THE IMPACT OF NUTRITION ON THE GLUTEN COMPOSITION AND PROCESSING QUALITY OF WHEAT

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November 2005 - October 2008

CONTENETS

ABSTRACT

Understanding nutrition is important for improving the quality and yield of crop plants but is also a vital aspect of increasing the sustainability of crop production through better resource use efficiency. This study used the Broadbalk experiment, which is the longest running agricultural experiment in the world, together with modern analytical techniques, to improve the understanding of wheat nutrition. Transcriptomics analysis, using Affymetrix Wheat Genechips®, showed that there are significant differences in gene expression profiles between wheat grain from plants treated with organic and inorganic fertilisers. Metabolomic fingerprinting using NMR spectroscopy and GC-MS showed that wheat crops grown with higher availability of nitrogen had increased levels of free amino acids in the developing endosperm and in the white flour. Analysis of the grain protein composition using SE-HPLC and SDS-PAGE revealed characteristic changes in protein composition associated with the availability of nitrogen, including an increase in the ratio of gliadin to glutenin subunits. These effects were related to changes in the functional properties of dough made from the white flour. Effects that were seen in samples from the Broadbalk experiment could be related to differences in commercially produced grain from organic and conventional farms. This demonstrates that nutritional treatments associated with these two cultivation systems are important in contributing to differences in end product quality. In addition, the effects of foliar urea treatment after anthesis were similar to the effects of increased nitrogen application rate to the soil much earlier in crop development.

ABBREVIATIONS

^1H	Proton
ANOVA	Analysis of Variance
AT	SE-HPLC Total Trace Area
BU	Brabender Units
cDNA	Complementary Deoxyribonucleic Acid
CNS	Carbon, Nitrogen and Sulphur
DEFRA	Department for the Environment Food and Rural Affairs
DM	Dry Matter
DNA	Deoxyribonucleic Acid
dpa	Days Post Anthesis
F	The F statistic
F1	Fraction 1 (SE-HPLC); HMW polymer enriched with HMW subunits of glutenin
F2	Fraction 2 (SE-HPLC); LMW polymer enriched with LMW subunits of glutenin
F3	Fraction 3 (SE-HPLC); ω-gliadins
F4	Fraction 4 (SE-HPLC); α-, β-, γ-gliadins
F5	Fraction 5 (SE-HPLC); albumins and globulins
FYM	Farmyard Manure
(F3+F4/F1+F2)	SE-HPLC, gliadin to glutenin ratio
GC-MS	Gas Chromatography Mass Spectrometry
GS	Growth Stage
HGCA	Home Grown Cereals Authority
HMW	High Molecular Weight
ICP AES	Inductively Coupled Plasma Atomic Emission Spectra
kg N ha^{-1}	Kilograms Nitrogen per Hectare
l ha^{-1}	Litres per Hectare
LMW	Low Molecular Weight
LSD	Least Significant Difference
maxcentre	Reomixer parameter; peak dough resistance
maxtime	Reomixer parameter; time taken to reach peak dough resistance
µmol kg^{-1}	Micromols per kilogram
mRNA	messenger ribonucleic acid
MW	Molecular Weight
NO$_3^-$	Nitrate
NH$_4^+$	Ammonium
N	Nitrogen
NABIM	National Association of British and Irish Millers
NMR	Nuclear Magnetic Resonance
NPK	Nitrogen, Phosphate, Potassium
OSC	Orthogonal Signal Correction
p	Probability
PCA	Principal Components Analysis
PLS	Partial Least Squares
r	Correlation Coefficient
REML	Residual Maximum Likelihood
Rmax	Extensograph parameter; peak resistance
RNA	Ribonucleic Acid
SAGE	Serial Analysis of Gene Expression

SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis
SE-HPLC	Size Exclusion High Performance Liquid Chromatography
t	Tonne
t	The t statistic
t ha^{-1}	Tonnes per hectare
tPS	the Predicted Scores
v/v	Volume/Volume
w/v	Weight/Volume

INTRODUCTION

Concerns about changing climate, land degradation and food quality are affecting the way in which modern agriculture is conducted and are placing new demands on research. One crucial component of improving the sustainability of agriculture will be to reduce carbon emissions, and from the perspective of crop nutrition this requires improving the efficiency by which fertilisers are utilised by the crop. Improved fertiliser use efficiency will contribute to reduced pollution of water courses with excess nitrates and reduced emission of nitrous oxides that are also potent greenhouse gases. In addition, the price of fertiliser has risen dramatically in the past year from approximately £180 per tonne (t) in November 2007 to £380 per t in November 2008 (prices are for ammonium nitrate (Datum, 2008)); therefore improving fertiliser use efficiency would also financially benefit farmers.

In recent years there has been an increased interest in low input farming systems including organic farming. The production of arable crops in an organic system requires the building of soil fertility through rotations and the use of manures that are derived either from animal or plant sources. These forms of nutrient delivery are based on improving the fertility of the soil before the crop is planted and do not permit the targeted application of nutrients during specific growth stages. This means that it is difficult for growers of breadmaking quality wheat to achieve sufficiently high grain protein content to meet the requirement set by UK millers. A better understanding of the different effects of organic and inorganic fertilisers will contribute to improvements in the nutrition of organic crops.

This project sought to improve our understanding of the nutrition of wheat in order to contribute to both of the above research needs. The set of approaches utilised in the project revealed impacts of nutrition on patterns of gene expression (the transcriptome), levels of metabolites (the metabolome), protein composition and end use quality of wheat grain for breadmaking.

The project used samples from the classical Broadbalk experiment at Rothamsted Research. Broadbalk was started in 1843 and is the longest running agricultural experiment in the world (Poulton, 2006). Originally Sir John Bennett Lawes began the experiment to investigate the nutrient requirements of wheat by growing it continuously on the same land. However, over the past 165 years Broadbalk has been used for studying weed populations, drainage water and soil conditions as well as for unforeseen purposes such as the investigation of climate change, past atmospheric conditions and radioactive fallout.

Figure 1 Aerial view of the Broadbalk experiment at Rothamsted Research.

Since it was first established in 1843 the Broadbalk experiment has undergone a number of modifications in order to maintain relevance to modern agricultural practices. Nevertheless, Broadbalk remains true to the original intent of Sir John Bennet Lawes and Sir Joseph Henry Gilbert in that it is principally a winter wheat experiment incorporating a range of nutrient treatments.

Over the three years of this study, from 2005 to 2007, Broadbalk was managed according to the plan shown in Figure 2. Throughout this time sections 2, 3, 4, 5 and 7 were managed according to a five course rotation of oats, forage maize and three years wheat. The remaining sections are all continuous wheat but are managed differently. Section 0 has the straw incorporated, section 6 receives no fungicides and section 8 no herbicides. Since 1996 Broadbalk has been planted with Hereward, a National Association of British and Irish Millers (NABIM) Group 1 variety that is highly regarded by millers and bakers, although it has lower yield performance and disease resistance than more modern cultivars (HGCA, 2008; National Association of British and Irish Millers, 2008).

Treatments from 2001 until present

FYM	Farmyard Manure at 35 t ha^{-1}
P	35 kg P as triple superphosphate
K	90 kg K as potassium sulphate
K2	180 kg K as potassium sulphate
K*	90 kg K as potassium chloride
Mg	12 kg Mg as Kieserite
Mg2	24 kg Mg as Kieserite
(Mg*)	30 kg Mg Kieserite until 2000

N as single applications of ammonium nitrate (Mid-April):
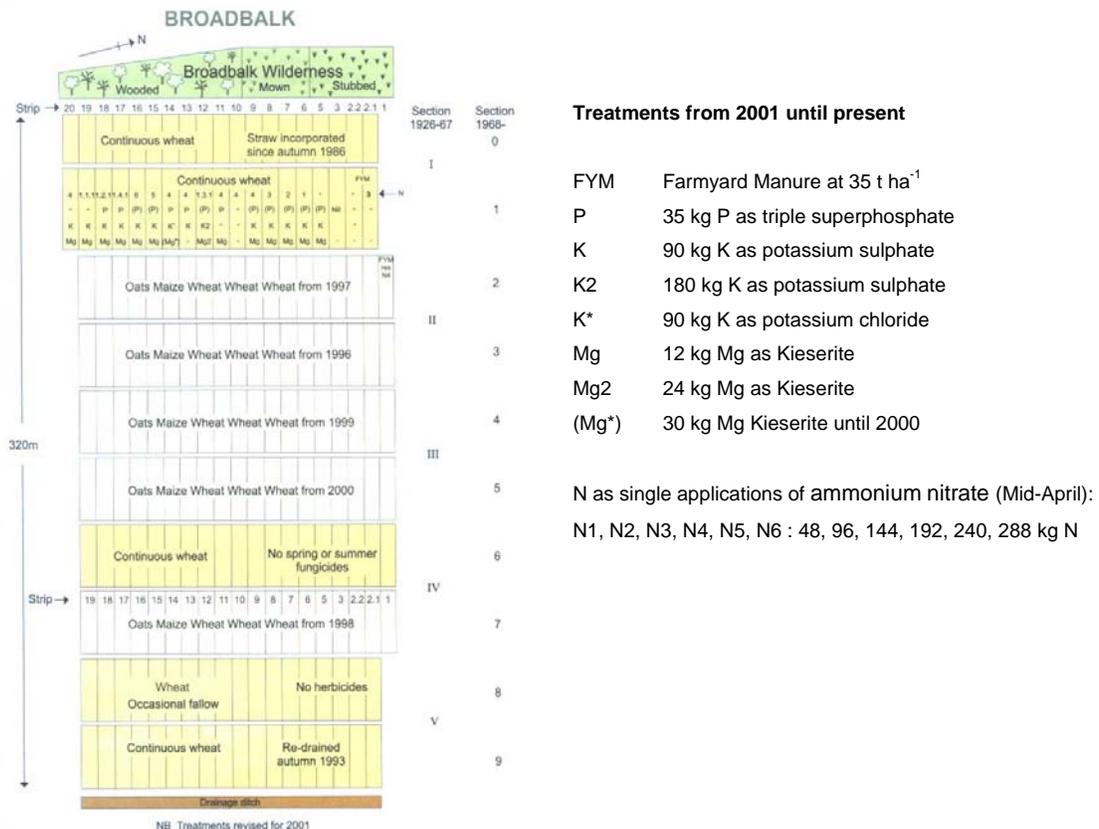N1, N2, N3, N4, N5, N6 : 48, 96, 144, 192, 240, 288 kg N

Figure 2 Plan of the Broadbalk winter wheat experiment at Rothamsted Research. Note that all treatments in the nitrogen application rate series incorporate 35 kg phosphorus (P) ha^{-1}, 90 kg potassium (K) ha^{-1} and 12 kg magnesium (Mg) ha^{-1}. FYM, farmyard manure; N nitrogen as ammonium nitrate.

