UNIVERSITY OF SOUTHERN QUEENSLAND

Measurement of Barley (*Hordeum vulgare*) feed quality parameters *In Sacco* and mapping of associated Quantitative Trait Loci (QTL) in Cattle

A Dissertation submitted by

Peter Wolfgang Gous

Bachelor of Science

For the award of

Master of Science

2009

Abstract.

Barley (*Hordeum vulgare*) is a major feed source for the livestock industry. Its competitiveness against other cereal grains such as wheat, oats, maize and grain sorghum depends largely on its price per unit of expressed feed quality. The traits which contribute to feed quality in barley are largely quantitative in nature but little is known about their genetic control and few studies have attempted to identify these quantitative trait loci (QTL).

A study to identify the QTLs associated with feed quality was performed using a F6 – derived recombinant inbred barley population from a Tallon x Scarlet cross. 117 unique lines from the Tallon x Scarlett population, for which a genetic map is available, were used. Samples from each line were incubated for three hours in the rumen of fistulated cattle, recovered, washed and dried for analysis. Both the original samples (pre-) and the post-digestion residue were analysed for key grain traits of feed quality, namely acid detergent fibre (ADF), starch, protein and *in sacco* dry matter digestibility. Analysis was performed using both analytical chemistry and NIR techniques.

The phenotypic results and data were used to identify genomic regions (QTL) associated with these traits. Putative QTLs were found on chromosome 2H, 3H, 5H and 7H. However, numerous suggestive QTLs were found throughout the barley genome. Genetic markers that define these QTL will be an effective tool for the selection and improvement of feed barley in the future. Additionally the research showed that the development of NIR calibrations appropriate for the detection of post-digestion nutrient measurement is essential for its establishment as a rapid, non-destructive feed quality measurement technique. This study also validated the ability of these QTL analyses to be performed under Australian conditions and with local feed grains and animals.

Declaration

I certify that the ideas, experimental work, results, analyses, software and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

______________________________ ____________________

Signature of Candidate Date

Peter Wolfgang Gous

ENDORSEMENT

______________________________ ____________________

Signature of Supervisor/s Date

Professor Mark Sutherland (USQ)

______________________________ ____________________

Signature of Supervisor/s Date

Dr. Glen Fox (DEEDI)

Acknowledgements

I commenced this Masters project in 2007 at the University of Southern Queensland's, Centre for Systems Biology under the joint supervision of Professor Mark Sutherland (USQ) and Dr. Glen Fox (DEEDI). This project was funded by the GRDC and by the former Queensland Department of Primary Industry currently the Department of Employment, Economic Development and Innovation (DEEDI).

I would like to thank members of the Centre for Systems Biology for their guidance and support over the last three years. I am particularly grateful to Dr. Bill Bovill and Dr. Anke Lehmensiek, for all their aid in getting settled and established in the laboratory. Their mentoring, encouragement and training helped in the completion of this research project. I would also like to thank Noel Knight for his aid in proof reading the thesis chapters.

I would like to thank members of DEEDI; Dr. Emma Mace and Dr. Bert Collard, for providing the genetic material, molecular map and a starting point for the molecular research of this project. Alison Kelly, biometrician with DEEDI, provided expert advice in the construction of the experimental design and data analysis. I would further like to extend my gratitude to Donna Hocraft and Jim Kidd for all their aid in sample preparation for the phenotypic trial.

To my supervisors Professor Mark Southerland and Dr. Glen Fox, thank you for your highly appreciated guidance, encouragement and active support which made this project possible. I would like to sincerely thank Professor Sutherland for giving me the opportunity to conduct this research and further my education.

To my family and friends thank you for being my constant and eternal support system. To all those that I may have neglected to mention my sincere apologies, and thank you for all your help with this project.

Contents

Abstract. ii
Declaration iii
Acknowledgements iv
Contents vi
Tables viii
Figures viii
Chapter 1 1
1. Literature review 1
1.1 History and Taxonomy 1
1.2 Types of barley 2
1.3 Production and breeding 5
1.4 Uses of barley 6
1.5 Requirements for animal feed 8
1.5.1 Ruminal nutrition and metabolism 9
1.5.2 Fibre 12
1.5.3 Starch 13
1.5.4 Protein 15
1.6 Genetic improvement of barley feed quality 16
1.6.1 Genetic control of quality parameters 18
1.7 Identification of QTL for traits influencing barley feed quality 19
1.7.1 Markers 19
1.7.2 Mapping populations 25
1.7.3 Statistical tools for genetic mapping 27
1.8 Research objectives 29
Chapter 2 32
2. Cattle experimentation. 32
2.1 Introduction. 32
2.2 Materials and methods. 34
2.2.1 Animal selection and adaptation 34
2.2.2 Experimental design 35
2.2.3 Sample preperation 35

2.2.4 Sample incubation 36
2.2.5 Residue preparation 37
2.2.6 Statistical analysis 38
2.3 Results 39
2.4 Discussion 41
Chapter 3 43
3. Wet chemistry analysis of pre- and post-digestion barley grain samples. 43
3.1 Introduction 43
3.2 Materials and methods 44
3.2.1 Protein 44
3.2.2 Fibre 45
3.2.3 Starch 46
3.2.4 Statistical analysis 47
3.3 Results 47
3.4 Discussion 56
Chapter 4 60
4. NIR analysis on pre- and post-digestion barley grain samples. 60
4.1 Introduction 60
4.2 Materials and methods 62
4.3 Results 64
4.4 Discussion 70
4.4.1. ADF 71
4.4.2. Protein 71
4.4.3. Starch 72
4.4.4. General 72
Chapter 5 75
5. Molecular mapping 75
5.1. Introduction 75
5.2. Methodology 78
5.3. Results 79
5.4. Discussion 87
Chapter 6 90
6.1. General discussion, Future directions and Conclusions. 90

6.1.1. General Discussion ... 90
6.1.2. Future Directions ... 92
6.1.3. Conclusions ... 93
7. References. ... 94

Tables

Table 2.1: The average daily gain (ADG) of each animal used in the DMD trial. ... 39
Table 2.2: Sources accounting for variance and standard error within DMD data set. ... 41

Table 3.1: The correlation between sources of variance attributing to protein content and digestion. ... 49
Table 3.2: The variance obtained within the pre- and post-digestion protein content. ... 50
Table 3.3: The correlation matrix between the various feed quality traits; pre-, post-digestion and amount digested. ... 51

Table 4.1: Correlations between feed quality traits both pre- and post digestion for Tallon x Scarlett, as measured with NIR. ... 67
Table 4.2: Variance observed for the feed quality traits of interest in pre- and post-digestion samples across the Tallon x Scarlett population. ... 68

Table 5.1: Summary of QTL associated with feed quality traits in a Tallon x Scarlett population. The phenotypic data was derived through analytical chemistry as described in Chapters 2 and 3. ... 81
Table 5.2: Summary of QTL associated with feed quality traits in a Tallon x Scarlett population. The phenotypic data was derived through NIR analysis as described in Chapter 4. ... 82

Figures

Figure 1.1: Shows the labelled cross section of a grain of barley (Haard & Chism 1996). ... 3
Figure 1.2: A representation of the digestive pathway of ingested feed through the four stomachs of a ruminant (Farm 2005). ... 10
Figure 1.3: An illustration of the digestive and synthetic pathway of protein in the rumen. This includes the recycling of free nitrogen in the form of ammonia which is utilized by microbes for protein synthesis (DFID 2006). ... 16

Figure 2.1: The phenotypic distribution of the dry matter digestability (DMD) after an 3 hour incubation period within the rumen for lines in the Tallon x Scarlett population. ... 40

Figure 3.1: Distribution of protein content per dry basis of the sample grains expressed as a percentage of the total grain weight. ... 48

Figure 3.2: Distribution of protein digested from the sample lines in the Tallon x Scarlett population, which can also be used to provide estimates of increased protein content due to the presence of bacteria and animal enzymes. 49
Figure 3.3: The distribution plot of pre-digestion ADF content in the Tallon x Scarlett population. 52
Figure 3.4: The distribution plot of ADF content digested from the sample lines in the Tallon x Scarlett population. 52
Figure 3.5: The distribution plot of pre-digestion starch content of the Tallon x Scarlett sample population. 53
Figure 3.6: The distribution plot of Starch content digested from the sample lines in the Tallon x Scarlett population. 54
Figure 3.7: Bi-plot representing 46% variance of the data set explained. The variance of the bi-plot is broken down into two components found on the axis representing 25% (x-axis) and 21% (y-axis). The correlation between two points observed is derived by the determination of the *cosine* of ϑ. The distance between the two points is interpreted as the variance between the points. 55

Figure 4.1: Flow diagram, of sample preparation and areas of NIR analysis and measurements. 62
Figure 4.2: Pre- and Post-Digestion population distribution for the percentage ADF component across the lines of the Tallon x Scarlett population. 64
Figure 4.3: Pre- and Post-Digestion population distribution for the percentage Protein component across the lines of the Tallon x Scarlett population. 65
Figure 4.4: Pre- and Post-Digestion population distribution for the percentage Starch component across the lines of the Tallon x Scarlett population. 66
Figure 4.5: Correlation between pre- and post-digestion Starch content across the Tallon x Scarlett population, with the nuisance variables statistically manipulated within the NIR data set. 69
Figure 4.6: Bi-plot representing 81% variance of the data set explained. The variance of the bi-plot is broken down into two components found on the axis representing 59.7 % (x-axis) and 21.3% (y-axis). The correlation between two points observed is derived by the determination of the *cosine* of ϑ. The distance between the two points is interpreted as the variance between the points. 70

Figure 5.1a: QTL analysis for feed quality traits in Tallon x Scarlett F6-derived RIL population (Chromosome 1H – 3H). 85
Figure 5.1b: QTL analysis for feed quality traits in Tallon x Scarlett F6-derived RIL population (Chromosome 4H – 7H). 86

<u>Chapter 1.</u>

1. <u>Literature review</u>

1.1 History and Taxonomy

Taxonomically barley is classified within the grass family Poaceae or Gramineae, tribe Triticaeae and genus Hordeum. The Hordeum genus contains at least 30 species, with approximately three fourths of these perennial in nature. The genus is divided into three distinct groups with the majority of the Hordeum possessing a diploid genome (2n=14), while the remainder are either tetraploid (4n=28) or hexaploid (6n=42) respectively (Bothmer 1992).

Archaeological evidence shows the birth of agriculture to be in the Near East during the Neolithic era with barley, along with emmer and einkorn wheats, amongst the first cultivated crops. This evidence suggests that barley was being utilised by approximately 16000 B.C. in the Nile River Valley in Egypt (Harlan 1968; Wendorf et al. 1979). The centre of origin and diversity of cultivated barley is thought to have been the Fertile Crescent of the Middle East (Zohary & Hopf 1988). Evidence reported by Harlan (1968) suggests barley cultivation was subsequently widespread across Ethiopia, Tibet, Afghanistan and India, but commencing considerably later than that in the Middle East.

Cultivated barley shows close similarity to a group of wild and weedy barley genotypes, which are traditionally grouped as *Hordeum spontaneum* or *H. Vulgare* . However cultivated barley varieties were selected to have non-brittle heads which stay intact after ripening. Brittleness is controlled by a mutation on chromosome 3H in either one of two tightly linked genes Btr 1 or Btr 2 (Nilan 1964; Takahashi 1955). Archaeological remains dating from 7000 to

6000 B.C. show that the dominant variety of this period was a two-rowed form, while six-rowed barley did not become widespread until well after 6000 B.C. (Zohary & Hopf 2000).

In this modern era, barley is the cereal with the widest and most diverse geographical distribution. It is cultivated from Norway, inside the Arctic Circle, to tropical Mali and is grown from sea level to an altitude of 4480 metres on the high plains of the Ganges in the Himalayas (Körnicke 1885, 1985). The minimum temperature for germination lies between 3-4°C, while optimal growth occurs at about 20°C. The maximum temperature at which barley can flourish occurs between 28-30°C.

1.2 Types of barley

Due to its wide distribution and adaptation, barley possesses a wide diversity of agronomic and morphologic properties. The inflorescence is a spike consisting, on each side of the rachis, of many vertical rows of groups of three spikelets, with each spikelet containing a single floret. If all are fertile, three rows of grain can be perceived on either side of the rachis. This type of barley is commonly referred to as six-rowed barley (Ried & Wiebe 1968). Many representatives of this type have rather lax spikes, in which case the lateral spikelets overlap slightly and give the appearance of having only four rows of grain. In other varieties only the median of the three spikelets at each node contains a grain, while the other two sterile ones are represented only by the glumes and reduced lemma and palea. Single mutations involving two genes commonly control row type in barley. The row type is controlled by a single recessive gene vrs1 on 2H which expresses the six-rowed type, while lateral floret fertility and size is controlled by another single recessive gene on 4H (Nilan 1964; Tanno et al. 2002).

Barley is divided into two additional structural groups, being those that are awned or awnless. The awn is a slender, apically tapering extension of the glumes and/or lemma (Ried &

Wiebe, 1968). The rough awns are usually barbed or have hairs that angle toward the apex. However in some cultivars the awns are replaced by a trifurcate (three-forked) appendage called a hood, and these barleys are more commonly referred to as "Hooded" barleys. It is believed that the awns also play a functional role in photosynthesis and transpiration, (Briggs 1978), since the photosynthetic structures and tissues of the awns greatly add to the area of the head (Briggs 1978). Genetic control of the phenotypic expression of the awn involves more than five genes of which the most important ones are: *lks1* (awnless) on 2H, *lks2* (short awn) on 7H, *kap1* (hooded awn) on 4H, *lks5* (short awn) on 4H and *raw1* (smooth awn) on 5H (Bothmer 2003).

