress was associated with both low YP/grain and low TGW. The low TGW was associated with high YP concentration but was mitigated by the lower YP/grain;
- In controlled environment experiments, variation in temperature and humidity had little impact on YP/grain but high temperature and low humidity were associated with low TGW and in turn high YP concentration;

Since the causative factors influencing YP concentration across environments were not able to be fully identified, despite a very large data set encompassing 46 site year combinations across five years, there are no obvious methods for manipulating YP with management or agronomic practices. Therefore, the most appropriate strategy for improving the quality of pasta processed from Australian durum is likely to be through breeding. Many of the outcomes of this thesis can be used to assist durum wheat breeders to develop varieties with improved YP concentration. Variation at *Psy1-B1* has been shown to be associated with variation in YP concentration in Australian germplasm and so breeders can target allelic variants at this gene to select for improved YP concentration. Conversely, breeders should not target allelic variants at *Psy1-A1* from Australian germplasm as allelic variation at this gene was shown to not be associated with variation in YP concentration. Opportunities may exist to target allelic variants at *Psy1-A1* from sources that have had an association between YP concentration and polymorphism at *Psy1-A1* verified. In addition to targeting *Psy1-1*, Australian durum breeders could target the QTL identified in this study as they were highly significantly associated with variation in YP concentration, behaved in an additive manner, and their effect was found to be stable across environments.

Due to the polygenic and additive genetic effects influencing YP concentration, the limited genotype by environment interaction observed in this study, and the availability of cheap, fast, safe and accurate phenotyping methods; a selection strategy integrating MAS in very early generations and selection based on phenotypic data in later generations is recommended. Grain weight was shown to be significantly associated with variation in YP concentration and with the amount of YP/grain; therefore breeders should take this relationship into account in selection strategies. In addition, end users may benefit from being aware of the relationship between TGW and YP concentration when sourcing grain.

There were no conclusive indications that any one region within Australia's wheat growing region consistently produces grain with significantly higher or lower levels of YP. This is important for end product manufacturers sourcing grain and for the durum wheat industry, including breeding companies, which may wish to expand the industry to new regions where durum wheat is currently not grown.

The complex influence of the environment on YP concentration has been simplified by showing that the amount of YP/grain is, not surprisingly, influenced by TGW, while simultaneously YP/grain is affected differentially from TGW in response to water stress and temperature variation. The relationship between TGW and both YP concentration and YP/grain; and the synthesis of YP in the grain in response to water availability and temperature; led to a hypothesis that YP concentration and YP/grain is influenced by the number and size of endosperm cells. This could be tested experimentally by counting endosperm cells and relating the data to YP content and grain weight data. Further areas of research were identified and discussed including;

- Characterising the effect of other climatic variables on YP concentration in addition to water and temperature effects, such as light intensity and duration;
- Development of a high throughput method of sorting single wheat grains according to YP concentration and grain size, perhaps utilising Near infra-red spectroscopy (NIR);
- Identifying the reason for allelic variation at *Psy1-A1* not having an association with variation in YP concentration, despite reports to the contrary;
- Confirming that the QTL on chromosome 7BL associated with variation in YP concentration in the WID22221/Tamaroi population is not due to allelic variation at *Psy1-B1*;
- Identifying candidate genes responsible for variation in YP concentration at the QTL identified in this study;
- Identifying further QTL associated with the remaining genetic variation in YP concentration;

All together, the improved understanding of the genetic and environmental control of YP concentration of durum wheat can assist breeders to select for this important quality trait more efficiently and effectively. Improved varieties will of course have flow on benefits for the whole industry and the pasta consuming community. In addition, the broader durum wheat industry may also benefit from the improved understanding of the genetic and environmental control of YP in durum wheat.

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