Despite the numerous investigations conducted on Broadbalk there is no record of the end use quality of the grain ever having been determined. Clearly an experiment of this longevity and with such appreciable effects of nutrition on crop yield and morphology should provide some interesting revelations about how nutrition is related to quality. To investigate this relationship, samples of grain from the first wheat section of Broadbalk, from several of the nutritionally different plots, were collected between 2005 and 2007. A broad range of analyses were used to determine the mineral composition, protein composition, transcriptome and metabolic fingerprint of the developing and mature endosperm of the grain samples. These data have been used to study the effects of nutrient treatment on plant metabolism in relation to the grain and to the breadmaking properties of the flour.

Of particular importance to this study was the series of nitrogen application rate treatments on Broadbalk. These treatments showed the effect of varying nitrogen availability on the metabolome, protein composition and end use quality of the grain. Broadbalk also includes a treatment of farmyard manure (FYM) that allowed comparisons to be made between this organic treatment and the inorganic nitrogen rate series.

The principal aim of the project was to determine the effects of organic and inorganic fertilisers on crop physiology and grain quality. Comparison of the results from the long term Broadbalk experiment with a survey of commercially produced organic and conventional grain was made in order to relate results from the experimental Broadbalk system to commercial production. Analysis focused on the transcriptome, metabolome, protein composition and dough functionality of the developing and mature grain in order to generate a comprehensive picture of the effects of nutrition on grain nitrogen metabolism, protein composition and breadmaking quality.

Another aim of the project was to determine the effects of late foliar urea on grain metabolism and quality. Treatment with urea is routinely used to improve grain nitrogen content in order to meet the requirements set by

mills for breadmaking wheat, however, little is known about the fate of this nitrogen and whether it contributes to improved breadmaking performance. Late nitrogen applications allow flexibility in how fertiliser is applied to the crop and they may also be a way for organic farmers to increase the nitrogen concentration of their grain, if acceptable organic alternatives could be found.

METHODS

Measuring nitrogen concentration and protein content

Nitrogen concentration was measured using a Leco carbon, nitrogen and sulphur (CNS) Combustion Analyser (Leco Corporation, St Paul, Minnesota). This instrument combusts samples in a pure oxygen environment and then measures the amount of nitrogen based on the thermal conductivity of the gases.

The protein content of the samples of white flour was determined using two different techniques. In the first a sample of dough is washed in a buffered solution of sodium chloride to remove the soluble components and leave the insoluble gluten matrix; the weight of the gluten as a percentage of the dough starting weight is then calculated. In the second method, protein is extracted from the flour using SDS solution and sonication and then separated using size exclusion high performance liquid chromatography (SE-HPLC) (as described below). The total area of the SE-HPLC trace (AT) provides an estimation of the total protein content of the sample.

Measuring sulphur concentration

Inductively coupled plasma atomic emission spectroscopy (ICP AES) is a technique used to quantify the presence of various minerals, including sulphur, in liquid samples. The sample material is introduced into the excitation chamber as an aerosol in an argon stream where it is vapourised, desolvated and atomised by an inductively coupled plasma torch. The energy supplied by the plasma is sufficient to excite the atoms

to higher energy levels; on returning to a normal state the atoms emit energy quanta in the form of electromagnetic radiation. These quanta are characteristic of particular elements and allow for their quantitative detection in the sample using a spectrometer.

Measuring dough functional properties

Three instruments were used to assess the dough functional quality of different flour samples. The Farinograph mixes a sample of flour and water to a peak resistance of 600 Brabender units (BU), the amount of water required to reach this level of resistance allows the calculation of the water absorption of the sample. The time taken to reach the peak resistance is the development time of the dough. The sample continues to be mixed for a further 12 min after peak resistance and the reduction in BU provides a measure of the degree of softening. The length of time from the dough reaching 600 BU and then subsequently dropping below it, gives a measure of the dough stability. These parameters are shown in Figure 3.
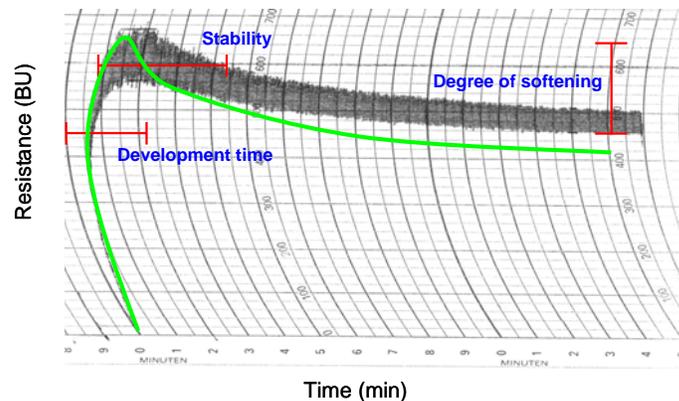

Figure 3 This Farinograph trace shows the three main parameters; development time, stability and degree of softening. This trace is for a flour sample that received adequate nitrogen, the green line shows the approximate shape of the trace for a sample grown with low nitrogen.

The Extensograph uses a sample of dough mixed to a resistance of 600 BU using the Farinograph. The sample is allowed to rest for 45 min before it is stretched on the Extensograph. The length to which the dough

stretches before breaking is its extensibility and the peak resistance to extension is termed Rmax.

The Reomixer uses a different mixing action to the Faringograph, but also measures the increase in resistance of flour and water as they are mixed to form dough. The instrument measures a number of different parameters associated with the dough development and breakdown, including the maximum resistance (maxcentre) and the time taken to reach maximum resistance (maxtime).

Measuring protein composition

Protein composition was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and SE-HPLC. SDS-PAGE is an electrophoresis technique that separates proteins based on their molecular weight. The samples are separated using porous gels through which smaller proteins can pass more easily and so migrate at a faster rate. The different protein types form bands on the gels that become visible after staining; the density of the staining can be measured and indicates the amount of a specific type of protein in the sample.

SE-HPLC is a chromatographic technique that separates proteins according to size. The chromatography column is packed with porous beads that retard the movement of large molecules to a greater degree than small molecules so that large molecules elute from the column first. Samples are prepared for SE-HPLC analysis by first extracting the soluble proteins using sodium dodecyl sulphate (SDS) and then using sonication to solubilise the polymeric gluten proteins. The SE-HPLC trace can be divided into 5 fractions that are associated with different protein types and so provides information about the protein composition of a sample.

Metabolomics

Metabolomics is the measurement of all the low molecular weight metabolites in a sample. Two of the most widely used approaches are nuclear magnetic resonance (NMR) spectroscopy and gas chromatography

mass spectrometry (GC-MS). GC-MS is a powerful technique that gives detailed information about the structure and amount of metabolites in a sample, however, it is only able to analyse compounds that can be volatilized. In this study it was used to measure the amounts of free amino acids in the samples of white flour and endosperm tissue. Proton (^{1}H) NMR spectroscopy provides a way of rapidly analysing complex mixtures of metabolites and can detect any proton-containing compound, but it does not provide the detailed quantitative data that are available from GC-MS. Often these two techniques are combined to build an accurate profile of the metabolome.

Transcriptomics

Transcriptomics aims to identify the genes that are expressed at a particular time point in a particular tissue. Different approaches to this challenge have been taken but all rely on profiling the messenger ribonucleic acid (mRNA) that is present, the so-called transcriptome. In this study, the transcriptome was profiled using Affymetrix Wheat Genechips®. Ribonucleic acid (RNA) collected from the sample is purified and then converted to complementary deoxyribonucleic acid (cDNA), this is hybridised to an array composed of oligonucleotide probe-sets that are representative of approximately 55,000 genes from wheat. Following hybridisation of the cDNA to the array, the slide is washed to remove the excess and then a fluorescent dye is applied that tags the cDNA so that it can be visualised. The array is then read by a computer that associates dye intensity at specific points with the level of expression of particular genes. In this study transcriptomics was used to profile gene expression in the developing endosperm of plants receiving different nutrient treatments and at different stages in development.

MATERIALS

The Broadbalk Experiment

Samples were collected from the first wheat section on Broadbalk in 2005, 2006 and 2007 (Figure 2). Three replicate samples were taken from each

plot for the treatments listed in Table 1. These samples were milled using a Brabender Quadrumat Junior mill and were used for all analyses except for the dough functional tests for which a larger sample of grain was required. This sample was taken from the harvested grain after routine measurements of yield and grain parameters had been made and a sub-sample had been archived. This larger sample was milled using a Chopin CD1 mill; this and the dough functional tests were carried out at Campden BRI.

Table 1 Treatments sampled from Broadbalk (2005 – 2007). FYM, farmyard manure; N, nitrogen as ammonium nitrate.