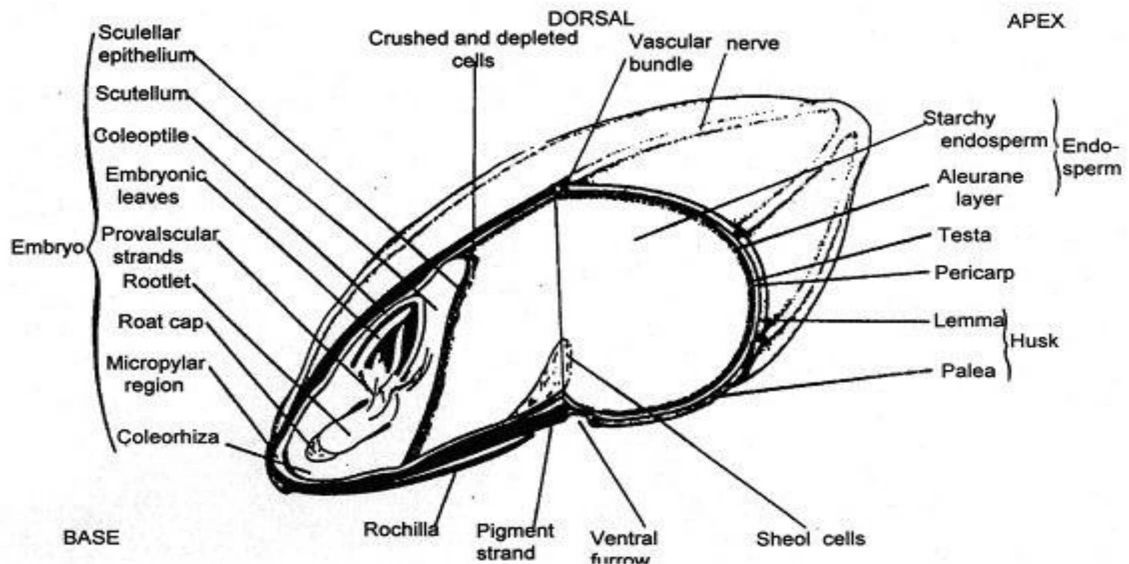

Figure 1.1: Cross section of a grain of barley (Haard & Chism 1996).

The barley kernel is a single seeded fruit called a caryopsis which is enclosed between the lemma and palea (Figure 1.1). The caryopsis consists of the pericarp, integuments, aleurone layer, endosperm and embryo (Ried 1985). The pericarp is a protective covering of the kernel, while the integuments are the outer cell layers enveloping the nucleus of the ovule (Figure 1.1). It is the integument that differentiates into the seed coat. Formed in the embryo sac after fertilization, the endosperm provides nutrition (starch, proteins) to the developing embryo. The surrounding aleurone layer contains protein bodies and enzymes involved with endosperm digestion (Briggs, 1978). In some barley cultivars, the lemma and palea adhere to the kernel, a desirable trait for malting (Burger & LaBerge 1985). However in other cultivars the grain threshes free of the lemma and palea and is thus referred to as hull-less or naked. Hull-less barley is more commonly concentrated and found in areas of the globe where barley is consumed as a food source by humans.

The aleurone is a layer of cells, usually two to four cells thick and is situated on the inside of the hull, together with the pericarp, testa, and nuclear tissue. These layers are part of and form the endosperm of the seed (Ried & Wiebe 1968). These layers are constituted of living cells that are filled with dense organelle rich cytoplasm, unlike the remainder of the endosperm. The aleurone layer does not just contribute to the hydrolysis of the endosperm during germination, but also contributes to the grain colour in barley (Kim et al. 2007). The grain colour of barley ranges from colourless to yellow to even blue or various shades thereof. The colour, which is influenced by the aleurone, is determined by the concentration of the accumulation of anthocyanin in this layer.

1.3 Production and breeding

Even with barley's versatility and ability to produce grain under various environmental locations and conditions, the best grain production is observed on well drained fertile loam soils with temperate temperatures (15-30°) and moderate (50-100 cm) annual rainfall (Nilan & Ullrich 1993). According to a USDA report (USDA 2008), the total area brought under barley cultivation in the world is calculated to be 57.2 million ha. Global production for the 2007-08 season was estimated at around 133 million metric tonnes. The major share of total global barley production was contributed by the EU with 43%, followed by Russia with 11.78% and Canada (8.28%), which constitutes 20% of the world's total barley harvested area. In contrast, the subtropical dry climate of Northern Africa produced only 1.6% of the world's barley production on 6% of the global barley cultivated area. Barley production represents 8% of the total harvested area of cereal crops in the world.

Australia produces high quality two-row spring barley, with annual production averaging 6 million tonnes per annum (Martin et al. 2009; Roberts, Haseltine & Maliyasena 2009). Barley is the second most widely produced cereal crop in Australia and occupies a land area of nearly 4.1 million hectares, ranging from south-west Western Australia to central Queensland. Thus comparatively Australia produces 1.7% of the total global barley production on an area constituting nearly 7.6 % of the cultivated area.

International trade in barley accounted for 15.61 million tonnes during the 2007-08 seasons. Australia contributes 33% or 3.1 million tonnes of this total, with the next major exporter being the EU at 22% of global trade. An estimated 54% of Australia's exported barley is for animal feed representing a $1.4 billion dollar contribution to Australia's economy (Martin et al. 2009; Roberts, Haseltine & Maliyasena 2009).

1.4 Uses of barley

Barley is generally grown for livestock feed or for human consumption, either directly as food or as brewing malt.

Food

Barley that is consumed as food is done so as either pearl barley or barley flour. Pearling is a polishing process that removes the outer husk and part of the bran layer of the kernel. Barley flour is used as an ingredient in baby foods and breakfast cereals, or is mixed with wheat flour in baking. Barley flat breads and porridges are widely consumed in Northern Africa and parts of Asia, where other cereals do not grow well due to the environmental limits such as latitude, low rainfall, soil salinity, high elevation and short growing season conditions (Nilan & Ullrich 1993). Hull-less barley is preferred for food applications, where a minimal amount of cleaning is required prior to processing. Barley is a good source of dietary fibre as it includes soluble and insoluble fibre fractions and contains inhibitors for cholesterol biosynthesis (Qureshi et al. 1996; Ranhotra et al. 1991). Barley consumption as a human food source is overshadowed by its use in livestock feed and malting (Smith 1995).

Malt

Barley production is primarily focused on malt with an average production of approximately 2.5M metric tonnes of malting barley. The average Australian malting barley selection rate is the highest of the world's exporting nations with around 35-40% of our national crop selected as malt. Domestically, malting barley demand is around 850,000 tonnes per year (Martin et al. 2009; Rebetzke 2007). Nearly 80% of malted barley is used for beer production and 14% for distilled alcohol (Dickson 1968). Both six-rowed and two-rowed barleys are utilised by the brewing industry. Brewers evaluate malt quality on a number of factors such as total

protein, soluble protein extract, final/coarse difference, diastatic power and alpha amylase content (Burger & LaBerge 1985).

Feed

Although the energy in barley is not easily utilised by animals, it does have a higher protein content than other feed cereals (Kellems & Church 2002a). Annually 4.1 M metric tonnes of feed barley is produced and domestic feed use is about 2 M tonnes each year (Martin et al. 2009; Rebetzke 2007). Malt sprouts and brewers by-products are also used as animal feed. The entire kernel is used generally after grinding or steam rolling to expose the endosperm. The amount of barley sold as feed is dependent on the relative price and availability when compared to other feed grains. The domestic demand for barley is driven by the livestock industries, which use cereal grain as a primary energy source and for finishing animals. An example of this is the fact that the beef feedlot, dairy and chicken industries have driven feed grain demand from 5.7 million tonnes in 1993 to 11.9 million tonnes in 2007, an average increase of 4.1% each year (Martin et al. 2009; Roberts, Haseltine & Maliyasena 2009). Cereal grain for animal feed is now the largest domestic market for Australian grain, representing 28% of grain production.

Not only has the demand for feed grain risen, but the rate of increase in the demand has been faster than the growth in grain production which has also been hampered by recent droughts. A large fluctuation in supply and price has placed pressure on the livestock sector and can result in feedlot production becoming uneconomic. Price increases are in part due to the lack of a dedicated feed grain industry in Australia, while the majority of such grains (such as sorghum and triticale), have had a static rate of production since 1993. With cereal grains for

human consumption fetching high prices, the feed market will remain a secondary consideration in Australia (Martin et al. 2009).

The lack of a dedicated feed grain industry in Australia differs markedly from its competitors such as the US and Brazil, where the dedicated feed grain production systems are well-established and based on crops such as maize and soybean. Lack of domestic competition for these grains for human consumption has reduced the precariousness between price and demand, resulting in stability to the livestock industries. In recent years, the demand for biofuels and grain as industrial raw material has created new markets that are competing with the livestock industries for grain (Martin et al. 2009).

Although wheat and barley are likely to continue to dominate grain production, the opportunity exists to produce varieties with feed characteristics matched to the needs of specific sectors of the livestock industries.

1.5 Requirements for animal feed

The nutrient requirements of animal feed include water and five major chemical groups. These groups are carbohydrates, fats, proteins, vitamins and minerals (Matsushima 1979). Water is obtained by drinking, food consumption and through metabolic processes via oxidation. Drinking water is a significant source for minerals; with cattle typically receiving 20-40% of NaCl, 7-28% of Ca, 6-9% of Mg and 20-40% of their sulphur requirements from water (Kellems & Church 2002b).

Most cereal feed grains contain between 8-14% crude protein, 1-6% fat and 41- 72% starch. In general however, barley contains a greater amount of total protein, higher essential amino acid levels and higher crude fibre content than maize and sorghum; conversely barley has a lower starch and lipid content (Kellems & Church 2002a). Carbohydrates are the primary

compounds found in feed grains and are divided into two categories namely: nitrogenous free extract (NFE) and fibrous carbohydrates. NFE is enzymatically degraded in the rumen into monosaccharides, disaccharides and starch (Cecava 1995b). In the small intestine, starch and disaccharides are broken down into 6-carbon sugars by amylase (Perry, Cullison & Lowrey 1999). Due to its composition, NFE is considered the most important component in cattle feedstuff as it represents the major energy source in finishing diets which tend to be dominated by cereal grains. Fibrous carbohydrates, crude fibre or cell wall carbohydrates include less degradable component such as cellulose, hemicellulose, pectin and lignin (Perry, Cullison & Lowrey 1999).

Ruminal micro-organisms are essential and mostly responsible for the degradation of feed proteins into peptides and amino acids. Protein is not essential for the survival of ruminant animals as long as their diets contain a source of nitrogen. This is due to the fact that the microbial organisms in the gastrointestinal tract can synthesize about 50% of both essential and nonessential amino acids required in the diet. (Bowman & Sowell 2002; Kellems & Church 2002b).

1.5.1 Ruminal nutrition and metabolism

Animals are separated and classified according to their digestive physiology into monogastrics or non-ruminant and ruminant animals. Non-ruminant animals (e.g.: pigs, rabbits, horses, poultry, cats and dogs) have a large single compartmented stomach and do not ruminate. Rumination is the process where ruminants move the rumen-reticulum contents back to the mouth for further particle size reduction by mastication. Ruminants have a multi-compartment stomach consisting of four chambers called the rumen, reticulum, omasum and abomasum (Perry, Cullison & Lowrey 1999). Another distinction between ruminants and non-ruminants, is

the production of salivary amylase which does not occur in ruminates thus there is no starch digestion in the mouth. Ruminants however are able to make effective utilization of high fibre feed by microbial fermentation in the rumen.

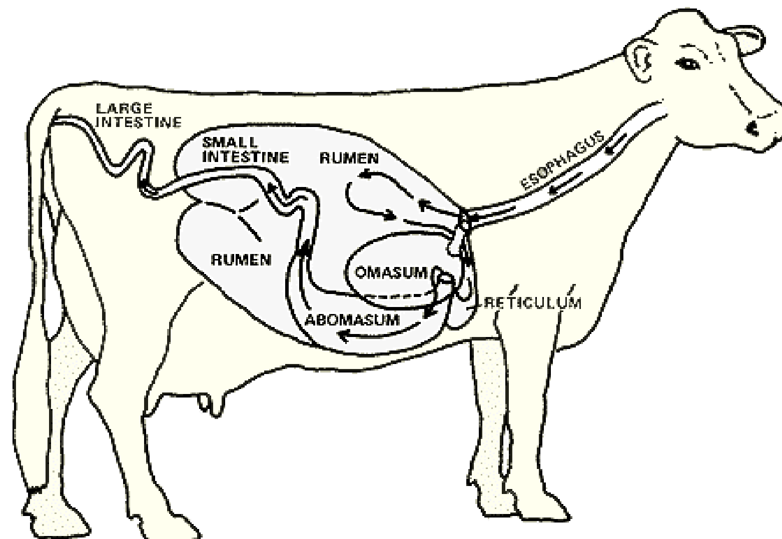

Figure 1.2: A representation of the digestive pathway of ingested feed through the four stomachs of a ruminant (Farm 2005).

1.5.1.1 Measure of ruminal digestion

In order to measure the nutritional supply of absorbed feedstuff, the digestibility of this feedstuff must first be determined. Several methods are available to determine the dry matter digestibility of these feedstuffs in the rumen. The first is the *in vivo* trial, where feedstuff is given to the animal in known amounts and the output of faeces measured (Matsushima 1979). This method has been extensively used in barley feed experiments (Boss & Bowman 1996; Bradshaw et al. 1996; Noon, Seoane & Scott 1998). *In vivo* analysis is accurate in the determination of digestibility. However, it is extremely expensive and not suitable for the evaluation of a large number of samples (Khorasani, Helm & Kennelly 2000) and it also requires a large amount of

grain per sample. The second method, *in vitro*, determines digestibility by simulating the intestinal enzymatic reactions and chemistry with hydrochloric acid and rumen fluid in a laboratory setting (McDonald et al. 1995). While being a rapid and inexpensive methodology, it does not replicate the ruminal physiology closely (McNiven et al. 2002). This results in generally lower digestibility when compared to *in vivo* techniques and the data needs conversions applied to relate the measures to each other (McDonald et al. 1995).

The *in situ* or *in sacco* method involves the insertion of non-digestible bags filled with whole or cracked grain, into the rumen of a cannulated animal for a certain incubation period. The amount of dry matter digested within the bag is measured (Herrera-Saldana, Huber & Poore. 1990; Khorasani, Helm & Kennelly 2000), providing digestion analysis within the rumen itself and reducing the need for ruminal simulation. The ruminal digestion and subsequent measurement of the incubated material in the nylon bag can be affected by a variety of factors which include the bag and sample size, bag material, pore size, sample processing, animal diet, feeding level and frequency, bag insertion and removal procedures, location of the bag in the rumen, rinsing procedure, microbial correction and incubation time (Vanzant, Robert & Titgemeyer 1998). After testing the *in situ* technique by utilizing various incubations and grain processing procedures, Bowman et al. (2001) formulated a standardized procedure for evaluating barley dry matter digestibility (DMD).

1.5.1.2 Barley feed quality characteristics

Feed quality of barley is influenced by numerous factors which include chemical and physical characteristics as an integral part. The complexity of these factors and their interactions has contributed to inconsistent data on feed quality of barley. Recent research identified factors such as protein content, starch content, acid detergent fibre (ADF) and dry matter digestibility (DMD)

are important barley feed quality traits (Surber et al. 2000). This research indicated that improving barley feed quality could be achieved by selection for lower ruminal DMD and other specific grain quality traits such as ADF. Thus the *in situ* measurements of DMD, ADF, protein and starch content of barley grain may contribute to our ability to gauge the performance of cattle fed high barley diets. The importance of ruminal and whole tract DMD is only apparent through the feed-intake of the animals (Zinn & Owens 1983). The alteration of levels of feed intake alters 1) bypass and supply of intestinally digested protein, 2) need for degradable N in the rumen, 3) efficiency of microbial growth and 4) ruminal fibre digestion. Both retention time and contingent characteristics of fermentation in the rumen appear to be involved in these alterations (Zinn & Owens 1983). The DMD referred to through the remainder of the thesis refers to ruminal dry matter digestibility.

1.5.2 Fibre

The hull of barley contributes approximately between 10-13% of the dry weight of barley grain (Bhatty, Berdahl & Christison 1975; Perry, Cullison & Lowrey 1999). Since the hull is primarily comprised of cellulose, hemicellulose, lignin and pectin (Bhatty 1993), hull-less varieties have a reduced fibre content but increased starch content (Bowman et al. 2001; Yang et al. 1997). This increase in starch content is reflected in the increase in digestibility and energy value for non-ruminants (Beames et al. 1996). However, a side effect of feeding ruminants' hull-less barley does occur, in the form of an increased rate of digestion which results in an accumulation of ruminal acid (Lehman et al. 1995). This increase in ruminal acid can lead to increased incidence of liver abscesses, bloat, acidosis and laminitis (Hunt 1996; Yang et al. 1997; Zinn, Montano & Shen 1996).

Fibrous carbohydrates tend to be retained within the rumen for varying periods of time up to 10 days, allowing for microbial fermentation and digestion of these components (Kellems & Church 2002b). A host of cellulolytic micro-organisms break down the cellulose into cellobiose and glucose (Flint & Forsberg 1995). The remaining cellulose is passed into the large intestine where it is further digested through microbial fermentation. The total crude fibre is estimated by the use of neutral detergent fibre (NDF) and acid detergent fibre (ADF) procedures. NDF is derived as the residue after extraction by boiling the sample with neutral solutions such as sodium lauryl sulphate and Ethylenediaminetetraacetic acid (EDTA). NDF consists primarily of lignin, cellulose and hemicelluloses, which is digested in neutral solutions. Whereas ADF is the residue after refluxing with 0.5 M sulphuric acid and cetyl trimethyl ammonium bromide (CTAB), and is comprised of the crude lignin and cellulose fractions (McDonald et al. 1995) which is not digested in acidic solutions. Both the NDF and ADF are effected by environment conditions and the genetic expression of the cultivar (Kong et al. 1995).

1.5.3 Starch

Barley grain consists of approximately 80% carbohydrates which is made up of starches, sugars and non-starch polysaccharides such as β-glucan, cellulose and arabinoxylans (MacGergor & Fincher 1993). Starch is the main carbohydrate constituent (NationalResearchCouncil 1996) and represents up to 65% of the kernel's dry weight (MacGergor & Fincher 1993). Starch content is affected by grain type, hull type (Kong et al. 1995; Li et al. 2001) and by environmental conditions (Kong et al. 1995). Most barley starches consist of two major components: amylopectin (74-78%) and amylose (22-26%) (Newman & Newman 1992).

Non-fibre carbohydrates are mainly degraded in the rumen. Digestion is dependent on the enzymatic activities of a range of micro-organisms, such as *Bacteroides ruminicola, B. amylophilus* and *Streptococcus bovis* found in the intestine (Kotarski, Waniska & Thurn. 1992; Russell & Rychlik 2001). There are a whole range of factors that influence the rate and extent of starch digestion in the rumen. These include the starch source, dietary composition, the extent of grain processing, amount of feed consumed per unit time and the ruminal microflora and fauna (Huntington 1997). The grain testa or husk affects the rate of digestion by the ruminal micro-organisms. Generally grains with a harder testa (maize and sorghum) are less affected by the ruminal micro-organisms and thus the rate of digestion tends to be slower than in cereals with a softer testa (wheat, barley and oats)(Herrera-Saldana, Huber & Poore. 1990). The processing of grain by steam rolling or cracking is essential in increasing the exposure and surface area of the endosperm to the microbes in the rumen; which allows for a more rapid digestion when compared to the whole grain. The rapid starch digestion in the rumen, may result in an increased risk of ruminal disorders such as acidosis (Kotarski, Waniska & Thurn. 1992; Owens et al. 1998) and bloat (Kellems & Church 2002b).

Little starch or other α-linked glucose polymers reach the small intestine on high forage diets (Heald 1952), while conversely considerable amounts of starch escape the rumen on high grain diets (Huntington 1997; Ørskov 1986; Sutton 1985). Starch digestion in the small intestine is through enzymatic hydrolysis; here the pancreatic amylase acts on starch to produce maltose (Huntington 1994). The intestinal wall also secretes maltase which hydrolyses the maltose to glucose (Perry, Cullison & Lowrey 1999)..

1.5.4 Protein

Protein digestion is more complex than that of carbohydrates. Ruminal microbes such as *Peptostreptococcus anaerobius, Clostridium aminophilum* and *C. sticklandii* are responsible for the degradation of some proteins into peptides and amino acids (Russell & Rychlik 2001). While protein is eventually degraded into ammonia, a small proportion is recycled within the rumen as urea in saliva (McDonald 1954). The nature of the diet markedly influences the extent of urea recycled to the rumen, both in saliva and by transfer across the rumen wall (Obara, Dellow & Nolan 1991). Ammonia is incorporated into amino acids by ruminal microbes resulting in the formation of microbial proteins. The extent of protein digestion in the rumen is affected by the rate of protein degradation and rate of passage of the digesta from the rumen (Cecava 1995a).

The rate of protein digestion is influenced by its physical and chemical properties, particle size of the ingested material and the speed of digestion within the rumen (Kellems & Church 2002b). The total protein in the intestine is comprised of microbial protein, intermediate protein breakdown products and dietary proteins that have escaped ruminal degradation. The proteins in the intestine are hydrolysed by trypsin and other proteases found in pancreatic juice to produce amino acids and intermediate protein breakdown products (IPBP). The remaining intermediate protein breakdown products are digested by intestinal peptidases to amino acids (Perry, Cullison & Lowrey 1999).

The absorption of proteins across the ruminal wall into the bloodstream is achieved in the form of ammonia and free amino acids. The absorbed ammonia is transported via the bloodstream to the liver where it is synthesised into urea. Urea may be transferred to the kidneys for excretion in the urine, pass into saliva and then return back into the rumen, or pass into the bloodstream and back into the gut (Cecava 1995a). Absorption of amino acids may follow two

distinct paths and outcomes. The first of these pathways is in the resynthesis of complex proteins and other nitrogen-containing compounds such as enzymes, hormones and milk proteins. Secondly, the amino acids can be deaminated in the kidneys and liver (Cecava 1995a) as seen in Figure 1.3.

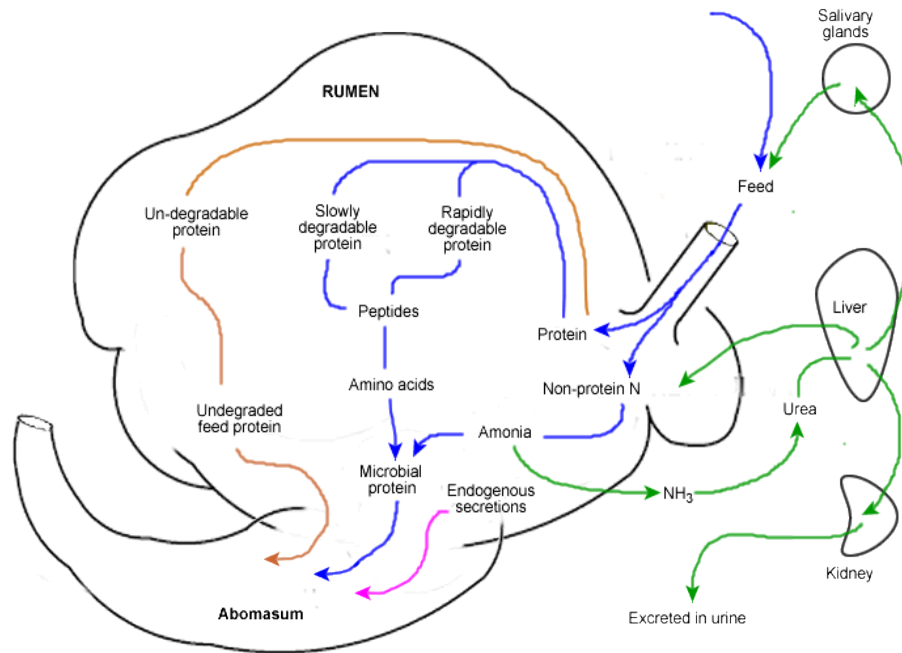

Figure 1.3: An illustration of the digestive and synthetic pathway of protein in the rumen. This includes the recycling of free nitrogen in the form of ammonia which is utilized by microbes for protein synthesis (DFID 2006).

Proteins account for 8-15% of the dry weight of the mature barley grain (Shewry 1993) and this percentage is affected by environmental and genetic factors (Kong et al. 1995). Barley contains more total protein and higher levels of lysine, tryptophan, methionine and cystine than corn (Kellems & Church 2002b).

1.6 Genetic improvement of barley feed quality

Improvement of barley varieties has focused on economically important traits such as increased yield, disease resistance and improvement of malt quality parameters (Anderson &

Reinbergs 1985). The past neglect of breeding for feed quality can be related to difficulties in the screening of breeding material for quality traits, the lack of well-defined feed quality parameters and lack of a premium in the marketplace for improved feed cultivars (Foster 1987).

More recently Bowman et al. (2001) concluded that selection for low DMD, low ADF, large particle size after dry rolling and high starch content will help the barley breeder improve feed quality by broadening the genetic variation of the selected parents. With these characteristics as a foundation Valier was released and was the first barley variety to have documented feed quality (Blake et al. 2002). The genetic improvement of barley has been accomplished by utilizing different sources of genetic variability within the primary, secondary and tertiary gene pools in the Hordeum genus (Bothmer 1992). The primary gene pool of cultivated barley is comprised of breeding lines, released varieties and their wild barley progenitor, *H. spontaneum*. There is no limitation to the extent of gene transfer between these forms; however the co-introduction of linked deleterious genes can create difficulties in the use of progeny as breeding materials (Bothmer, Jacobsen & Jorgensen 1992).

H. bulbosum comprises the secondary gene pool and is a commonly used breeding material. Hybridisation between *H. vulgare* and *H. bulbosum* results in high meiotic pairing and it is believed these two species share a basic common genome (Jakob, Meister & Blattner 2004; Kakeda et al. 2008; Subrahmanyam & Bothmer 1987) . The remainder of the Hordeum genus makes up the tertiary gene pool. Crosses between the tertiary gene pool plants and cultivated barley varieties predominantly produce sterile offspring. Domestication of barley as with many other crop species has limited the capture of genetic variation for economically important traits (Hayes et al. 1997; Martin, Blake & Hockett 1991). Hence even though feed barley has variation

in feed quality parameters, this variation is limited when compared to the world collections of barley genotypes (Bowman et al. 2001; Khorasani, Helm & Kennelly 2000; Surber et al. 1998).

To increase the available variation of DMD and other feed quality parameters, Bowman et al. (2001) surveyed the USDA world barley core collection. It was found ruminal DMD values of the core collection varied from 8.2% to 62.1%, while a diverse array of commercial cultivars varied from 42% to 56%. After the USDA core collection was screened for DMD values, a subset with low DMD was taken to evaluate trait stability and to correlate DMD and starch content, ADF and particle size. Significant correlations were observed among these characteristics with individual lines showing unique combinations of these specific traits.

1.6.1 Genetic control of quality parameters

Traits such as diastatic power, grain yield, lodging and plant height are considered to be quantitative. A study performed on the genetic heritability of these characteristics across a range of populations concluded that these characters varied from high 82% (diastatic power) to medium 66% (lodging score and plant height) and low 44 % (grain yield) (Hockett & Nilan 1985). Previous research has however not endeavoured to gauge the genetic variation of barley feed quality parameters such as DMD, starch content, protein content and ADF. It is nonetheless known that several genes such as *amol* on chromosome 2H and *stb* on chromosome 5H impact on starch content (Hockett & Nilan 1985).

While the starch content of the barley grain is affected by its genetics it is also determined by the environment (Kong et al. 1995), although the reported G x E interaction effect is low (Molina-Cano et al. 1997). Based on the study of five varieties at six locations, the broad sense heritability of starch was determined to be 33% (Molina-Cano et al. 1997). Similarly ADF is affected by environment and genotype (Bowman et al. 2001; Kong et al. 1995). Frègeau-Ried

et al. (2001) detected a significant G x E interaction for crude fibre, which contradicted the work of Molina-Cano et al. (1997) who concluded that there was no G x E interaction for crude fibre. Molina-Cano et al. (1997) further suggested that the selection for low crude fibre can start in early generations as it has a high percentage of heritability (80%). Frègeau-Ried et al. (2001) reported that additive x additive epistasis was detected for NDF and ADF, and selection intensity for these traits should not be too severe early in the selection program.

1.7 Identification of QTL for traits influencing barley feed quality

Marker assisted selection (MAS) is a process whereby a marker (morphological, biochemical or molecular (DNA)) is used for the indirect selection of a trait of interest. Barr et al. identified nine key steps in the identification of molecular marker(s) linked to traits be it qualitative or quantitative for use in MAS. A quantitative trait locus (QTL) is the chromosomal location of a gene that affects a trait, measured on a quantitative or linear scale (Barr et al. 2001; Frègeau-Ried et al. 2001; Tanksley 1993). QTL identification and analysis is comprised of four major components: generation of a segregating population; secondly identification of segregating genetic markers in the population of interest; thirdly, measurement of phenotypic data on the traits of interest for each individual of the population; and lastly the correlation of the phenotypic data with the occurrence of particular marker alleles using an appropriate statistical approach (Spooner, Treuren & Vicente 2005; Tanksley 1993).

1.7.1 Markers

Morphological markers - Have been utilised over the last 50 years; in the construction of numerous barley genetic maps (Brown 2006; Franckowiak 1997; Hockett & Nilan 1985; Nilan 1964). These markers are distributed throughout the seven chromosomes and are

associated with various traits such as *msg1* (genetic male sterile), *fch7* (chlorina seedling) on 1H, *yst4* (yellow streak*), log1* (elongated outer glume), *Vrs1* (two/six rowed spike) *fch14* (chlorine seedling) on 2H, *alm1* (albino lemma), *als1* (absent lower laterals) on 3H, *glf1* (glossy leaf), *yhd1* (yellow head) on 4H, *raw1* (rough awn), *srh1* (short rachila hair) on 5H, *rob1* (orange lemma) on 6H and *wax1* (waxy endosperm), *nud1* (naked caryopsis), *lks2* (short awn) on 7H (Franckowiak 1997). Even though morphological markers are easy to score, some are hard to use in mapping studies as they are often recessive in nature, may exhibit epistatic effects and pleiotrophy and their expression may be affected by the environment.