Treatment Code	Treatment
N0	0 kg N
N1	48 kg N ha^{-1}
N3	144 kg N ha^{-1}
N4	192 kg N ha^{-1}
N5	240 kg N ha^{-1}
N6	288 kg N ha^{-1}
FYM	35 t FYM ha^{-1}
-S	No Sulphur (192 kg N ha^{-1})

The Urea Experiment

The urea trial was conducted as part of the Broadbalk experiment by utilising the end part of some of the plots in the section comprising the first wheat in the rotation, and treating them with a foliar urea spray. In 2006 and 2007, the last 5 m of the N4 and FYM plots were sectioned off as shown in Figure 4. The end part of the plot was sprayed at approximately 7 days after anthesis with an application of foliar urea that delivered 40 kilograms nitrogen per hectare (kg N ha^{-1}) as a 4.3% weight/volume (w/v) urea solution. This was sprayed at a rate of 1000 litres per hectare (l ha^{-1}) on two consecutive days. The application was made at this late stage in development because this is the time at which the greatest effects on grain nitrogen concentration have been reported (Dampney and Salmon, 1990; Turley et al., 2001).
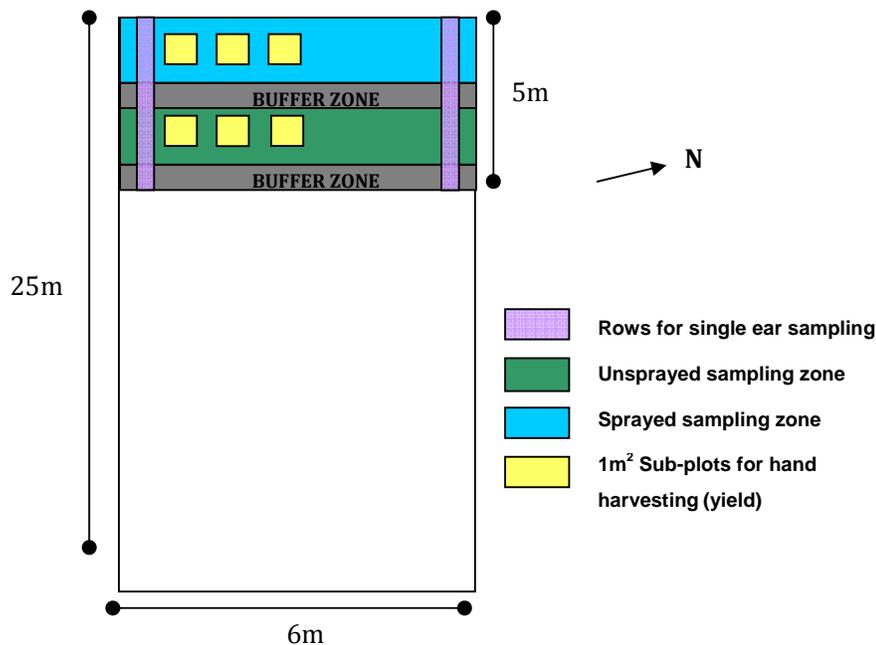
Figure 4 Diagram of the Broadbalk urea experiment, showing an example single plot. In 2006 and 2007 the western end of the N4 (strip 9) and FYM (strip 2.2) plots were sectioned off and divided into control and treatment areas divided by a buffer strip. 40 kg N ha^{-1} was applied to the sprayed section as a foliar urea spray.

Developing endosperm tissue was collected from the grains at various stages following anthesis by making a small incision in the grain and then squeezing the endosperm from the caryopsis and dropping it immediately into liquid nitrogen. These samples were stored at -80 ˚C until required for metabolomic and transcriptomic analyses.

Three replicate samples of mature grain were also collected from each of the sub-plots and were milled to provide white flour for further analysis. Each of the subplots was then cut by hand to provide enough grain for dough functional tests.

Conventional and Organic Experiment

Samples of breadmaking wheat, cv. Hereward, were obtained in 2006 and 2007 from cooperating farms and grain merchants across England. The aim was to obtain a representative sample of organically and conventionally produced grain grown commercially for sale to the breadmaking industry. In fact, whilst Hereward remains a popular variety

with millers and is commonly grown under conventional practices, many organic farmers struggle to achieve adequate protein content with this variety and instead choose to grow the spring variety Paragon. For this reason it was difficult to source samples of organic Hereward grain and in both 2006 and 2007 a larger number of conventional samples were included in the analysis than organic samples (Table 2).

Table 2 Sources of organic and conventional samples in 2006 and 2007.

Year	Organic	Conventional	
2006	Worcestershire Derbyshire Derbyshire Berkshire Suffolk Worcestershire Lincolnshire Somerset Suffolk	Lincolnshire Gloucestershire Oxfordshire Derbyshire Wiltshire Lincolnshire Northamptonshire Warwickshire Oxfordshire	Oxfordshire Staffordshire Lincolnshire East Anglia Oxfordshire Buckinghamshire 4 from unknown sites
2007	No information	Kent Wiltshire Warwickshire Berkshire South Lincolnshire Norfolk Staffordshire Oxfordshire West Sussex Cambridgeshire	

The organic and conventional samples were essentially regarded as treatments for the purposes of the analyses. This was because our aim was to determine whether there were characteristic differences between grain samples produced using these cultivation practices, rather than to analyse the effects of specific practices used under the umbrella of organic or conventional.

RESULTS & DISCUSSION - BROADBALK

The Broadbalk experiment has proved to be an excellent resource for studying the effects of nutrition on metabolism and end-use quality. The experiment is deficient in terms of modern experimental design because it lacks the elements of randomisation and replication; however, its longevity means that the nutritional treatments have produced well-defined effects on the soil and these are clearly apparent in the physiology and biochemistry of the crop.

Nitrogen and protein concentration in the white flour

Nitrogen is the most important nutrient in terms of wheat yield and quality and it was not surprising to find that for many of the results there were clear trends related to the availability of this nutrient. Most prominent was the increase in white flour protein concentration with increasing application rate of nitrogen fertiliser. The six levels of nitrogen rate that were compared, revealed a clear sigmoidal response of white flour nitrogen concentration to nitrogen application rate (Figure 5). The lowest application of 48 kg N ha^{-1} resulted in dry matter accumulation at a rate that out-stripped the increase in protein accumulation such that the overall concentration of nitrogen in the white flour fell. In contrast the N0 treatment plot had received no nitrogen fertiliser since at least 1967 and the resulting crop was extremely nitrogen deficient. At very high applications of nitrogen fertiliser a levelling of the response curve occurred demonstrating that increasing quantities of nitrogen fertiliser do not lead to indefinite increases in white flour nitrogen concentration. In the linear phase of the curve, for applications between 48 and 240 kg N ha^{-1}, an increase of 48 kg N gave an increase of approximately 0.3% nitrogen in the white flour. This is equivalent to an increase in protein of 1.7% (using a conversion factor of 5.7 (Mosse, 1990)). In agreement with these values, the wet gluten content of the dough and the AT value also increased significantly with nitrogen application rate (probability (p) < 0.001).
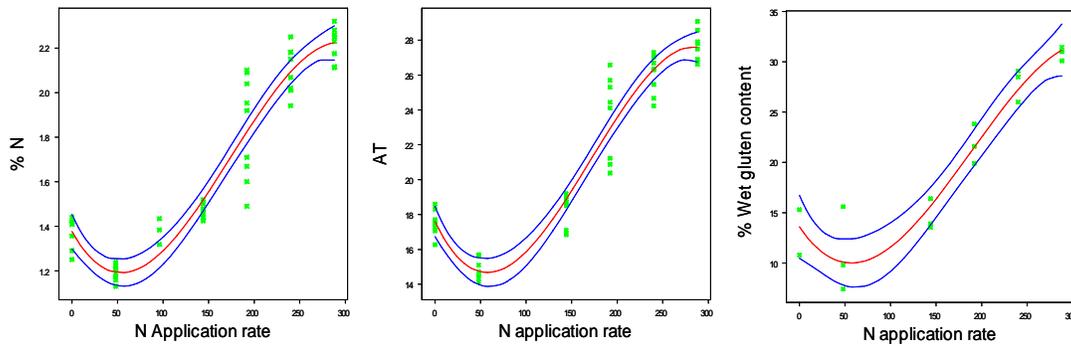
Figure 5 Regression plots of various parameters associated with white flour protein content. N application rate is presented as kg N ha^{-1}. Samples are from the Broadbalk experiment. Correlation values are as follows; %N, p < 0.001, r = 0.90; AT, p < 0.001, r = 0.90; % Wet Gluten, p < 0.001, r = 0.91.

The three years over which the study was conducted included a wide range of weather conditions, which not surprisingly led to significant effects on the parameters being measured. 2006 had the highest values for white flour nitrogen concentration and this was probably due to the hotter and drier weather in this year. It has been shown that hotter, drier conditions result in grain with higher grain nitrogen concentration (Daniel and Triboi, 2000; DuPont et al., 2006; Gooding and Davies, 1997). This is because high temperatures appear to reduce the flow of both nitrogen and carbon to the grain, but the flow of carbon is more affected resulting in a change in the overall concentration of nitrogen (Daniel and Triboi, 2000).

The nitrogen to sulphur ratio of the white flour samples increased significantly with increasing nitrogen application rate (p < 0.001), from a value of 12.31 for the N0 treatment up to a value of 15.34 for the N6 treatment.

Protein Composition of the white flour

Increasing nitrogen application rate increased the amounts of all protein fractions measured by the SE-HPLC and SDS-PAGE analyses. However, the rate of increase was not the same for each protein fraction leading to changes in the protein composition of the samples. Specifically there was a significant increase in the ratio of the gliadin to glutenin proteins (F3+F4/F1+F2), as measured by the SE-HPLC (p < 0.001) (Figure 6).