Biochemical markers - Include isozyme and storage proteins and are based on separation of enzymes or proteins followed by specific staining of distinct protein subclasses (Brown 2006; Weising et al. 1995). In isozyme analysis, the polyacramide gel is stained with an enzyme by adding a substrate and dye under optimal conditions, resulting in a band formation. The band forms in the position where the enzyme has migrated, which is in accordance to its polypeptide size and charge. Isozymes have been utilised in the construction of linkage maps for barley (Hockett & Nilan 1985; Wettstein-Knowles 1992). Isozymes such as *Pgd2* (6-Phosphogluconate dehydrogenase), *Pre1*, *Pre2* (Peroxidases) and *Est1*, *Est2* (Esterases) are located on 1H, 2H and 3H respectively on the barley genome (Shin et al. 1990). The main storage proteins of barley seed are hordeins whose biochemical characteristics and migration in SDS-PAGE allows for the differentiation of the various types (Shewry & Morell 2001). B-hordeins, C-hordeins and D-hordeins are encoded by the *Hor2*, *Hor1* and *Hor3* loci on chromosome 1H, respectively (Blake et al. 1996). Biochemical markers are advantageous in that they are relatively inexpensive (Brown 2006; Burow & Blake 1997) and are co-dominant in nature, which allows for the distinction of heterozygotes and homozygotes. The disadvantages

associated with biochemical markers, especially isozymes, in linkage mapping are the limited numbers of identified markers, which are unevenly distributed on the genetic map (Nielsen & Scandalios 1974). Additionally, assay methodology is time and labour consuming with the possibility of environmental conditions affecting the protein and/or enzyme expression.

Genetic (Molecular) markers - DNA level and structure is not affected by environmental conditions, contributing to the fact that DNA based molecular markers are the most widely used marker types. A variety of methods can be used to detect variations within the DNA. Consequently molecular markers are categorised in accordance to their methodology. These categories are: hybridization-based DNA markers (RFLP), PCR-based DNA markers (RAPD, SCAR, STS, SSR and AFLP) and DNA chip-based micro array such as SNP (Brown 2006).

Restriction fragment length polymorphism (RFLP) - Relies on the digestion of the DNA into specific fragments by the use of restriction enzymes. This is achieved by the recognition of specific base pair combinations and cleaving of the DNA at these sites by the restriction enzyme (Brown 2006). Mutations such as insertions, deletions and translocations may cause changes at the restriction sites (Botstein et al. 1980; Brown 2006). These cleaved fragments are then separated by means of electrophoresis and then blotted to membrane by means of Southern blotting. The result is hybridized by labelled DNA that recognizes and binds to specific sequences. Polymorphisms are detected as variations in length. The advantage of RFLPs is they are a co-dominant and highly reproducible marker which have no phenotypic effect. They are particularly useful as an anchor marker in a comparative linkage map construction of different plant species (Ahn & Tanksley 1993; Hernández et al. 2001). Unfortunately RFLP analysis requires large amounts of DNA, is labour intensive, time consuming and potentially hazardous as

radioactive probes are used (Brown 2006). Originally used in the human genome studies (Botstein et al. 1980), RFLP's were adopted for plant genome mapping (Weber & Helentjaris 1989). A genetic map for barley has been constructed using RFLP markers (Kleinhofs et al. 1993; Shin et al. 1990). The North American Barley Gene Mapping Group (NABGMP) constructed a comprehensive barley genome map using the Steptoe x Morex population, which a 1325 RFLP loci (Kleinhofs & Graner 2001).

Random amplified polymorphic DNA (RAPD) - RAPD markers are generated by using a single, short (usually 10 base pairs (bp) long) arbitrary sequence oligonucleotide to detect complementary sites across relatively short distances within the genome (Brown 2006; Williams et al. 1990). The presence of complementary sites within a genotype will result in amplification of the DNA fragment by the primer. The absence of a complimentary site will however result in the absence of any DNA amplification. Distinct advantages of RAPD are that little DNA is required as it is a PCR base marker, as well as the availability of a large (10 bp = 410) number of different primers. Since it is based on short (10 bp, 40 – 60% GC) random primers, no target information is required (Brown 2006; Weising et al. 1995). It is also a relatively quick and simple process. RAPD is however not without its drawbacks. These include the fact it is a dominant marker only, thus PCR products are either present or absent and being based on short primer sequences PCR becomes unreliable. RAPD also exhibits a lack of reproducibility between laboratories due to instability of PCR (Brown 2006; Weeden et al. 1992). RAPDs have however been used in the construction of linkage maps in many plant species including barley (Costa et al. 2001; Hernández et al. 2001; Tacconi et al. 2001).

Amplified fragment length polymorphism (AFLP) – Is a technique which was described by Vos et al. (1995) and combines the restriction site recognition of RFLP analysis with the

exponential amplification aspects of PCR based markers. AFLP is advantageous as relatively little DNA is required (more than RAPD, but less than RFLP). As the PCR is based on long adaptor sequences, it makes this method more predictable and relatively reliable (Brown 2006; Vos et al. 1995). As no prior sequence knowledge is required, this technique is appropriate for analysis of germplasm, biodiversity and genetic relationship studies (Ellis et al. 1997). Though the markers are dominant, they can be scored co-dominantly (Brown 2006; Castiglioni, Ajmone-Marsan & Wijk 1999; Waugh et al. 1997), making this technique suitable for genetic mapping studies. With modest effort high marker densities can be obtained (Qi, Stam & Lindhout 1998). The use of several different restriction enzymes and primers provides a high degree of flexibility allowing for the efficient scanning of the genome for polymorphism. The high reproducibility and reliability of AFLPs allows for the construction of high-resolution maps in barley (Becker et al. 1995), as well as identification of closely linked genetic markers for a specific phenotype such as the *Mla* locus in barley (Schwarz et al. 1999).

Sequence tagged site (STS) – STS analysis is comprised of PCR amplification of a genomic region by using a primer set (18-22 bp), which directs the amplification of a sequence for a specific locus Olson et al. (1989). STS primers are designed based on cloning and sequencing mapped RFLP (Brown 2006; Larson et al. 1996; Tragoonrung et al. 1992), AFLP (Brown 2006; Shan, Blake & Talbert 1999) and RAPD products (Brown 2006; Naik et al. 1998; Olson et al. 1989). STS polymorphisms can be read directly from an agarose gel, while point mutations can be identified by digestion with restriction enzymes after PAGE (Tragoonrung et al. 1992). STS is easy to detect with moderate-resolution analytical techniques and the sequence-tagged-sites provide co-dominant markers. A disadvantage of STS development includes the time and labour required to sequence a large number of RFLP markers. Using primers developed from RFLP

probes sequences, Blake et al. (1996) amplified a series of 135 barley-specific markers using a set of 115 STS primers. STS markers have been used in the construction of barley linkage maps (Kleinhofs et al. 1993; Larson et al. 1996; Mano et al. 1999; Shin et al. 1990).

Simple sequence repeats (SSR) - Also known as simple sequence length polymorphism (SSLP), SSRs are short tandem repeats that repeat across the whole genome. SSRs, also commonly known as microsatellites, were first reported in plants by Condit and Hubbel (1991). SSR development involves the construction of a small-insert genomic library followed by screening with a number of microsatellite probes (Condit & Hubbel 1991). This methodology has led to the discovery of numerous barley SSRs (Ramsay et al. 2000). The high development cost and need for extensive DNA sequencing and cloning is a major drawback of SSR markers. However the co-dominant nature of SSRs, the relative ease in genotyping and their use as an anchor marker between species is extremely advantageous (Brown 2006). SSR have proven to be multi-allelic; the number of alleles varied between 5-15/microsatellite (Struss & Plieske 1998). The extensive use of SSRs in barley genetic analyses includes linkage mapping (Liu, Biyashev & Maroof 1996; Pillen et al. 2000; Ramsay et al. 2000), genetic diversity (Macaulay et al. 2001; Struss & Plieske 1998) and varietal discrimination (Russell et al. 1997).

Single nucleotide polymorphisms (SNP) – SNPs are based on point mutation, which is the random substitution of a single nucleotide for another. Single point mutations are classified into two groups, being transition or transversion mutations. Transition mutations are where a purine is replaced with a purine or a pyrimidine with a pyrimidine (A to G, T to C etc.). Transversions are similar, but a purine is replaced with a pyrimidine and vice versa. Kowk and Gu (1999) defined the development of SNP markers as a six step process as follows: acquisition of DNA sequences surrounding the SNP, development of STS markers, identification of the SNP,

mapping the SNP to a unique location in the genome, determination of the allelic frequencies of the SNP in the population and development of a genotyping assay for the SNP. Various methods are available for the genotyping of SNPs such as gel electrophoresis-based genotyping, fluorescent dye-based genotyping and DNA chip-based micro-array methods (Kowk & Gu 1999). Gel-based detection of SNPs, which allows for the characterization of PCR products such as size and fluorescence, has been developed (Kanazin et al. 2001; See et al. 2000). With the use of this method, Kanazin et al. (2001) was able to distinguish and identify 38 barley loci containing single nucleotide polymorphisms.

1.7.2 Mapping populations

The construction of plant genetic maps are based on one or more specific mapping populations. The selection of a mapping population is reliant on many factors; such as the goal of the mapping project, choice of parents for crossing, population size, cross advancement and generations used for genotypic and phenotypic analyses (Young 2008). There are various types of populations that can be studied in this way. F2 generations, backcrosses (BC), recombinant inbred lines (RIL) and doubled haploids (DH) are those most commonly used (Devaux & Kasha 2008).

The advantages of producing doubled haploids in a self-pollinating species such as barley, are the immediate fixation of a homozygous genotype, the elimination of non-additive genetic variations and the speed with which new varieties are produced (Bjørnstad, Skinnes & Thoresen 1993). Drawbacks include the variation in varietal responses to *in vitro* culture techniques, the possibility of soma- and gametoclonal variation and the lower cost-efficiency compared with more conventional procedures (Bjørnstad, Skinnes & Thoresen 1993).

The development of a RIL population is achieved by the repeated selfing of an individual per line per generation for five or more generations beyond the F2 (Brown 2006; Burr et al. 1988), with single seed descent being a commonly used approach. The numerous meiosis cycles required for reaching homozygosity can result in additional recombination events occurring, resulting in increased differentiation from the parents. The differences in each line provide a basis for linkage analysis (Young 2008). The production of RILs when compared to other mapping populations is time consuming. However they have the advantage of representing six or more independent recombination events (meioses) which result in many more crossing over events per chromosome and the ability to map marker order and distances more accurately.

Specific segregation ratio at each locus is obtained in the different mapping populations. Polymorphic dominant and co-dominant markers within the F2 population segregate in a 3:1 and 1:2:1, respectively, while the segregation ratio is 1:1 in BC, DH and RIL. The maximum amount of information for a co-dominant marker can be obtained by using a F2 population, whereas the use of DH or RIL can maximize the information obtained by dominant markers (Kang 2002). The source of tissue for DNA or protein is limited in BC and F2 lines as these populations are not perpetual (true breeding). Both DH and RIL populations are capable of bulk seed production from each unchanging genotype enabling each line to be evaluated repeatedly (Burr et al. 1988; Kang 2002). Also, increasing the number of progeny evaluated or the number of replicates allows for the detection and mapping of additional regions with relatively smaller effects (Kang 2002). The use of DH and RIL is advantageous as all the information obtained from mapping is accumulative, can be shared amongst laboratories and may result in the map detail being continually improved with additional markers.

1.7.3 <u>Statistical tools for genetic mapping</u>

The identification of QTL is achieved via statistical procedures correlating the genotypic and phenotypic data to specific regions of the genome at specified levels of statistical probability (Collard et al. 2005). The mapping QTL is not as simple as mapping a gene that affects a qualitative trait. Single marker analysis has traditionally been the tool used to detect a QTL in the vicinity of a marker studied individually. That approach was first described when the linkage between seed colour and weight of beans was studied by Sax in 1923. The differences in the phenotypic means present an approximation of the phenotypic effect of each allele at a specified QTL. The basis of this method is the classification of the offspring into one of two pending the genotype at the marker. Calculation of the mean trait value of each offspring class and the subsequent comparison of the mean trait values is done to obtain significant differences. Simple statistical tests such as t-tests, analysis of variance and regression analysis are used to determine whether an inferred phenotypic effect is significant (Collard et al. 2005; Liu 1998). Any significance obtained with these statistical tests indicates that a QTL is linked to the marker. The farther a QTL is from the marker, the less likely it is to be detected statistically due to crossover events between the marker and gene.

A complete molecular linkage map is not required for single point analysis. Single point analysis has drawbacks in it is labour intensive and it has the decreased power to detect a QTL between markers. Point analysis is incapable of distinguishing between the tight linkage to a QTL with small effect and loose linkage to a QTL with a large effect (Lander & Botstein 1989). Software available for statistical analysis is able to detect QTLs by identifying associations between the marker genotype and the quantitative trait phenotype. This is achieved by a single marker analysis approach.

Simple interval mapping (SIM) is another approach for QTL analysis, with a well known example being Mapmaker/QTL developed by Lincoln et al. (1993). Interval mapping is based on the principle of testing a model for the presence of a QTL at various positions between two mapped maker loci. The model utilises regression and the maximum likelihood method which assumes the QTL is located between two markers (Lander & Botstein 1989; Lincoln, Daly & Lander 1993). The maximum likelihood method involves the searching of QTL parameters that give the best estimate for quantitative trait distributions observed for each marker class. An evaluation of the model is performed by computing the probability of observed distributions with and without fitting a QTL effect. The position of the QTL is derived by the maximum likelihood; from the distribution of the likelihood values calculated for each locus. The LOD scores are statistical measurements of linkage as revealed by pedigree analysis. These LOD scores are the ratios of the probability that an effect occurs because of linkage to probability the effect occurs by random chance. It has been found when multiple QTLs per linkage group are present, this method could fail to detect any effect or could detect a phantom (suggestive) QTL (Collard et al. 2005; Martinez & Curnow 1992). A QTL with a LOD score of greater than three (>3) is significant while a suggestive QTL may occur on the flanking regions of a significant QTL and has generally a LOD score ranging between 1.8 and 2.9. The LOD Score of suggestive QTL can be influenced by numerous factors such as experimental error, environmental conditions and masking by significant QTL. Interval mapping by regression (Collard et al. 2005; Haley & Knott 1992) was primarily developed as a simplification of the maximum likelihood method. As the genotype of the QTL is unknown it is replaced by probabilities estimated from the nearest flanking markers. Regression mapping gives estimations of QTL position and effect; that are frequently identical to those given by the maximum likelihood method. One of the factors

weakening interval mapping is fitting the model for a QTL at only one location. This approach is problematic because the effects of additional QTLs contribute to sampling variance and linkage of multiple QTL will cause biased estimates. It also allows for a QTL with large effects to mask other QTLs with smaller effects.

Composite interval mapping (CIM) was developed as a solution to the drawbacks of the simple interval mapping (Collard et al. 2005; Jansen & Stam 1994; Zeng 1994). QTL analysis in composite interval mapping is similar to interval mapping, except variance from other QTLs is accounted for. This is achieved by the inclusion of partial regression coefficients from markers in other regions of the genome. CIM gives more power and precision than SIM because the effects of other QTLs are not present as residual variance. CIM is able to remove any bias caused by QTLs linked to the position being tested. Currently numerous software packages are available that can perform composite interval mapping. These programs are also able to perform simple interval mapping such as MQTL, a program for composite interval mapping in multiple environments (Tinker & Mather 1998).

1.8 Research objectives

The sample population of interest for this project consisted of a 117 double haploid lines that were derived from a F6 – derived recombinant inbred population of Tallon x Scarlet. This Tallon x Scarlett population was developed as part of the National Barley Molecular Marker Program during the 1990's in Australia, using a cross between two malting varieties. Tallon is a malting variety developed at the Hermitage Research Station in Queensland while the other parent, Scarlett, is a well established malting variety from Europe. The Tallon x Scarlett population was originally used for the possible determination, identification and mapping of QTLs associated with malt quality. The Tallon x Scarlett population was specifically used in this

feed quality analysis because of the availability of large quantities of sample grain, field trial data and a molecular map from the Queensland's Department of Employment, Economic Development and Innovation (DEEDI). The further use of this population by DEEDI may result in specific comparative studies and a more complete understanding of genotypic and phenotypic attributes of interest.

The first objective of this project was to test the feasibility of using *in sacco* protocols developed in the USA at the University of Montana (Bowman & Blake 1996), for feed grain quality analysis within Australian cattle breeds and under Australian conditions. A successful trial would pave the way for Australian research in cereal grain feed quality to routinely apply these protocols with a great degree of accuracy. The experimental work involved the measuring of the degree of digestibility and nutritive value of lines in the test population.

The second objective was to conduct nutritive analysis on both pre- and post-digestion samples for the criteria considered to affect feed quality and assess the degree of correlation between these measurements. The nutritive analysis was performed using both analytical chemistry techniques and Near Infrared Spectroscopy (NIR). The latter technique was included due to its rapid, accurate and non-destructive nature in measuring nutrient content. In addition the lack of application of this technique to post-digestion nutrient residues, suggested a fruitful line of enquiry. *Last sentence doesnt quite make sense here*

The third objective was to establish markers for pre- and post-digestion traits based on both wet chemistry and NIR data. The first step was to determine if these pre- and post-digestion feed quality markers mapped to the similar chromosomal regions of the barley genome. Then to determine if analytical chemistry-derived and NIR-derived phenotypic data mapped QTLs to same regions in the barley genome.

The results obtained from this research, should aid in the rapid and accurate acquisition of phenotypic data to be used in QTL analysis. The identification of robust feed quality QTLs could potentially be used in MAS breeding programs for the development of an industry specific barley feed grain, allowing the industry to grow and develop. Additionally the extensive use of NIR for data collection may aid in the future development of NIR calibration for processed and post-digestion samples.

Chapter 2.

2. Cattle experimentation.

2.1 Introduction.

In order to measure the nutritional value of absorbed feedstuff, its digestibility must first be determined and is expressed as dry matter digestibility (DMD). DMD is frequently expressed as a percentage of the whole, and is calculated using the formula:

$$DMD = 100\% - \left(\frac{Dry\ Residue\ Sample\ Weight}{Dry\ Feed\ Sample\ Weight}\right) \times 100\%$$

Several methods are available to determine the DMD of feedstuff in the rumen. The first is the *in vivo* trial, where feedstuff is given to the animal in known amounts and the output of faeces measured (Matsushima 1979). This method has been extensively used in barley feed experiments (Boss & Bowman 1996; Bradshaw et al. 1996; Noon, Seoane & Scott 1998). *In vivo* analysis is accurate in the determination of digestibility, however it is expensive and not suitable for the evaluation of a large number of samples (Khorasani, Helm & Kennelly 2000) as it also requires a large amount of grain per sample. The second method, *in vitro*, determines digestibility by simulating the intestinal enzymatic reactions and chemistry with hydrochloric acid and rumen fluid in a laboratory setting (McDonald et al. 1995). While being a rapid and inexpensive methodology it does not replicate the ruminal physiology exactly (McNiven et al. 2002). This results in generally lower digestibility when compared to *in vivo* techniques and the data needs conversions applied to relate the measures to each other (McDonald et al. 1995).

The *in situ* or *in sacco* method involves the insertion of multiple non-digestible porous nylon bags, filled with whole or cracked grain, into the rumen of a cannulated animal for a certain incubation period. This method allows the amount of dry matter digested within the bag

to be measured (Herrera-Saldana, Huber & Poore. 1990; Khorasani, Helm & Kennelly 2000) and provides digestion analysis within the rumen itself, reducing the need for ruminal simulation. The ruminal digestion and subsequent measurement of the incubated material in the nylon bag can be affected by a variety of factors which include the bag and sample size, bag material, pore size, sample processing, animal diet, feeding level and frequency, bag insertion and removal procedures, location of the bag in the rumen, rinsing procedure, microbial correction and incubation time (Vanzant, Robert & Titgemeyer 1998). After testing the *in situ* technique by utilising various incubations and grain processing procedures Bowman et al. (2001) formulated a standardised procedure for evaluating barley DMD.

Observed genotypic effects on *in vitro* DMD (Kemalyan et al. 1989), *in situ* DMD (Khorasani, Helm & Kennelly 2000) and *in vivo* DMD (Boss & Bowman 1996) have resulted in an increase of barley selection based on DMD. It was reported *in situ* DMD was affected by the barley grain's genotype and particularly spike type (Bowman et al. 2001). The results indicated an improved barley feed quality could be achieved by selecting for lower DMD and other specific grain quality traits such as ADF.

The Tallon x Scarlett population was developed as part of the National Barley Molecular Marker Program during the 1990s in Australia. Each state breeding program contributed three populations to the national program. The Tallon x Scarlett population was a cross between two malting parents. The one parent Tallon was a malting variety developed by the Queensland Breeding Program and the other parent Scarlett a malting variety from Europe. These two parents were crossed to identify malting quality QTLs. The reason why the Tallon x Scarlett population was selected for feed quality analysis was that DEEDI had previously generated field trial data and some malt QTL data for this population. Currently their molecular program is also

looking at feed quality of this population, because of the dominant position of the cattle livestock industry in Queensland. In addition, it is known brewers' byproducts are utilized by feedlots as animal feed.

The present research would allow comparisons of the phenotypic data as well as potential QTLs to malting data and QTLs. The aim of this experiment was to identify the lines within the Tallon x Scarlett population with the greatest DMD and to derive a digested residue that could be used in further analysis. Additionally this research aimed to help establish testing protocols in Australia to be used in subsequent studies.

2.2 Materials and methods.

2.2.1 Animal selection and adaptation

An application for the ethical clearance for research involving animal experimentation was submitted to the Queensland Department of Primary Industry. Ethical approval number: SA 2008/04/246 was granted for the evaluation of barley grain genotypes for high-grain feedlots diets of cattle. After approval was granted, a group of seven three-year-old rumen-fistulised *Bos taurus* cross-bred steers with average paddock weight of 479.3 kg were selected for the study. The steers were fitted with large diameter rumen cannulae, with the surrounding area clipped and cleaned. Each animal's weight was recorded before they were housed in individual pens for the duration of the study. Because the animals' diets were forage based, individual body weights were used to calculate the required feeding rations to gradually obtain the 50% grain/hay diet required for the trial. The gradual increase in barley grain content over a 10 day period allowed for the adaptation of the animals' physiology and intestinal micro flora to a grain based diet (Soest 1994). From the 11th day till the end of the trial the animals remained on the 50% grain

diet. The average daily weight gain of the animals was recorded to ensure animal welfare and performance.

2.2.2 Experimental design

A two phase experimental design of five replications of 140 grain genotypes with partial balance across the trial was drawn up. Phase 1 consisted of the field grain replicates, while the second phase consisted of the cattle used in the phenotypic trial with complete blocks representing each day and incomplete blocks represented by the animals. The trial was performed over a five day period using six animals per day with 30 nylon sample bags per animal. These nylon bags contained 30 unique samples, so no duplication of a sample per day per animal occurred. The six animals by 30 bags therefore contained 180 field plots per day, comprising of 140 genotypes with the partial duplication of 40 genotypes. Each day would form a complete replicate of the material, with animals used as an incomplete block. The selection of seven animals placed on a 50% diet allowed for a spare animal, should an animal be taken ill and have to be removed from the trial.

2.2.3 Sample preperation

The grain samples from the 180 field plots (140 genotypes and 40 replications) were rolled through a brewer's hand mill (Schnitzer's CAMPO Flockenquetsche). This cracked the grain to expose the endosperm and is a method similar to that used by the feedlot industry. French stitched Monofilament polyester bags with dimensions of 24 x 10 cm and a pore size of 45 µm were clearly numbered 1 to 900. The clean numbered empty bags were checked for holes and placed in a forced-draught drying oven at 55°C for 24 hrs prior to use. This ensured no residual moisture was present in the bags. The bags were then removed and cooled to room

temperature in desiccators prior to weighing. The empty bag weight was recorded before approximately 5 g of rolled grain was placed in the weighed bag. The bag number, bag weight and combined bag x grain weights were recorded. The filled bag was allocated to a particular day and animal according to the experimental design. The top of the filled weighed bag was folded length-ways three times over itself, and then with a twisting motion folded down, thus sealing the top of the bag. A 4-inch cable tie was placed along the fold of the bag with the knob of the tie facing inwards and a size 16 rubber band was tightly wrapped around the tie and top of the bag. The sealed samples were place in plastic bags and marked according to their day of incubation and corresponding animal. Sub-samples of the feed material were collected at the start, middle and end of the bag filling process. These sample weights were recorded prior to being placed into a drying oven at 105°C for 48 hrs. The dried sub-samples were removed from the oven and placed in desiccators for cooling. The cooled samples were weighed and average dry matter percentage calculated for each feedstuff. This result was later used in determining the rate of degradability.

2.2.4 Sample incubation

The sample bags were attached to a chain with a cable tie (Appendix 2), so each link of the chain would contain an even amount of sample (four samples per link). This allowed the chain to securely hold the required 30 samples per day for the particular animal. The chain, with the sample attached, was then soaked for 3-5 minutes in clean water prior to insertion into the animal. The animal was secured in a crush to allow safe and easy access to the cannula. The plug was gently removed from the animal and the chain holding the samples was inserted. It was essential to ensure the chain and the samples were pushed down below the feed raft to ensure maximum exposure to microbes and digestive enzymes. A string, attached to the end of the chain

was left to hang outside of the animal with a tag attached to its external end. The plug was then inserted and secured to the cannulae to ensure that the rumen content did not spill. The time of the insertion of the samples was recorded as the timing of sample removal was based on this time. The procedure was repeated for the other animals with each insertion time individually recorded.

2.2.5 Residue preparation

The samples were digested in the animal for a 3 hr period before removal. The animals were secured once again in the crush and the plug removed. The chain and bags were gently removed from the animal. The chain was flicked to remove any excess solid rumen content from outside of bags. The chain and bags were lowered into bucket of cold water to stop any further digestion of the samples. The time of the removal was recorded and the area around the bung was cleaned before resealing. With the aid of blunt nose scissors the cable ties were cut and removed to free the bags from the chain. The rubber bands were left on the sample bags as the bags were washed under cold water, by giving them 10 – 25 squeezes in the process. The samples were placed into underwear bags and weighed down. The samples were washed in a washing machine using cold water for two 6-min cycles and then spin dried. The samples were removed and dried by placing them in a force-draught oven at 55°C for 48 hrs, keeping bags from different animals and days separate. The bags were removed from the oven and allowed to cool in a desiccator. Once cooled, the rubber bands were removed and any dried rumen content was removed from the outside of the bags. The bags were quickly weighed and the weight recorded. The residue was removed from each bag and placed into a labelled 250 ml screw top jar for later analysis. The empty bags were washed in Lux soap flakes and given two deep rinses.

The DMD of each line was calculated using the dry sample residue weight and the dry sample weight according to the following formula:

- A = Dry Bag Weight
- B = Dry Bag Weight + Feed Sample Weight
- C = Dry Bag Weight + Dried Sample Residue Weight
- D = Average Dried Feed Sub- Sample Dry Matter %
- Dry Feed Sample Weight $= \frac{(B-A)\times D}{100}$
- Dry Residue Sample Weight = C – A
- $\text{DMD} = 100\% - \left(\frac{\text{Dry Residue Sample Weight}}{\text{Dry Feed Sample Weight}}\right) \times 100\%$

2.2.6 **Statistical analysis**

The use of the two phase experimental design allows the identification and manipulation of specific sources of variance from the data i.e. days and animals. This was accomplished by using the Residual Maximum Likelihood (REML) approach of ASReml-R (Gilmour, Thompson & Cullis 1995; Patterson & Thompson 1971).

The REML approach is a form of analysis using the maximum likelihood estimation, which does not require the use of a full set of available observations, but instead relying on a likelihood function calculated from a transformed data set. This method is used to fit linear mixed models as in this project. REML, unlike other conventional maximum likelihood estimation procedures, can produce an unbiased estimation of variance and covariance parameters (Gilmour, Thompson & Cullis 1995; Patterson & Thompson 1971), therefore allowing the identification of variance caused by the genetic components *per se.*

2.3 Results

The Average Daily Gain observed during the trial is consistent with expected results of adapting to a new diet over a two week period. During the first week there was an initial decline in the average daily weight gain of the steers of 6.83 kg. During the second and third week the average daily weight gain increased in the majority of the steers used (Table 2.1). The ADG does not indicate how the animal performed to a specific line but rather to the barley diet in general.

Table 2.1: The average daily gain (ADG) of each animal used in the DMD trial.

Animal	Weight (kg):				ADG (kg) For:		
	Initial	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
1	476	477	470	484	1	-7	14
2	516	508	508	516	-8	0	8
3	496	488	486	482	-8	-2	-4
4	494	486	492	499	-8	6	7
5	458	458	466	468	0	8	2
6	436	418	421	427	-18	3	6

After trial completion, the percentage DMD for each line of the population was plotted. A normal distribution curve was observed across the population with parental lines lying across the median position of the plot as observed in Figure 2.1.