(Analysis of the protein composition using the SDS-PAGE also showed an increase in the ratio of gliadin to glutenin but this was only significant at p = 0.052).
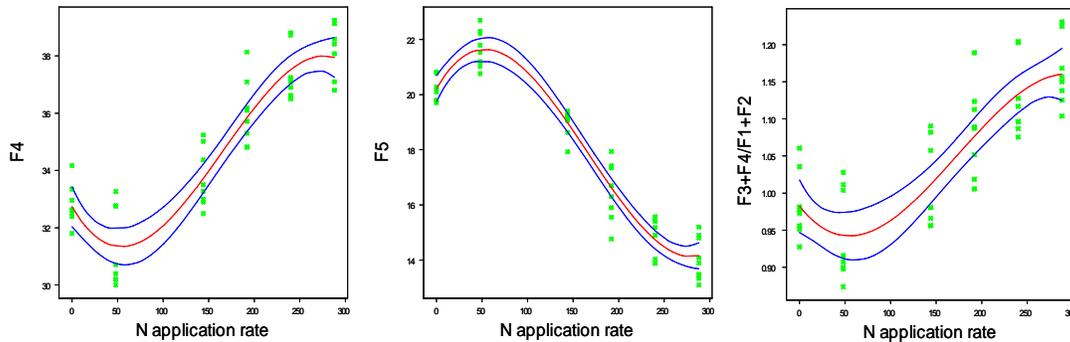

Figure 6 Regression analysis of SE-HPLC fractions with nitrogen application rate (presented in kg N ha^{-1}). Samples are from the Broadbalk experiment. F4, α-, β- and γ-gliadins; F5, albumins and globulins; F3+F4/F1+F2, ratio of gliadin to glutenin.

The SE-HPLC analysis revealed a strong positive relationship between increasing nitrogen application rate and the % area of the F3 and F4 fractions (representing the ω-gliadins and α-, β- and γ-gliadins respectively) (F3, p < 0.001, correlation coefficient (r) = 0.80; F4, p < 0.001, r = 0.87). In contrast the % area of the F5 fraction (albumins and globulins) decreased significantly with increased nitrogen application rate (p < 0.001, r = -0.92), showing that the levels of the albumins and globulins, which are metabolic and structural proteins in the endosperm, did not increase at the same rate as the gliadins and glutenins, which are storage proteins (Figure 6).

Similar changes in protein composition were also shown using SDS-PAGE; however, there was an increase in the HMW glutenin subunit content of the flour as well as increased gliadin proteins.

These results are in agreement with other studies that showed increases in the proportion of gliadins to glutenins with increasing nitrogen application rate, accompanied by decreases in proportion of albumins and globulins (DuPont et al., 2006; Gupta et al., 1992; Wieser and Seilmeier, 1998). However, in these reports the increased gliadin content arose

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primarily from increases in ω-gliadins, whereas the data presented here shows increases due to the levels of the α-, β- and γ-gliadins. The change in protein composition with increased nitrogen fertiliser application rate is usually ascribed to increases in the nitrogen to sulphur ratio that favour the formation of sulphur-poor rather than sulphur-rich proteins (DuPont et al., 2006; Zhao et al., 1999). However, this does not explain the results presented here that show increases in the sulphur-rich gliadins as the nitrogen to sulphur ratio increases.

Dough functionality

The results from the analysis of dough functionality show an increase in dough strength with increases in nitrogen application rate as shown by greater stability, development time, extensibility and maxcentre and reduced Rmax, degree of softening and maxtime. This increase in dough strength is expected with increased protein content of the flour.

Metabolomic analysis of the white flour

The NMR analysis of the Broadbalk samples revealed a significant effect of year on the metabolome of the grain, caused principally by changes in the levels of maltose, sucrose and glycine betaine. This effect was more significant than the effect of the different nutritional treatments and was most likely related to the different weather conditions in the three years.

The free amino acids alanine, aspartate, isoleucine and valine all increased significantly in concentration in response to increased nitrogen application rate ($p < 0.05$). In contrast, glycine betaine, maltose and sucrose showed increases in concentration with nitrogen application rates up to N3-N4 levels, followed by decreases up to N6 (Figure 7). The average effect of treatment over the three years does not take into account the year to year variation in the results, which was significant ($p < 0.001$).
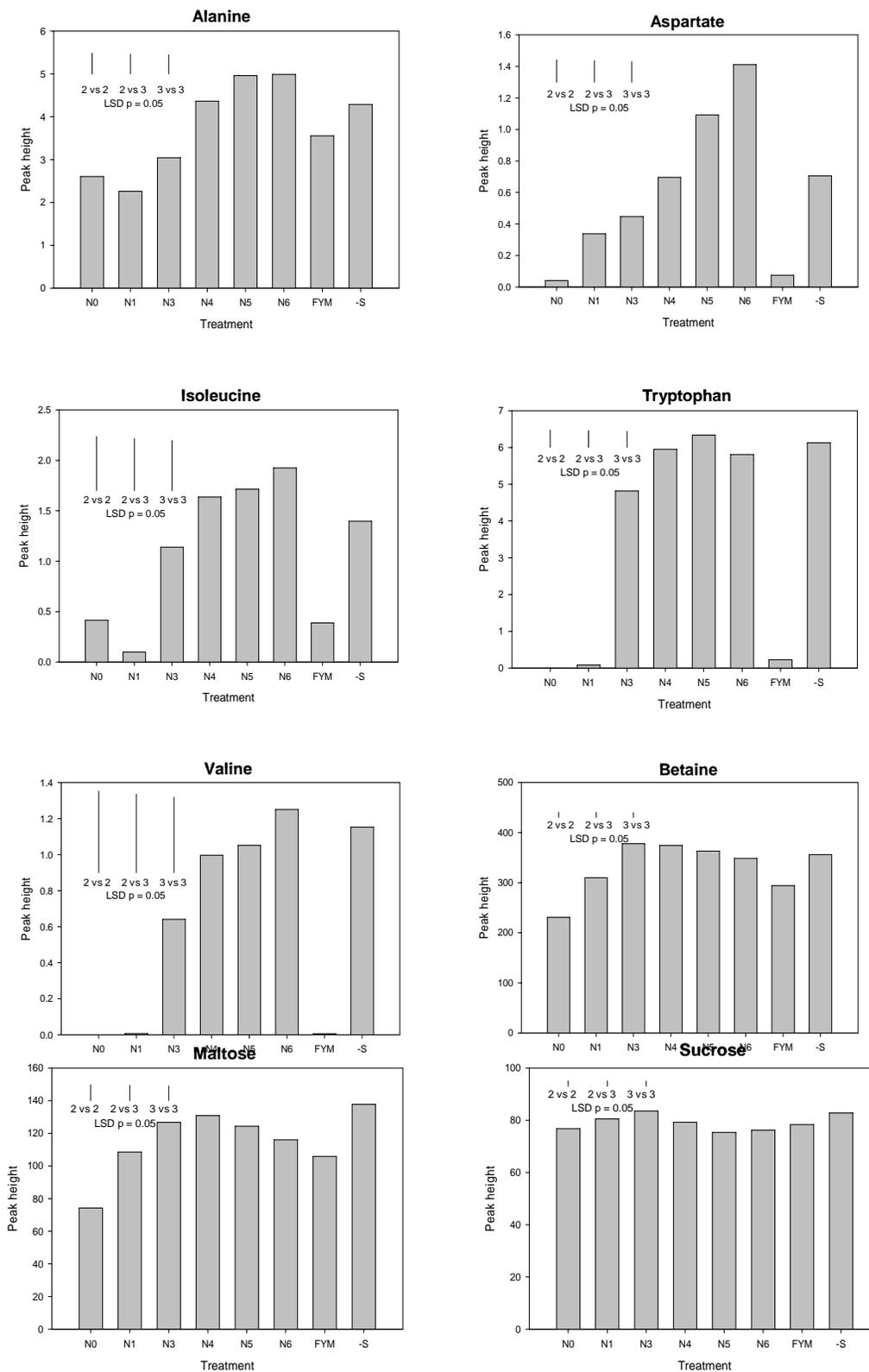
Figure 7 Bar charts show the mean average metabolite levels in each treatment across the three years of the study (2005-2007). In 2005 there were only 2 replicates for treatments N0, N3, N5 and N6, in all other cases there were three replicates. Least significant differences (LSDs) refer to this disparity in replication. Peak height values are relevant for treatment comparisons for a given metabolite but not for inter-metabolite comparisons.

These results show that increasing the available nitrogen to the plant not only increases the total white flour nitrogen concentration and protein content, but also the concentration of free amino acids in the white flour. This suggests that higher applications of nitrogen fertiliser increase the pool of labile nitrogen in the plant and that ultimately more of this persists as free amino acids in the mature grain endosperm. In particular it is interesting to see the strong positive relationship between nitrogen application rate and levels of free aspartate. Aspartate is a major transported amino acid and much of the nitrogen transported to the grain probably arrives in this form and as glutamate (Hayashi and Chino, 1986; Lea and Ireland, 1999).

Transcriptomic analysis

Samples of developing endosperm tissue and white flour from the plants treated with FYM and N4 were compared using transcriptomic analysis. These two treatments were chosen as they most closely reflect the different nutritional treatments used by farmers to produce breadmaking wheat in organic and conventional systems respectively. 12,320 genes varied significantly in expression in the developing endosperm over the period between 14, 21 and 28 days post anthesis (dpa). This list revealed that the expression of genes involved in cell division and protein synthesis decreased over this time period whilst the expression of genes involved in defence and stress responses increased (Figure 8). This result agrees with other studies that have studied wheat grain development using both micro-arrays and serial analysis of gene expression (SAGE) (McIntosh et al., 2007; Wan et al., 2008). The increase in the proportion of genes involved with stress and defence related roles shows agreement with the high levels of glycine betaine in the white flour as shown by the metabolomics data.

A total of 762 genes also differed significantly in expression between the two nutrient treatments. The majority of these genes were more highly expressed in the FYM treated samples than in the N4 treated samples, including 14 transcription factors. Some of these transcription factors have been shown to increase in response to pathogen attack. Several auxin-

responsive proteins that have been linked to senescence processes were also more highly expressed in the FYM samples. The increased expression of these elements combined with overall increases in the proportions of expressed genes involved in storage protein synthesis and with stress and defence-related roles indicates that the FYM samples represent a later stage in grain development than the N4 samples. This may be related to nitrogen status, as it is known that higher nitrogen availability can delay senescence and this in turn may extend grain development (Gooding and Davies, 1997). Such an effect would be expected to result in extended grain development in the N4 treated plants as these have higher nitrogen status. However, this explanation does not satisfactorily account for the greater number of genes being more highly expressed in the FYM treated samples.