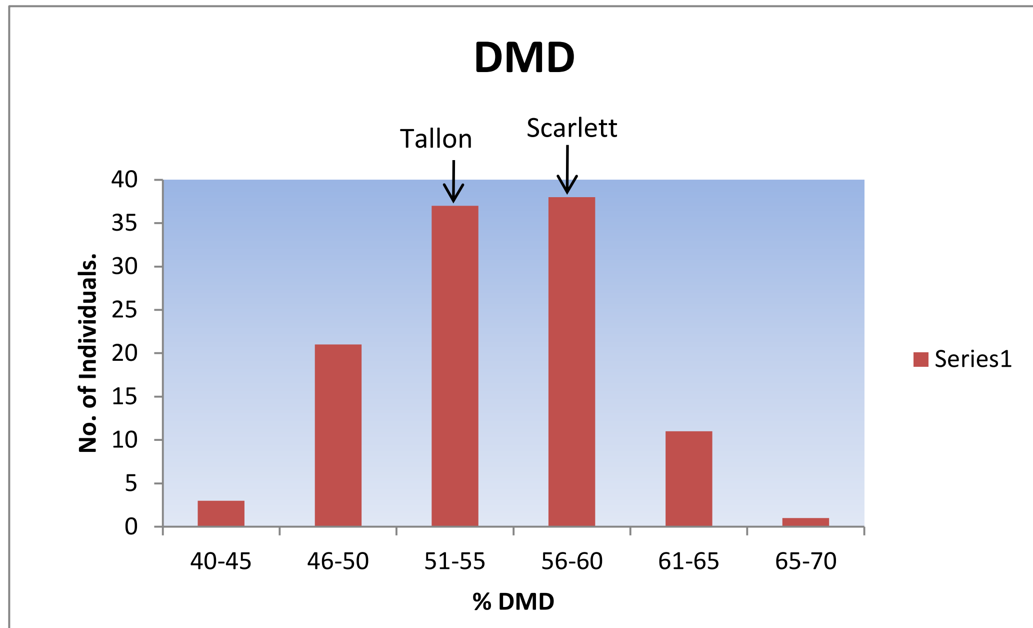

Figure 2.1: The phenotypic distribution of the dry matter digestability (DMD) after an 3 hour incubation period within the rumen for lines in the Tallon x Scarlett population. The parental scores are indicated.

The Residual Maximum Likelihood (REML) of the data was analysed by using ASReml-R. The source of variance for sample DMD was determined. The analysis indicated that experimental error (Comp = 33.7%) had the greatest contribution to the observed variance within the DMD data set. The day the trial was performed also contributed to the source of variance (Comp = 21.0%) observed, closely followed by the day/animal interactions with a contribution of 20.1% to the total variance (Table 2.2). The statistical manipulation of the factors attributing to variance allowed for the observation of the expressed genetic variance.

Table 2.2: Sources accounting for variance and standard error within DMD data set.

Source of variation	Component	Standard Error
Genotype	10.2	3.1
Field Plot	5.5	2.5
Day	21.0	17.5
Animal	9.3	8.7
Day x Animal	20.1	6.8
Random Variance	33.7	1.8

2.4 Discussion

The meat of barley-fed animals tends to be more marbled and richer in colour than corn-fed animals (Beauchemin et al. 1997), a characteristic which is desired by the industry. Both the physical and chemical characteristics of barley and their interactions with each other influence animal performance. Animal performance is often measured in average daily gain, carcass grade and feed efficiency. The ADG from different barley varieties varies and is linked to DMD, especially the digestible starch intake (Boss & Bowman 1996), which results in differences in energy content.

The observed ADG in the steers is consistent with other studies in which animals are placed on an adaptation diet for a two week period (Bowman & Blake 1996). An initial drop in animal weight is expected when moved from green pasture to a rationed barley grain and hay diet. During the high hay and low barley grain concentration period of the adaptation process the steers lost on average 6.83 kg of weight. This weight loss is due to the physiological and microbial changes undergone in the rumen as part of the adaptation to the new diet.

The significant loss of weight in animal six during the first week of the trial was subsequently explained by the removal of a considerable amount of bailing twine and plastic

from the rumen during the first week. During the second week of the dietary adaptation process the majority of the animals showed positive weight gain, indicating that ruminal adaptation to the new diet was taking place. During the *in sacco* trial of Week 3 there was a steady weight increase of the animals due to the high energy content of the feed. There was an exception to this weight increase as found in animal 3 during the third week of the trial. The decline in weight and subsequent ADG was due to the removal of ingested twine and plastic from the rumen.

The distribution curve of the DMD percentages suggests transgressive segregation for this trait occurs within the Tallon x Scarlett population (Figure 2.1). This implies there are multiple genetic regions which influence DMD and that both parents contribute unique loci to this mix. Due to the two phase design of the trial it is impossible to speculate about the nutritive value of each line, but line performance in terms of DMD was determined. However the experimental design does allow for the attribution and subsequent manipulation of variance in the data. The manipulation of variance, results in the variance expressed within the DMD data to be genetic in nature. The low genetic variance for DMD in the Tallon x Scarlett population lines suggests a low heritability of this trait. This low heritability and observed genetic variance may be the result of the shared pedigree of the two parental lines. DMD by itself without the relevant nutritive content of the cereal grain does not express feed quality, thus prompting nutritional analysis.

Chapter 3.

3. Wet chemistry analysis of pre- and post-digestion barley grain samples.

3.1 Introduction

The DMD results discussed in the previous chapter is one dimensional as it only identifies the lines in the population with the greatest rate of digestibility. This measure of absolute mass digested from the sample does not help to identify and quantify the nutrient content digested. Both the physical and chemical characteristics of cereal grains and their interactions with each other influence animal performance and ruminal digestion. This notably affects the degree of nutrient digestion and available energy of feed grains.

There is no consensus in the feedlot industry as to what constitutes feed quality. Traditionally, feed quality is measured in terms of animal performance, which is based on the average daily weight gain of the animal. Recent research by Surber *et al.* (2000) has identified four basic factors that constitute and affect feed quality. These are starch, protein, fibre and dry matter digestibility (DMD). More accurately the fibre component of interest consists of acid detergent fibre (ADF). These fibrous components of feed grains are indigestible even when suspended in an acidic solution such as the rumen.

In order to measure the nutrient content digested and to identify quantitative trait loci (QTLs) associated with feed quality, the nutritive composition of the feed grain needs to be determined firstly through analytical chemistry techniques followed by the dry matter digestibility of the feedstuff. The latter is achieved with the aid of the *in sacco* trials and analytical chemistry performed on the whole grain and the grain residues. The nutrient

concentrations digested from the grain samples can be calculated through simple arithmetical means.

Results obtained, through this experimental sequence should be able to validate previous research which found that the improvement of barley feed quality could be achieved through the selection of lower ruminal DMD and other specific grain quality traits such as ADF. This phenotypic data can then be used in the identification of quantitative trait loci, associated with the four major feed quality traits in the Tallon x Scarlett population.

3.2 Materials and methods

Whole grain from each of the Tallon x Scarlett lines were individually milled with a Cyclotec 1093 mill from Foss-Tecator to pass through a 1 mm screen. Similarly, residues of the same lines after *in sacco* trials were also milled. The resulting barley flour pre- and post-digestion samples were stored in dry, labelled, air-tight containers. An experimental sample population of a 117 unique genotypes were selected from the original Tallon x Scarlett population (n = 140).

3.2.1 <u>Protein</u>

The protein content of the 117 pre- and post-digested samples were analysed with the automated Vario Max N/CN Macro elemental analyser according to the manufacturers' specifications. The analysis of the samples is achieved through the Dumas principle. This principle involves the combustion of the sample at 900-1150 $^{\circ}$C in an oxygen rich environment. A 500 mg dry weight sample of milled grain was weighed and placed in a stainless steel crucible which was then loaded into a carousel. The sample weights were recorded in the appropriate location within the VarioMax software. The robotic 'gripper arm' lifts the crucible from the

carousel and lowers it into the combustion chamber. In the combustion chamber oxygen is introduced during the sample combustion through jet injection directed above the sample. The high velocity of the oxygen stream & its positioning results in an excess of oxygen at the point of combustion, without requiring large amounts of oxygen to be used. Helium acts as a carrier gas and carries the combusted gasses through the reduction furnace. In the reduction furnace NO is reduced to N_2 & excess O_2 is released and absorbed by a series of catalysts. Instead of the conventionally used copper as the reducing agent the Vario Max utilises Tungsten, which has three to four times the absorption capacity of copper. Nitrogen travels after additional gas scrubbing to the thermoconductivity detector (TCD) where it is quantitatively measured (Elementar 2009). The TCD reading of the nitrogen is expressed as a nitrogen percentage. To ensure accuracy L-Glutamic acid with known nitrogen content is used as a standard. The standard was run after every 25 samples. Two repetitions were performed on both the pre & post digestion samples. To further ensure accuracy, random repeats were done after every ten readings. The nitrogen content was recorded and stored on the appropriate software.

3.2.2 Fibre

The ADF content of the pre & post digestion samples was determined following the method of Van Soest et al (1991) using the Fibre Tec 2021 Fibrecap system. The concept behind the fibre analysis is that plant cells can be divided into less digestible cell wall and readily digestible cell content. These components can be separated by using two detergents: a neutral detergent and an acidic detergent. The neutral detergent solution is aided by the use of acetone, EDTA (Ethylenediaminetetraacetic acid) and sodium lauryl sulphate which dissolves protein, starch, cellulose, hemicellulose and lignin (Soest, Robertson & Lewis 1991). For the determination of ADF, which is a good indicator of digestibility and thus energy intake, the

samples are boiled in an acidic solution. This solution consists of sulphuric acid and the detergent cetyl trimethyl ammonium bromide (CTAB). The remaining hemicellulose and cell wall proteins are dissolved from within the NDF residue containing cellulose, lignin, lignified nitrogen, cutin, silica and some pectin. The cellulose, lignin, silica and cutin residue that remains is termed ADF. ADF analysis was performed using 0.5g of sample in the Fibrecap system(Soest, Robertson & Lewis 1991), which was dried after analysis and weighed. The ADF content is thus simply the dry weight of the residue expressed as a percentage of the original sample dry weight. This was performed for both the pre- and post-digestion samples.

3.2.3 Starch

The starch analysis was performed by using the Megazyme total starch analysis kit. The procedure followed was according to the manufacturer's specification on the pre and post digestion barley flour that was milled using the Foss-Tecator Cyclotec 1093 with a 1mm screen. The Megazyme kit procedure allows for the measurement of total starch in most cereal products, being either natural or processed. The Megazyme kit is based on an assay format in which the starch hydrolysis proceeds in two phases. In the first phase, starch is partially hydrolysed and totally solubilised. During the second phase, the starch dextrins are quantitatively hydrolysed to glucose by amyloglucosidase. For most samples complete solubilisation of starch can be achieved by heating in the presence of thermostable α-amylase. However, for samples containing high levels of resistant starch (high amylose), complete solubilisation and dextrinisation requires pre-treatment with dimethyl sulphoxide at 100°C. Samples containing high levels of glucose and maltodextrins are washed with aqueous ethanol before analysis to aid with dispersion and start starch digestion (Wrolstad et al. 2004). The starch is broken down into glucose, whose concentration is measured and recorded through the use of a spectrophotometer. Conversion

equations are applied on the observed glucose concentration to convert it into the expressed starch percentages (Megazyme 2009; Wrolstad et al. 2004).

3.2.4 Statistical analysis

The samples were randomly selected across the population of interest and experimental analysis performed. The use of the two phase experimental design discussed in the previous chapter allowed for the statistical manipulation of known variance from the data set. This was accomplished by using the Residual Maximum Likelihood (REML) approach of ASReml-R (Gilmour, Thompson & Cullis 1995; Patterson & Thompson 1971).

REML was used to derive an unbiased estimation of variance and covariance parameters, therefore allowing the identification of variance caused by the grain's genetic component (Gilmour, Thompson & Cullis 1995; Patterson & Thompson 1971). The feed data obtained from experimental analysis was used to fit a linear mixed model (McLean, Sanders & Stroup 1991). However, the lack of a sufficient number of replications during experimental analysis (due to time and financial constraints) generally resulted in the observation of phenotypic variance and correlations rather than genotypic. The variance and correlations observed between the feed quality data could then be visualised in a Bi-plot and expressed on a correlation matrix.

3.3 Results

A normal distribution across the sample population was observed for the pre-digestion protein content of the sample grain, with both parents in the distribution curve falling on either side of the median (Figure 3.1). The sample population's absolute protein mass content ranges from 7.5 – 12.0 mg of protein per 500mg of sample, whilst the range of the percentage distribution ranges between 1.4 – 2.3%.

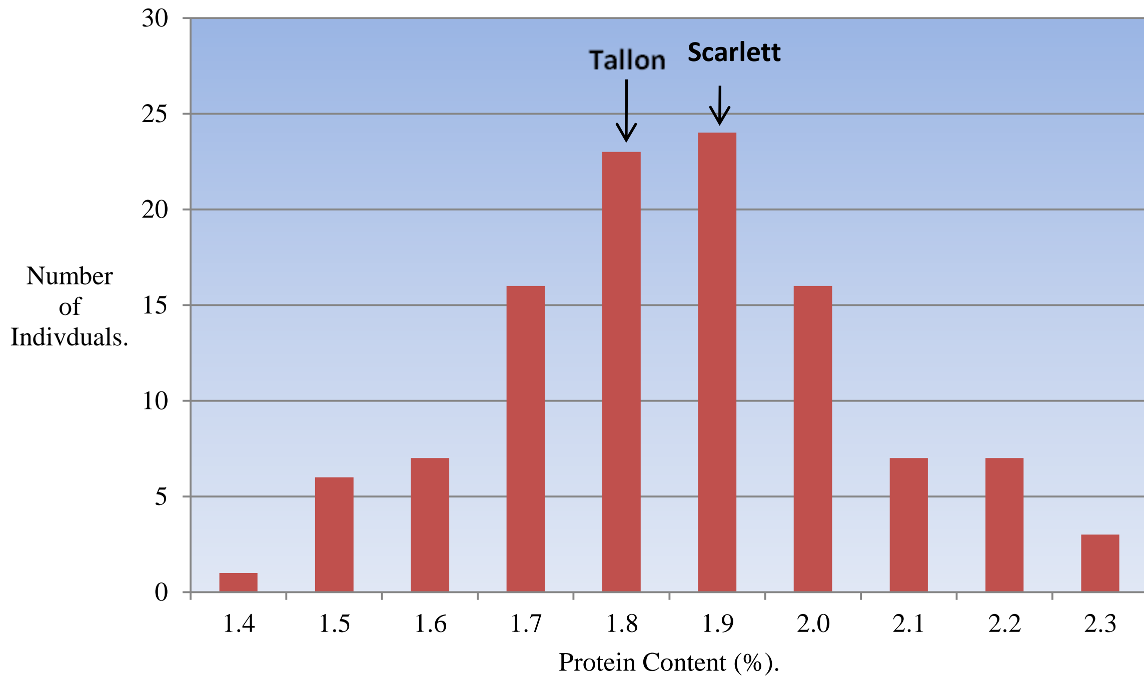

Figure 3.1: Distribution of protein content per dry basis of the sample grains expressed as a percentage of the total grain weight.

Figure 3.2 represents the percentage protein digested from the grain samples after a three hour incubation period in the rumen. The absolute amount of protein extracted from the grain is of importance for the animal, however this amount may not be perfectly correlated with the initial protein content of the sample.

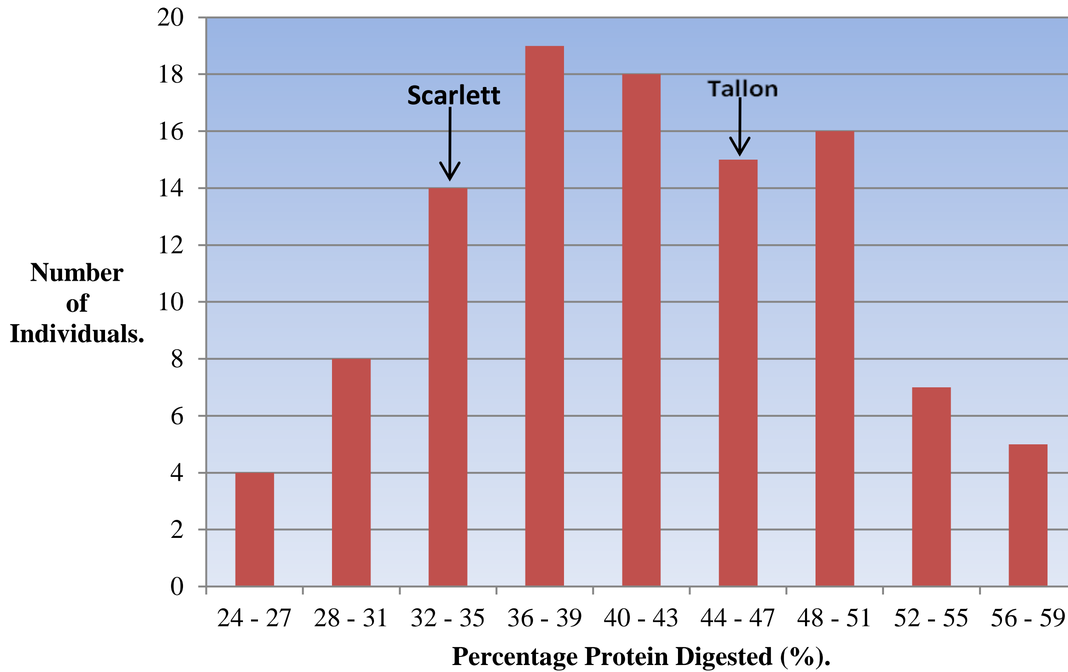

Figure 3.2: Distribution of protein digested from the sample lines in the Tallon x Scarlett population,*belongs in discussion*

A linear mixed model was constructed testing for sources of variance from a variety of factors (Table 3.1). The genotype and field-plot location of the barley lines within the trial did not affect the genotypic correlation observed between the pre- and post-digestion protein content for each trait. Thus the significant correlation for the samples' genotype (r = 1) and field-plot location (r = 0.9396) does not contribute and affect the rate of protein digestion.

Table 3.1: The correlation between sources of variance attributing to protein content and digestion.

Source of Variance	Variance component
Genotype*	~1 Thus no genotype by digestion interaction.
Field-plot**	0.9396

* Genotype does not and is not affected by digestion.
** The Field-plot variance component for nutrient content is taken into account, which allows the variance between pre- and post-digestion content to be directly compared.

However the field-plot location was the greatest contributor to the source of variance observed within the pre-digestion data set and similarly to the post-digestion data set. The small measure of observed variance was greatest in the post-digestion results, with the exception of the variance cause by genotype in the post-digestion data set (Table 3.2). The greatest contributor to variation in protein content across the sample population was attributed to field-plot effects rather than genotype. The relatively low contribution of genotype to variance of the protein content may be attributed to the shared pedigree of the parental lines.

Table 3.2: The variance obtained within the pre- and post-digestion protein content.

Source of Variance	Variance component
Genotype variance Initial	0.0036
Genotype variance Digested	0.0010
Field-plot variance Initial	0.0287
Field-plot variance Digested	0.0472
Residual variance Initial	0.0002
Residual variance Digested	0.0019

As seen in Table 3.3, a significant correlation was found for pre- and post-digestion protein content (r = 0.639), pre-digestion starch concentration and amount of starch digested (r = 0.655) and between post-digestion ADF and ADF digested with r = 0.954.A negative correlations was observed between feed traits such as pre-digestion protein concentration and DMD (r = -0.537). A strong negative correlation was found when post-digestion starch content was compared to the amount of starch digested (r = -0.722). Numerous other moderate and weak correlations were also determined and expressed in the correlation matrix (Table 3.3), some of which will be referred to in the discussion.

Table 3.3: The correlation matrix between the various feed quality traits; pre-, post-digestion and amount digested.

Correlations	**Protein Pre**	**Protein Post**	**Protein Digested**	**Starch Pre**	**Starch Post**	**Starch Digested**	**ADF Pre**	**ADF Post**	**ADF Digested**	**DMD**
Protein-Pre	1.000									
Protein-Post	0.639*	1.000								
Protein-Digested	-0.311	0.483*	1.000							
Starch-Pre	-0.141	0.007	0.192	1.000						
Starch-Post	-0.150	-0.219	-0.146	-0.005	1.000					
Starch-Digested	0.027	0.118	0.173	0.655*	-0.722*	1.000				
ADF-Pre	-0.085	-0.017	0.072	0.152	-0.061	0.133	1.000			
ADF-Post	0.032	0.032	0.002	-0.103	0.159	-0.171	0.060	1.000		
ADF-Digested	0.057	0.037	-0.020	-0.144	0.173	-0.205	-0.243	0.954*	1.000	
DMD	-0.537*	-0.248	0.212	0.159	-0.008	0.071	0.223	0.019	-0.049	1.000

* A significance level of 0.05 was used in the correlation analysis.

A normal distribution across the sample population was observed for the pre-digestion ADF content of the sample grain, with both parents in the distribution curve falling on or near the median (Figure 3.3).

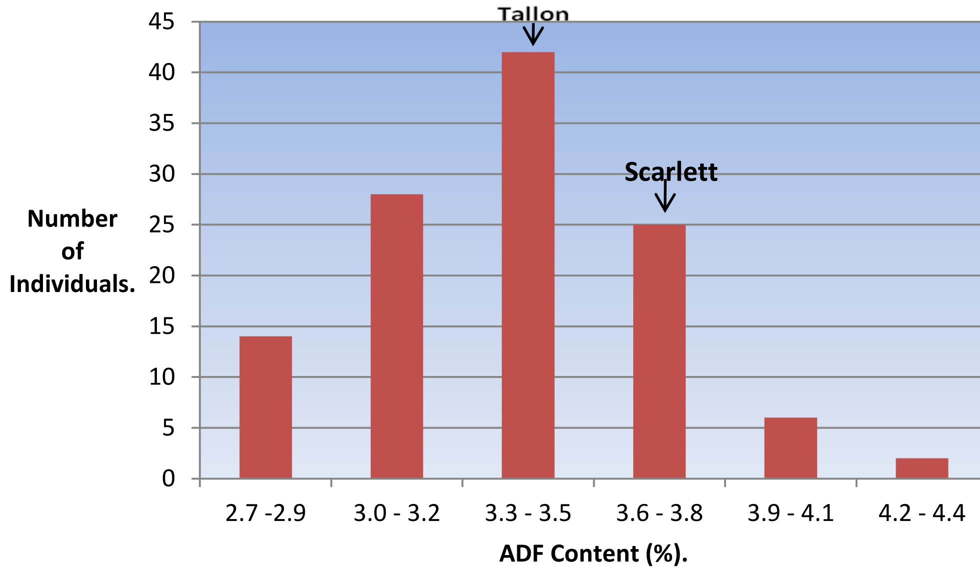

Figure 3.3: The distribution plot of pre-digestion ADF content in the Tallon x Scarlett population.

The observed normal distribution (Figure 3.4) of the ADF digested from the sample population with both parents falling just to the right of the median.

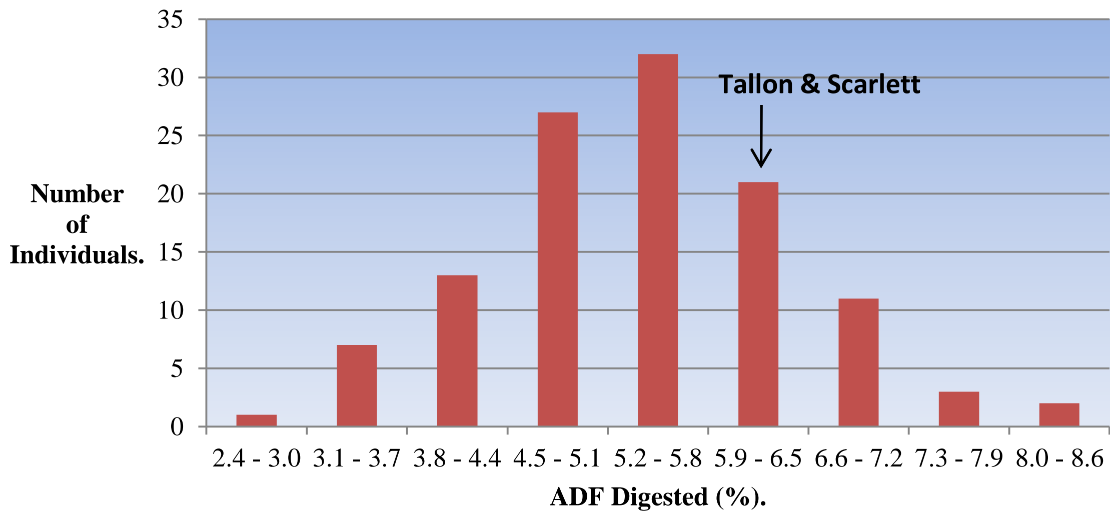

Figure 3.4: The distribution plot of ADF content digested from the sample lines in the Tallon x Scarlett population.

A positively skewed (to the right) bi-modal distribution was observed for the Starch content across the Tallon x Scarlett sample population lines (Figure 3.5). Tallon was found in the trough of the distribution while Scarlett was found on the second mode.

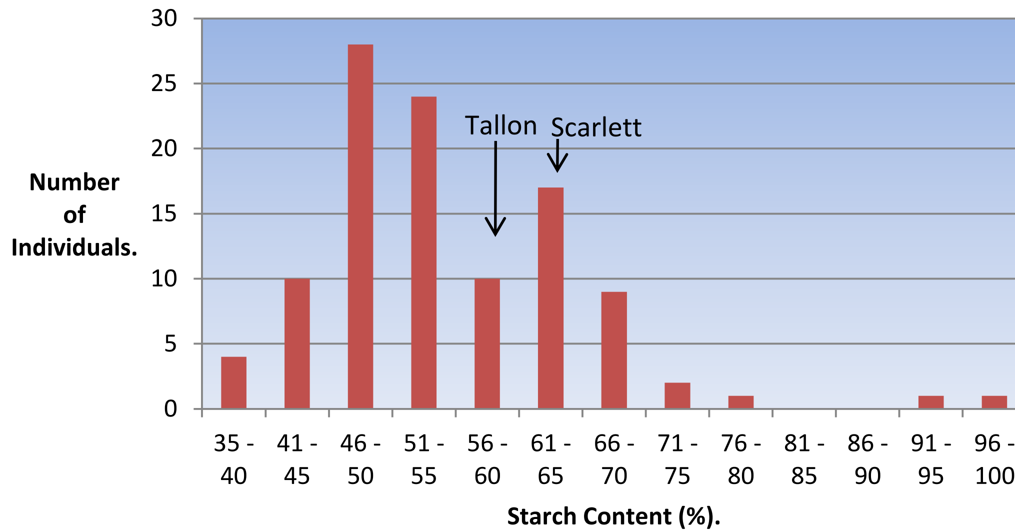

Figure 3.5: The distribution plot of pre-digestion starch content of the Tallon x Scarlett sample population.

A positively skewed distribution is also observed for the amount of Starch digested (Figure 3.6) from the lines in the sample population. A Positively skewed distribution is left-leaning. For each type of skew, the mean, median, and mode diverge.

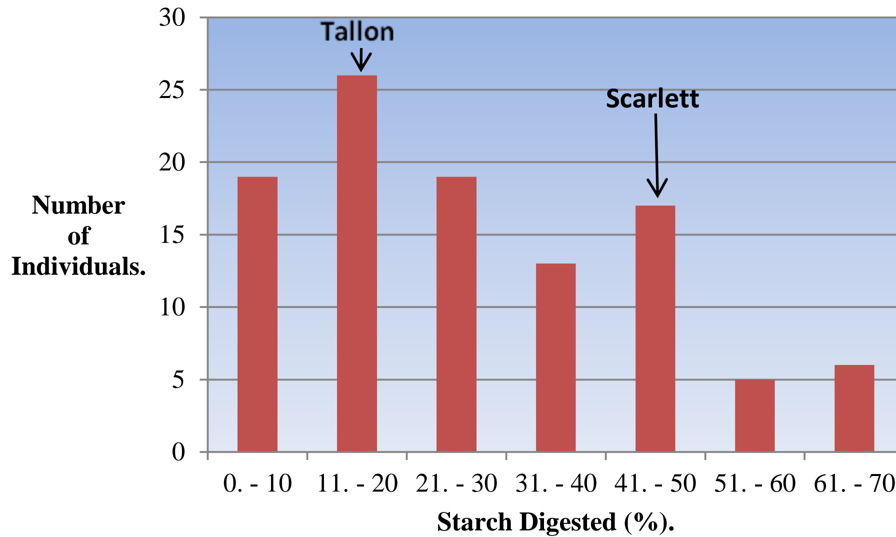

Figure 3.6: The distribution plot of Starch content digested from the sample lines in the Tallon x Scarlett population.