Comparison of the FYM treatment with the nitrogen application rate series

The nitrogen application rate series provided important baseline data against which to compare data from other treatments and experiments. The flour from the FYM treatment on Broadbalk was most similar to that from the inorganic N3 treatment in terms of protein content and composition and dough functionality (Figure 9). However, in terms of metabolite profile, the flour from the FYM treatment was more similar to the low nitrogen treatments such as N1 (48 kg N ha^{-1}). This suggests that the FYM treated crop used the nitrogen liberated by senescence in a more efficient manner and converted a greater proportion into storage proteins. This small difference in metabolism is unlikely to have much of an effect on the overall crop performance or white flour quality; particularly as the free amino acid fraction accounts for such a small proportion of the total grain nitrogen content (approximately 0.12% of total nitrogen in the endosperm (Pomeranz, 1988)).
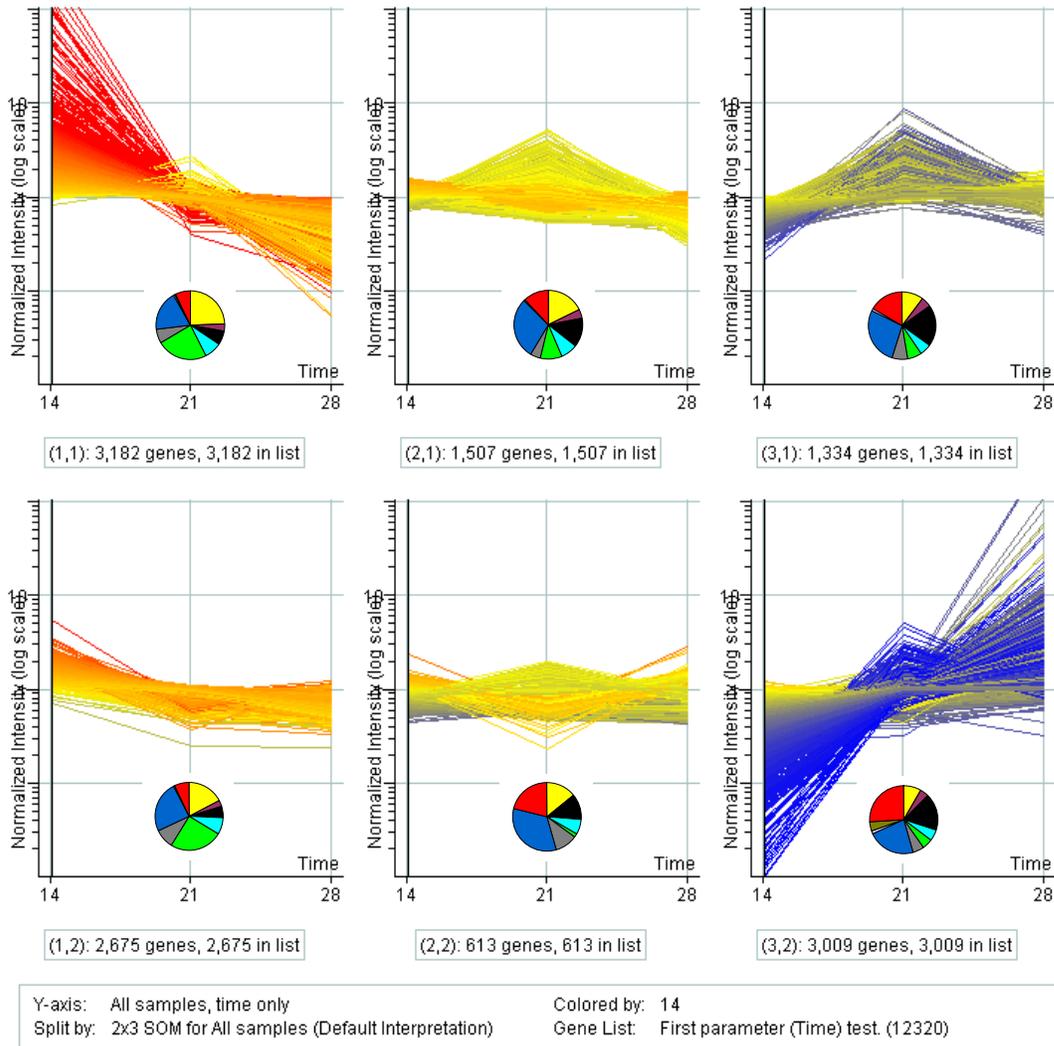
Figure 8 Clustering of genes with similar expression patterns using the Self Organising Map algorithm. The starting gene list was genes changing significantly with time in both the FYM and N4 treated samples. Graphs show normalised gene expression versus days after anthesis. Pie charts show the proportion of genes with specific functions in each cluster.
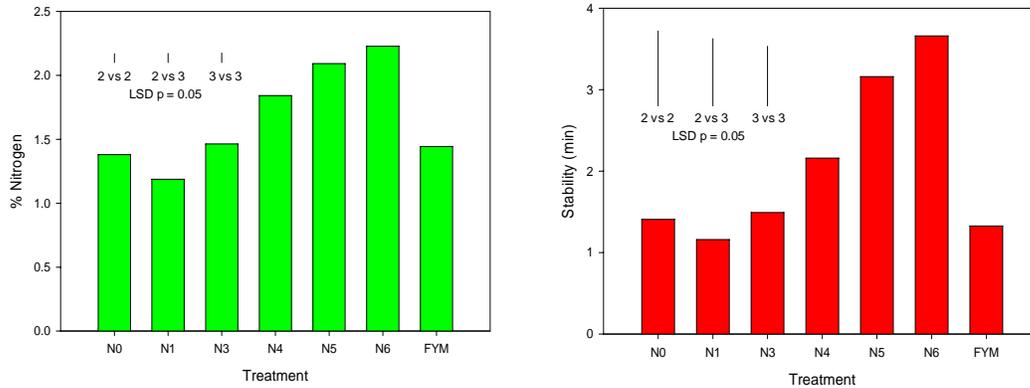
Figure 9 Bar charts show the % nitrogen in the white flour and dough stability as measured on the Farinograph. Note the similarity between the FYM treatment and the N3 and lower nitrogen treatments. In 2005 there were only two replicates for treatments N0, N3, N5 and N6. LSDs refer to this disparity in replication.

The application of 35 tonnes per hectare (t ha^{-1}) FYM delivers approximately 250 kg N ha^{-1}, but much of this nitrogen is in organic forms that are not readily available to the plant (Poulton, 2008). It is estimated that the proportion of nitrogen that is NO_3^- or NH_4^+ in the FYM is approximately 1-2%. In addition the application of FYM must be made in the autumn before planting, unlike the inorganic fertiliser applications that can be made in the spring when most benefit will be gained in terms of yield and grain nitrogen concentration. During the winter months up to 25% of the total nitrogen in the FYM application is leached, this is because of high rates of organic nitrogen mineralization that are poorly utilized by the crop at this early stage in development (Goulding et al., 2000). The FYM treated crop has a lush appearance and a high yield potential due to this abundant nitrogen early in development, but later the availability of nitrogen is not sufficient to fulfil this potential and consequently the grain quality and yield are affected. The combined influence of these effects is that the grain nitrogen content of the FYM treated samples is most similar to a sample that has received 144 kg inorganic N ha^{-1} (N3) rather than 250 kg N ha^{-1}.

RESULTS & DISCUSSION – THE UREA EXPERIMENT

Clear effects of post-anthesis foliar urea treatment on the transcriptome, metabolome and breadmaking quality of the grain were observed. In this experiment, developing endosperm tissue was collected as well as white flour from the mature grain, and this enabled the nitrogen dynamics within the grain during development to be studied. It was clear that the effect of urea on the grain was similar to that of increasing the nitrogen application in the spring (as was determined from the nitrogen application rate series on Broadbalk). However, there were also some differences in the way in which this late nitrogen was utilised.

Nitrogen and protein concentration of the white flour

Urea application resulted in a significant increase in the nitrogen concentration of the flour ($p < 0.001$), which was similar in magnitude to that resulting from an equivalent amount of nitrogen applied in the spring, as shown in the Broadbalk data. The AT value also increased significantly with urea treatment ($p < 0.001$), demonstrating that the additional nitrogen was incorporated into protein.

Protein Composition

The composition of the grain protein was also affected by the application of urea. The F2 (comprising low molecular weight (LMW) polymers enriched in LMW subunits of glutenin), F3 (ω gliadins) and F4 (α-,β- and γ-gliadins) fractions separated by SE-HPLC all increased in response to the urea treatment, while the F5 (albumins and globulins) peak decreased. The F1 peak (comprising high molecular weight (HMW) polymers enriched with HMW subunits of glutenin) increased in response to urea in the N4 treatment but not in the FYM treatment. These effects of urea on protein composition are similar to those seen with increasing nitrogen application rates in the spring, as shown in the Broadbalk experiment. However, in the Broadbalk samples the F2 peak decreased with N rate rather than increased.

Overall, the gliadin to glutenin ratio (F3+F4)/(F1+F2) was significantly increased by the urea treatment ($p < 0.001$), as it was for the increase in spring nitrogen application rate (Figure 10).
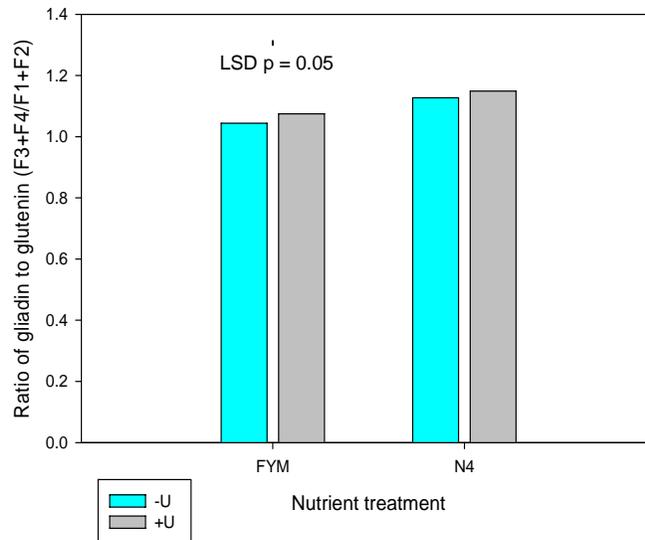

Figure 10 The effect of foliar urea treatment on the gliadin to glutenin ratio as estimated from the SE-HPLC analysis (F3+F4/F1+F2). The actual amounts of all protein groups increased in response to the urea treatment as shown by increases in the peak areas of the SE-HPLC separations and in the optical densities of the SDS-PAGE analyses.

Dough functionality

The data from the Reomixer analysis also supported the observation that the nitrogen from the urea treatment was incorporated into functional protein in the grain. The urea treated samples showed significantly increased maxcentre and significantly decreased maxtime when compared with the untreated controls ($p < 0.001$) (Figure 11). The same effect was seen in response to the increased nitrogen application rate in the spring, as shown in the Broadbalk experiment. This result confirms that nitrogen from late applications of foliar urea is not merely contributing to increased grain nitrogen concentration, but is being incorporated into protein that affects dough functionality and therefore breadmaking quality.
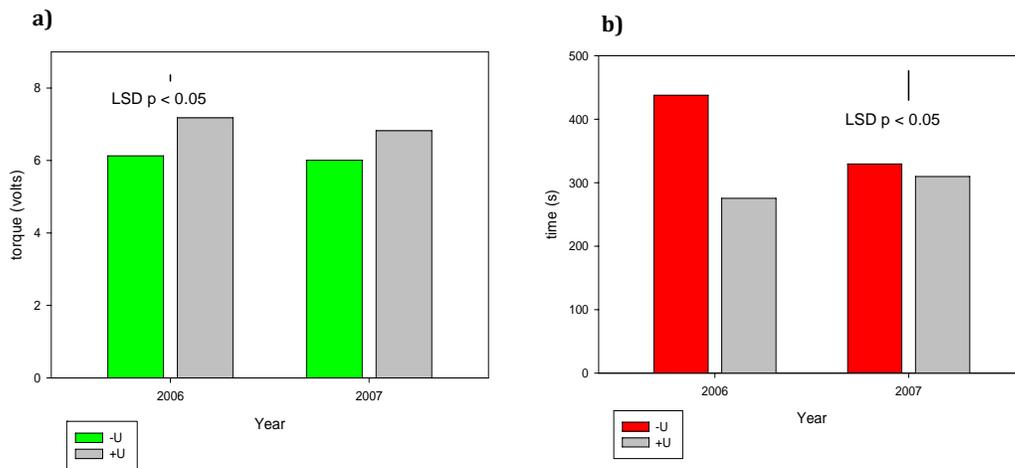
Figure 11 The effect of urea treatment on maxcentre (a) and maxtime (b) in different years, as measured by the Reomixer.

Metabolomic analysis of the developing endosperm and white flour

The metabolome of the developing and mature endosperm was analysed using NMR and GC-MS. Both analyses showed that the main influence on the metabolite profile was the developmental stage of the endosperm rather than the nutritional or urea treatments. A number of amino acids were present at significantly greater levels in the 14 dpa endosperm tissue compared with the 21 dpa endosperm tissue. The levels of glycine betaine were also higher at 14 dpa as were sucrose and maltose. The GC-MS data also showed that the amounts of all of the free amino acids decreased with time between 14 dpa and 21 dpa and between 21 dpa and maturity except for glutamic acid and tryptophan (Figure 12). These results suggest that the grain was progressing from a highly metabolic state, with nitrogen being delivered to the grain in the form of amino acids, to a state where many of the free amino acids and sugars were being incorporated into starch and protein and the grain was preparing for dormancy.

The second most important factor distinguishing between the samples in the NMR and GCMS analyses was the nutritional treatment. In both analyses samples the N4 samples had higher levels of many free amino acids at both 14 and 21 dpa than the FYM (Table 4). The levels of free amino acids in the white flour were also higher in the N4 samples, but the effect was not as great.

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In the NMR analysis, the effect of the urea treatment on the metabolite profile of the developing endosperm was not as great as either sampling time or nutritional treatment. However, the urea did lead to significant increases in the levels of some free amino acids and sugars in the endosperm.
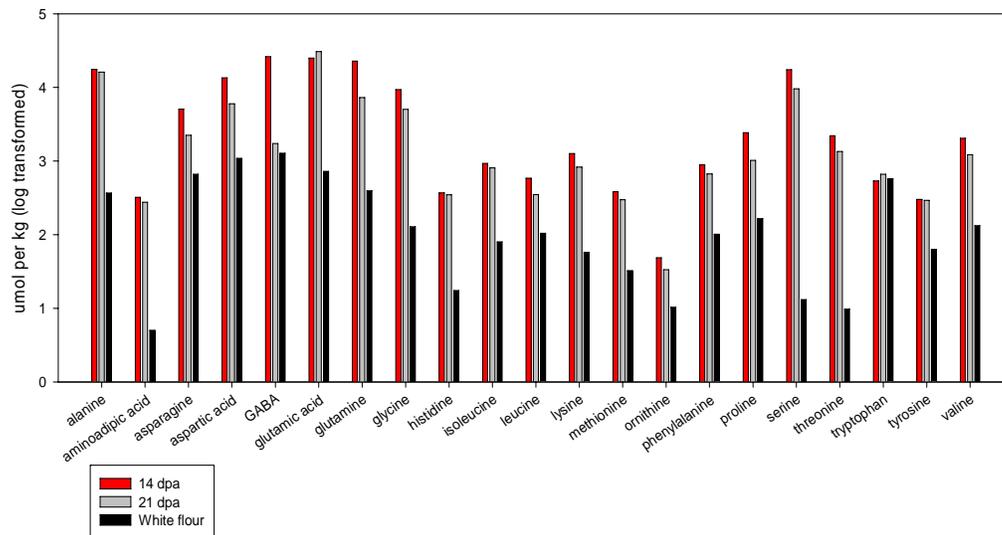
Figure 12 Concentrations of free amino acids in developing endosperms sampled at 14 and 21 dpa, and in white flour as measured by GC-MS. The data have been log_{10} transformed so that they can be easily represented in this figure (this prevents the presentation of estimates of variability such as LSD). The effect of time was significant for all amino acids at $p < 0.001$. Actual values for the concentration of free amino acids and corresponding LSDs are presented in Table 3.

Table 3 The concentrations of free amino acids in the developing endosperm at 14 and 21 dpa and in the white flour from the mature grain as measured by GC-MS. Data are presented in μmol kg^{-1}.

Free amino acid	14 dpa endosperm	21 dpa endosperm	White flour	p value	LSD
alanine	17551.1	16113.9	367.6	> 0.001	523.90
aminoadipic acid	322.4	275.5	5.0	> 0.001	25.48
asparagine	5058.0	2236.9	659.9	> 0.001	451.20
aspartic acid	13511.7	6001.3	1085.9	> 0.001	655.10
GABA	26128.7	1727.9	1272.8	> 0.001	1164.00
glutamic acid	24958.9	30632.0	724.5	> 0.001	1885.00
glutamine	22761.2	7274.9	395.0	> 0.001	1362.00
glycine	9335.3	5037.3	127.9	> 0.001	243.70
histidine	370.1	348.0	17.5	> 0.001	29.43
isoleucine	929.8	806.7	80.0	> 0.001	69.89
leucine	585.2	351.2	104.5	> 0.001	39.54
lysine	1265.2	827.9	57.4	> 0.001	61.56
methionine	382.7	298.3	32.5	> 0.001	17.95
ornithine	48.4	33.5	10.3	> 0.001	2.86
phenylalanine	885.9	665.8	101.1	> 0.001	34.44
proline	2419.4	1018.6	164.8	> 0.001	135.30
serine	17372.7	9560.6	13.1	> 0.001	728.60
threonine	2198.7	1350.2	9.7	> 0.001	93.29
tryptophan	535.0	663.4	575.0	> 0.021	92.03
tyrosine	301.5	293.1	63.2	> 0.001	23.68
valine	2036.1	1221.0	132.3	> 0.001	63.43

Table 4 Free amino acids significantly higher (p < 0.05) in FYM or N4 treated endosperm samples at 14 or 21 dpa as measured by GC-MS.

Significantly higher in N4 (p < 0.05)		Significantly higher in FYM (p < 0.05)	
14 dpa	21 dpa	14 dpa	21 dpa
alanine	alanine	lysine	None
aminoadipic acid	asparagine	tyrosine	
asparagine	glutamic acid		
aspartic acid	glutamine		
GABA	glycine		
glutamic acid	histidine		
glutamine	isoleucine		
glycine	leucine		
isoleucine	methionine		
leucine	ornithine		
methionine	phenylalanine		
ornithine	serine		
proline	threonine		
serine	tryptophan		
threonine	tyrosine		
tryptophan	valine		
valine			

GC-MS analysis did not show a significant effect of urea on the levels of any free amino acids in the white flour; however, the levels of many free amino acids, including alanine and valine, increased in response to urea in the developing endosperm samples (Table 5). In contrast, tryptophan and aminoadipic acid decreased in the developing endosperm. Several amino acids showed interactions between urea treatment and year. Valine, leucine, isoleucine, phenylalanine, tyrosine and histidine increased in response to urea in 2007 but decreased in response to urea in 2006. In contrast, aspartic acid and glutamic acid showed the reverse relationship and increased in response to urea in 2006 but decreased in response to urea in 2007. It is not known why there should be an interaction effect between weather conditions and urea treatment in this way, but it could

be related to the effects of temperature and water availability on the metabolism of carbon and nitrogen, as has been discussed previously.

Table 5 Amino acids changing significantly with urea treatment in the developing endosperm only. Only those amino acids changing consistently in both years are reported here.

Increasing with Urea	Decreasing with Urea
Alanine ($p < 0.001$)	Aminoadipic acid ($p < 0.001$)
GABA ($p < 0.001$)	Tryptophan ($p < 0.001$)
Glutamine ($p < 0.001$)	
Glycine ($p < 0.001$)	
Leucine ($p < 0.001$)	
Methionine ($p < 0.001$)	
Proline ($p < 0.001$)	
Serine ($p < 0.002$)	
Threonine ($p < 0.001$)	

Overall trends in the metabolite data collected using GC-MS and NMR spectroscopy, showed that levels of free amino acids in the endosperm decreased as the grain developed, but were greater when nitrogen was applied either in the spring or after anthesis. It is possible that the increased nitrogen availability reduced the rate of senescence in the plants treated with N4 or urea but further experiments would be needed to show whether this occurred and if so whether it was correlated with the differences in metabolite levels occurring during developmental.

Transcriptomic analysis

The results of the transcriptomic analysis were in agreement with the other datasets, showing that the effect of the nutritional treatment on the samples (ie. N4 vs FYM) was greater than the effect of the urea treatment. 1056 genes varied significantly with nutritional treatment and 983 of these were more highly expressed in the FYM samples than in the N4 samples. No genes varied significantly in expression level with urea treatment, however, a separation of the urea treated and control plants was observed when the samples were clustered into a condition tree,

suggesting that there was an effect of the treatment but at a level too low to be detected by the statistical test. Data from additional years and a greater number of biological replicates would provide further information on the effects of the urea on the transcriptome.

Organic and Conventional comparison

The analysis of the conventional and organic samples clearly showed that the conventional samples had a higher nitrogen status than the organic samples. This was apparent in the results of all of the analyses when they were considered in the context of the nitrogen application rate series from the Broadbalk experiment. This is in agreement with the results of other surveys of the nitrogen content of organic wheat (Samuel and East, 1990), and is consistent with the fact that it may be difficult to obtain high protein breadmaking wheat using organic cultivation practices in the UK.

Nitrogen and protein concentration of the white flour

The nitrogen concentrations of the conventional flour samples were significantly higher than those of the organic flour samples ($p < 0.001$). This was also confirmed by determination of wet gluten contents and by SE-HPLC (Figure 13). Comparison of the conventional and organic flours with the flours from the nitrogen application rate series on Broadbalk showed that the organic flours were most similar to the N3 and N4 treatments from Broadbalk whereas the conventional flours were most similar to the N5 treatment.

The nitrogen to sulphur ratio was significantly higher in the conventional flour samples ($p < 0.002$). The flours from the 2007 harvest had significantly higher nitrogen to sulphur ratio than the 2006 flours ($p < 0.001$), which could have resulted from the wetter weather conditions during the early summer of 2007. Wet conditions can increase the uptake of nitrogen leading to increased nitrogen yield, although the nitrogen concentration of the flour is likely to fall because of greater carbohydrate accumulation. This increased nitrogen uptake could lead to a change in

the nitrogen to sulphur ratio in the flour if sulphur uptake is not also increased.
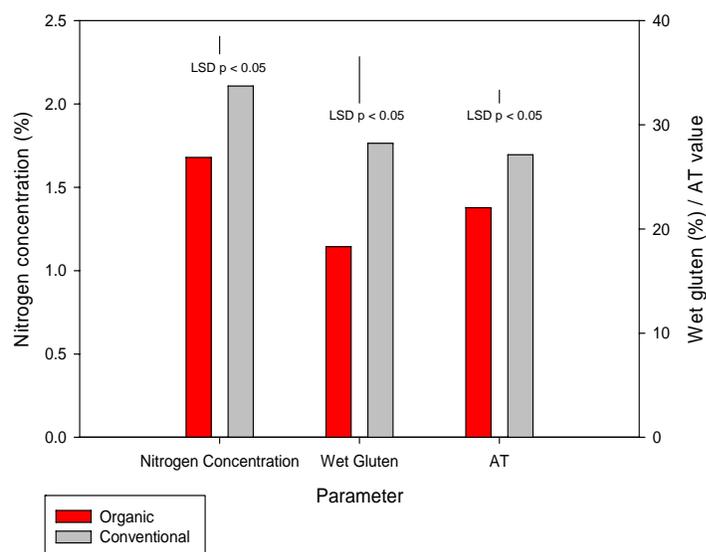

Figure 13 Mean values of nitrogen concentration, wet gluten content and AT for Organic and Conventional flour samples collected from across the UK in 2006 and 2007.

Protein Composition

The protein compositions of the conventional and organic flours also reflected the difference in nitrogen status with the conventional flours having a higher average ratio of gliadins to glutenin polymer (F3+F4/F1+F2) than the organic flours. However, there was large variation in the organic sample sets from both years and some organically grown samples were similar in protein composition to the conventional samples. This indicates that whilst the average organic sample generally had low nitrogen status, some farmers were succeeding in producing organically grown Hereward of good breadmaking quality.

The conventional flours had higher total amounts of all proteins determined by SE-HPLC and SDS-PAGE (Figure 14). The proportion of HMW glutenin subunits was lower in the conventional samples than in the organic samples whilst the proportion of LMW glutenin subunits was higher. This is the opposite of the pattern that may be expected from comparison with the Broadbalk nitrogen application rate series, in which higher nitrogen status crops had higher proportions of HMW glutenin

subunits and lower proportions of LMW glutenin subunits. Comparison of the actual peak heights of the different fractions from the SE-HPLC indicated that the organic flours were most similar to the N4 treatment from Broadbalk and that the conventional flours were most similar to the N5 and N6 treatments.
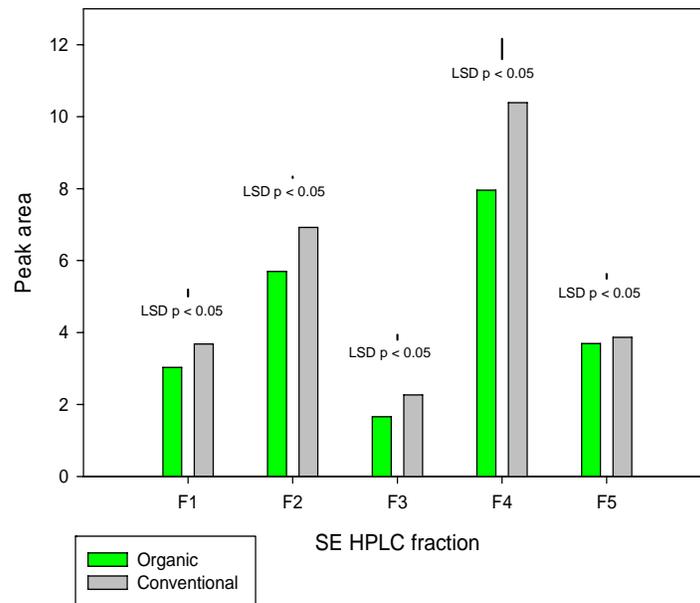

Figure 14 Mean values for total peak area of different SE-HPLC fractions, samples are organic and conventional flours sampled in 2006 and 2007. F1; HMW polymer enriched in HMW subunits of glutenin. F2; LMW polymer enriched in LMW subunits of glutenin. F3; ω gliadins. F4; α, β and γ gliadins. F5; albumins and globulins.

Dough functionality

The results of the dough functionality studies supported the conclusion that the conventional flours had a higher nitrogen status than the organic flours, clearly showing that the conventional flour produced stronger dough. This was evident in the greater stability, development time, extensibility, water absorption and maxcentre and in the lower degree of softening and maxtime (Table 6). Interestingly, the organic flours were most similar in performance to the N4 – N6 treatments from the Broadbalk experiment and the conventional flours were considerably stronger than any of the treatments from the Broadbalk experiment. This is an interesting result considering the similarity between the organic and

N4 treatment and the conventional and N5 treatment in terms of protein content and composition.

Table 6 REML analysis of functional parameters of white flour from organically and conventionally produced wheat (2006 and 2007).

	Mean		LSD ($p < 0.05$)	F probability
	Organic	Conventional		
Water Absorption (%)	47.94	50.79	1.515	0.001
Development Time (min)	1.78	3.06	0.650	0.001
Stability (min)	2.72	4.39	1.184	0.009
Degree of softening (BU)	163.90	134.40	44.490	0.180
Rmax (BU)	496.20	495.00	57.350	0.964
Extensibility (cm)	14.51	18.53	2.647	0.006
Maxcentre (volts)	6.30	7.91	0.561	0.001
Maxtime (min)	7.08	4.73	1.202	0.001

Metabolomic analysis of the white flour

The NMR data showed similar differences between the organic and conventional samples as between the low nitrogen and high nitrogen treatments from Broadbalk. In both years the conventional samples had higher levels of free amino acids and lower levels of sugars such as sucrose and maltose, than the organic samples (Figure 15). This further supports the conclusion that the conventional samples generally had a higher nitrogen status than the organic samples. It was clear from these data and from the results of the other analyses that there was greater variability among the organic samples than among the conventional samples. This reflects the variety of different cultivation practices that are employed in the production of organic wheat as opposed to the relatively consistent practices used in conventional production.
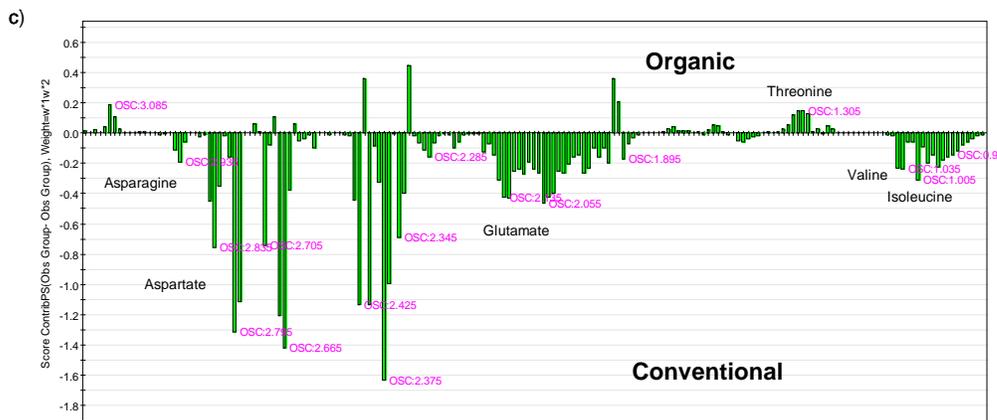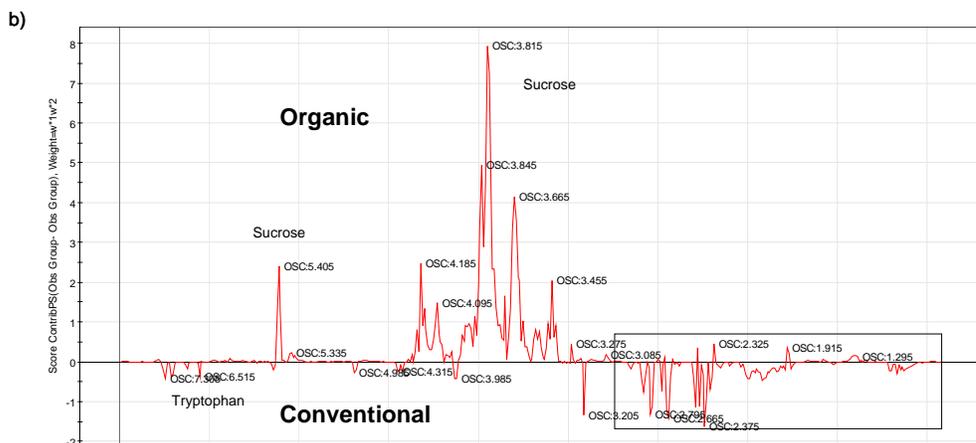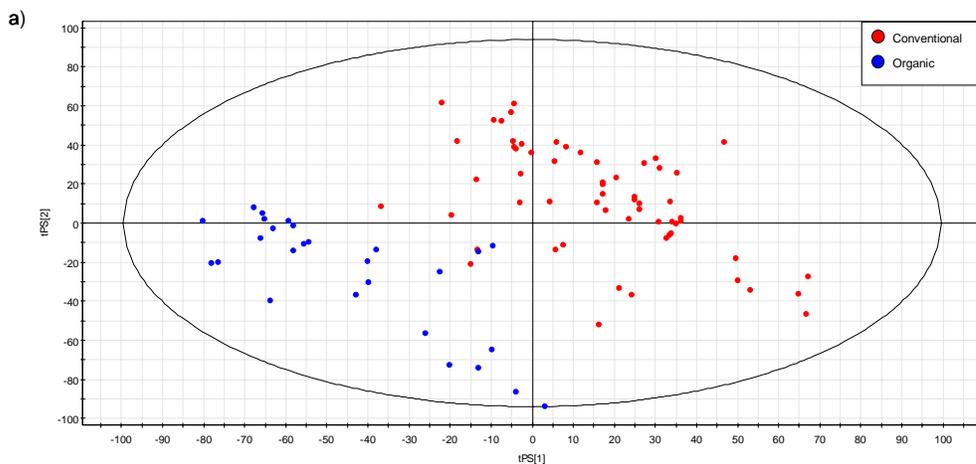
Figure 15 Partial least squares analysis (PLS) of NMR data from conventional and organic flour samples following orthogonal signal correction (OSC) filtering (2006 samples), training and validation data from the OSC model were included in the prediction set used to create this PLS plot. 44.3 % of sums of squares were retained by the OSC filter; the predicted scores 1 (tPS1) R2X = 0.286; tPS2 R2X = 0.175. a) PLS plot showing separation of conventional and organic sample groups. b) Scores contribution plot for separation of conventional and organic groups in the PLS, showing areas of the trace that contributed to the separation of the two treatment groups. c) Detail of the aliphatic region of the contribution plot highlighted in b) showing higher levels of most free amino acids in the conventional samples.

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These results reflect the acknowledged difficulty of growing breadmaking quality wheat, to meet UK standards, using organic methods (DEFRA, 2003; Starling and Richards, 1990). However, it is worth stressing again that this project has only considered the variety Hereward and in fact many organic farmers have now changed to growing the spring variety Paragon, which reportedly gives higher protein content under organic practices.

GENERAL CONCLUSIONS

The set of approaches utilised in the project has revealed impacts of nutrition on the transcriptome, metabolome, protein composition and end use quality of wheat grain for breadmaking.

The results from the Broadbalk experiment showed that a number of parameters were associated with nitrogen application rate. In particular, increasing the level of inorganic nitrogen applied to the crop increased the nitrogen concentration and protein content of the white flour, increased the gliadin to glutenin ratio, increased the levels of free amino acids and altered dough functional properties leading to overall dough strengthening. The use of a large range of nitrogen application rates enabled the identification of clear correlations with these parameters and also demonstrated that for very low and very high application rates the relationship was often non-linear. Many of the effects of increasing nitrogen rate on wheat quality are well known, but the use of the Broadbalk experiment revealed particularly clear relationships between the nitrogen rate and the various parameters measured.

For most of the parameters analysed, the FYM treatment on Broadbalk was most similar to the inorganic N3 treatment (144 kg N ha^{-1}). The FYM treatment consists of 35 t ha^{-1} applied to the soil in the autumn, which contains approximately 250 kg nitrogen in total (Poulton, 2008). The fact that such a large amount of nitrogen in the form of FYM is equivalent to a much smaller quantity as inorganic nitrogen is most likely due to the fact that much of the nitrogen in the FYM is in the form of organic compounds that are not readily available to the plant and because the supply of

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nitrogen to the crop does not match demand throughout development due to the early nature of the application. This was revealed in effects on the development of the plants; the FYM treated crop grew well early in the season but fell left behind the N4 treated crop when the inorganic fertiliser was applied in the spring.

Further comparisons were made between the N4 and FYM treatments on Broadbalk because these are most representative of the nutritional practices used by farmers growing breadmaking wheat in the UK. As expected, this comparison revealed that the N4 treatment resulted in a higher nitrogen status crop than the FYM treatment with greater white flour protein content, characteristic changes in protein composition and free amino acid content, and increased dough strength.

Analysis of the transcriptome revealed that the expression of a number of genes differed between the FYM and N4 treated samples, 60% of these genes were more highly expressed in the FYM treatment. However, a comparison of the differently expressed genes did not reveal any pronounced association between gene function and expression except that a higher proportion of the genes that were up-regulated in the FYM samples were involved with stress and with storage protein synthesis. It is possible that the difference between the FYM and N4 samples occurred because the N4 samples senesced more slowly because of their higher nitrogen status.

Late foliar urea (at approximately growth stage (GS) 75) may be applied by conventional farmers to increase the nitrogen concentration of their grain. The results presented here show that this affects the properties and quality of the grain in a similar way to application of additional nitrogen in the spring (i.e. at the time when the standard application of nitrogen is made to the Broadbalk plots). The effects of the late foliar urea treatment were similar to the effects of the spring application of ammonium nitrate for all of the parameters analysed. This suggests that nitrogen applied as a late foliar urea spray, is transported to the developing grain together with nitrogen that is released from the vegetative parts of the plant during

senescence and remobilisation. The application of late foliar urea therefore represents a useful way of increasing the quality of breadmaking wheat at a late stage in development. However, there is a limit to the amount of nitrogen that can be delivered in this way due to the risk of leaf scorch at high concentrations of urea, and clearly nitrogen must also be supplied to the plant much earlier in development in order to produce a healthy crop with high yield potential.

Late foliar urea also increased the quality of the FYM-treated crop, indicating that if an organic alternative to urea could be identified it would be capable of improving the breadmaking quality of organically-grown wheat.

In general the organic and conventional samples collected from farms across England showed similar differences to those between the Broadbalk FYM and N4 treatments. This indicates that nutrition strongly affects the quality of organic and conventional wheat for breadmaking, and suggests that this is principally due to nitrogen availability. The higher nitrogen status of the conventionally grown samples was evident from their higher contents of protein and free amino acids; their higher gliadin to glutenin ratio and their increased dough strength. However, it should be pointed out that there was considerable variability in the dough properties of the organic samples, with some being similar to the conventionally grown wheat. In contrast, the conventional samples were less varied.

Table 7 Summary table of effects of different treatments on the different parameters measured.

Parameter	Effect of		
	Increasing N rate	Post-anthesis foliar urea treatment on grain parameters	Conventional cultivation on grain parameters compared to organic cultivation
Nitrogen concentration	Higher	Higher	Higher
Wet gluten content	Higher	No significant effect	Higher
SE-HPLC trace area (AT)	Higher	Higher	Higher
Nitrogen to Sulphur ratio	Higher	No significant effect	Higher
Gliadin to glutenin ratio	Higher	Higher	Higher
Development time	Higher	*	Higher
Degree of softening	No significant effect	*	No significant effect
Stability	Higher	*	Higher
Water Absorption	Higher	*	Higher
Extensibility	Higher	*	Higher
Rmax	No significant effect	*	No significant effect
Max centre	Higher	Higher	Higher
Max time	Lower	Lower	Lower
Free amino acid content	Higher	Higher for most amino acids	Higher
Sucrose content	Increased up to N3 then decrease	*	No significant effect
Maltose content	Increased up to N3 then decrease	*	Lower

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