Bi-plots summarise and exhibit similar correlations to that of the correlation matrix. These correlations are presented two dimensionally on a graph explaining the observed variance in the data set. The variance observed in Figure 3.7 is 46% which can be broken down into two major components. These components are 25% and 21% respectively and are represented on each of the major axes of the graph. Bi-plots can also be three dimensional in their construction, with the y-axis as another component of observed variance. This third dimension helps to distinguish between what can be observed as points occupying the same place in a two dimensional construction. Correlations between two observations in Bi-plots are determined by the cosine θ. The variances between these observations are further determined by the distance from the origin. When observations are perpendicular to each other they are found to be independent, while those that are opposite to each other are said to be negatively correlated. A positive correlation was found between pre- and post-digestion protein concentration. Both the pre- and post-digestion protein content was negatively correlative to the DMD of the samples in

the data set. Additionally a significant correlation was observed between post-digestion ADF content and ADF digested. Protein digested and pre-digestion starch content is also correlated to each other, but negatively to post-digestion ADF content and ADF digested.

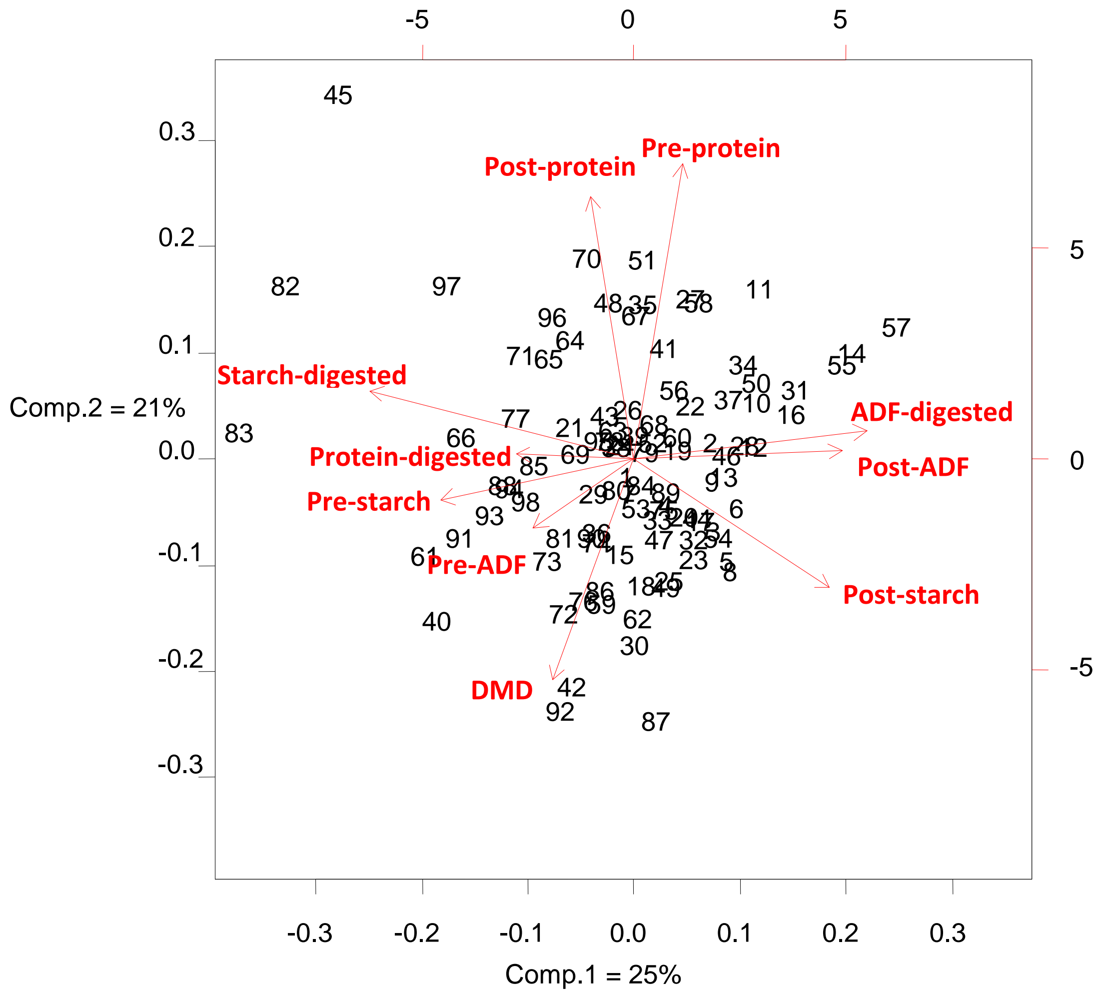

Figure 3.7: Bi-plot representing 46% variance of the data set explained. The variance of the bi-plot is broken down into two components found on the axis representing 25% (x-axis) and 21% (y-axis). The correlation between two points observed is derived by the determination of the *cosine* of θ. The distance between the two points is interpreted as the variance between the points.

3.4 Discussion

There are numerous factors that influence the rate and extent of digestion in the rumen of cattle. These factors include nutritional source, composition, processing of feed, feed consumption and microbial cultures. Also well know is that the grain testa or husk, determines and affects the rate of digestion (Pauly, Spörndly & Udén 2006). The distributions of observed feed values when plotted indicated there was transgressive segregation occurring within the population. This implies that the feed values were not purely influenced by environmental factors alone, but also by underlying genetic controls that affect feed quality. Numerous sources of variance were identified within the population. The identification of these sources of variance was not further pursued as financial constraints have limited sufficient replications of starch and ADF analysis.

The protein analysis had an adequate number of replications which made it possible to attribute sources of variance to the data set. The greatest contribution to variance was found to be field-plot replications while the Tallon x Scarlett genotypes did not contribute to the total variance. Protein concentrations in grain can vary due to environment. Thus the variance observed in field-plot replications was not unexpected. Additionally, the variance in post-digestion samples was greater than their wholegrain counterparts. This increase in variance was due to pooling of post-digestion samples. This pooling of samples has effectively doubled the observed variances in the data set. To overcome these limitations, the correlation analysis was thus performed on samples where specific sources of variance were manipulated for protein concentration and DMD. Additionally bacterial and animal enzymes are high in protein (nitrogen), thus the absorption (for digestion) of these by the sample temporarily increase the

protein content. However the loss of starch could be the main factor responsible for the increase of protein content and observed variance.

Nutritional digestion of samples was influenced by their nutritional and chemical characteristics (Kellems & Church 2002b). Therefore correlation analysis was performed to understand the interaction and composition of the sample grain. The positive correlation (r = 0.639) detected between pre- and post-digestion protein content and not with the other feed traits (pre-and post- digestion starch and ADF) suggests complex interactions within the rumen during digestion (Annison & Bryden 1998; Faichney 1996). However the correlations observed between pre- and post- digestion starch and ADF is best explained by the fact that the majority of the starch was digested, irrespective of the amount initially present in the samples.

A moderate negative correlation (r = -0.311) between protein digested and pre-digestion content indicates that increasing the protein content in the grain does not result in increased protein digestion, thus the rate of protein digestion is constant irrespective of concentration. This might indicate that the protein structure is involved in the inhibition of enzymatic activity from microbes. This results in an alteration of the proteolytic activity of microbes by affecting the availability and susceptibility of the protein peptide bonds (Stern, Bach & Calsamiglia 2006). The fore mentioned process is essential as it indicated that the digestion of proteins in the rumen was facilitated mostly by microbes. This allows the industry to potentially shift the site of protein digestion from the rumen to the small intestine where it is much more beneficial to the animal (Chalmers, Cuthbertson & Synge 1954).

Comparatively a positive correlation (r = 0.483) was found between post digestion protein concentration and amount of protein digested. It must be noted that the protein digested is a percentage of the total weight and not percentage of composition. It is also likely microbial

digestion of the husk results in protein synthesis (Pathak 2008) affecting the post-digestion nitrogen content as determined by the Vario Max N/CN Macro elemental analyser. The total nitrogen content was measured and the protein concentration was calculated accordingly. This can be problematic as there may be alternate sources of nitrogen in the samples other than protein.

The negative correlation (r = -0.219) between the post-digestion starch and protein suggests that one acts as an inhibitor of digestion for the other. The interaction between starch and protein is well known. The structure of the protein matrices prevents the binding of enzymes to the α-starch granules and β-starch granules (Ipharraguerre, Clark & Freeman 2005; Meissner & Preez 1996). This inhibitory effect of proteins could be a possible benefit to feedlots as the rapid digestion of starch in the rumen leads to a number of severe disorders (acidosis and bloat) that can result in animals loss (Hunt 1996).

The pre- and post-digestion protein content was negatively correlated to DMD with r = -0.537 and r = -0.248 respectively. Increasing the grain weight by increasing protein content does not alter the DMD of the grain. This is due to the need of enzymes and microbes to overcome the husk of the grain before digestion of the nutrient (starch and fibre) rich endosperm can occur. Additionally the more easily digestible starch is digested first from the samples, within the relative short incubation period used in this trial. The moderate correlation (r = 0.212) between protein digested and DMD could be attributed to the fact that the protein digested is a percentage of the total mass rather than its composition. This interaction can also be explained by the fact that protein contribute significantly to the barley kernel weight (Meissner & Preez 2006; Rebetzke 2007).

Starch is essential as an energy source for animals and is thus highly desirable by industry as a feed component. The significant correlation (r = 0.655) between pre-digestion starch content and starch digested is evident of this. It stands to reason the greater the initial starch content the greater the amount of starch digested from the sample, the less starch remains in the residue as observed in the negative correlation (r = -0.722) between post- digestion starch content and starch digested.

ADF digested was negatively correlated (r = -0.205) to starch digested. This is to be expected as the hydrolytic enzymes have to penetrate the fibrous husk before endosperm digestion can occur. The increase of ADF digestion in the rumen could have potential benefits in shifting the site of starch digestion to the small intestine, thus limiting adverse effects. The negative correlation (r = -0.243) between the initial ADF and ADF digested should be noted. According to the correlation a decrease in the initial ADF will result in the increase of ADF digested. The most probable explanation for this is that when the amount of substrate decreases and the reagent concentration remains the same the reaction time will be shortened. The expression of pre- and post-digestion ADF and ADF digested values is also misleading, as the comparisons made in the correlation analysis is between percentage composition and absolute weight loss rather than absolute mass *per se*.

Lastly the moderate correlation (r = 0.233) between the ADF content and DMD is of interest as it describes the metabolism of fibre in the rumen. Microbial hydrolytic and proteolytic activity has to break down the fibrous grain husks (testa) in the rumen which is a time consuming process. Increasing the ADF content will result in a minor increase of DMD. The increase in ADF content also has a secondary impact by decreasing feed intake and thus reducing the available energy to animals.

Chapter 4

4. NIR analysis on pre- and post-digestion barley grain samples.

4.1 Introduction

Near infrared spectroscopy (NIR) was developed in the 1950's as a means for quantitative analysis of grain quality and by the late 1970's it was routinely used for protein and feed quality (starch and fibre) analysis in forage (Norris et al. 1976). NIR analysers detect wavelengths within the electromagnetic spectrum between 730 and 2500 nm, which falls the near infrared region, making it particularly well suited to quantitative analysis (Givens & Deaville 1999). The NIR analysis is dependent on the detection of the presence and intensity of spectral bands resulting from overlapping overtones of corresponding chemical bonds, such as C-H, O-H and N-H. The main absorption bands of water are at 1940 and 1450 nm, aliphatic C-H bond absorption occurs at 2310, 1725, 1400 and 1210 nm, while O-H bonds are detected at 2100 and 1600 nm and N-H bonds at 2180 and 2055 nm (Givens & Deaville 1999). NIR data is generally subjected to a mathematical pre-treatment to reduce interference and measurement errors caused by sample particle size, moisture content, temperature and light scatter (Barnes, Dhanoa & Lister 1989). Additional mathematical treatments are performed on the data, as the NIR spectra absorbance is non-linear in nature and consist of a log/reflectance (Szalay et al. 2005).

The success of the technique relies heavily on the calibration set used to derive prediction equations. For the setup of the NIR calibrations, several different multivariate calibration methods can be used. This relates the spectral data from a sufficiently large and representative sample set to the primary, 'wet chemistry' data (Blanco et al. 1997). Finally, calibrations are subjected to validation procedures with an independent set of samples. A simple monitoring procedure has been developed to minimise NIR analysis errors (Shenk, Westerhaus & Abrams

1989). NIR calibrations should be based on at least 50 known samples (Deaville & Flinn 2000). However, many more are required (>150) for very high NIR reading accuracy.

Givens and Deaville (1999) point out that 'NIR is largely a secondary technique requiring calibration using samples of known composition determined by using standard methods (primary techniques)’. It has been stressed that consistent sample preparation is required, as variations in particle size, residual moisture content and packing density can adversely affect NIR spectra. Researchers are now aiming to predict directly from NIR spectra the functional properties of animal feeds with regard to nutrient supply and production responses such as live weight gain, milk, fat and protein or meat composition, rather than measuring feed components (Wrigley 1999).

NIR provides simultaneous, rapid and non-destructive predictions of components in many organic substances. On the other hand, the method requires a large number of samples for calibration and the high cost of the instrument limits the expansion of the methodology elsewhere. However, the technique is applicable to many food and agricultural commodities and is widely used in the cereal, oilseed, dairy and meat processing industries (Osborne, Fearn & Hindle 1993). NIR was specifically used for the estimation of barley protein concentration by Fox *et.al*. (2008) in a barley feed quality study. Barley starch, fibre and DMD have also been extensively measured by NIR (Osborne 2006). Thus NIR is widely in use at grain receival points for the assessment of moisture and energy, and given its unrivalled combination of speed, accuracy and simplicity (Osborne, Fearn & Hindle 1993), it holds the greatest potential of any strategy for the assessment of livestock feed ingredient quality.

The major objective of this segment of the research project was to use NIR as a rapid, non-destructive alternative analytical method for the prediction of phenotypic data collection.

These phenotypic parameters include dry matter digestibility (DMD), protein, starch and acid detergent fibre (ADF) concentration, which are all considered to be critical feed quality traits. The calibration equations used in the NIR analysis were internally developed by DEEDI for wholegrain samples. Even though the NIR calibration equations was whole grain specific, processed and post-digestion samples were also scanned to provide indicative feed quality data for individual lines in the Tallon x Scarlett RIL population.

4.2 Materials and methods

Barley grain (100 g) from each line was cracked with a Campo hand mill (Schnitzer, Germany) and the samples were stored in dry, air-tight containers. Subsets of the whole grain samples were milled with a “Rotor disk mill” at a 1 mm grind and separately stored in labelled and sealed containers. The dried post-digestion residue sample subsets were similarly milled and stored as indicated in Figure 4.1.

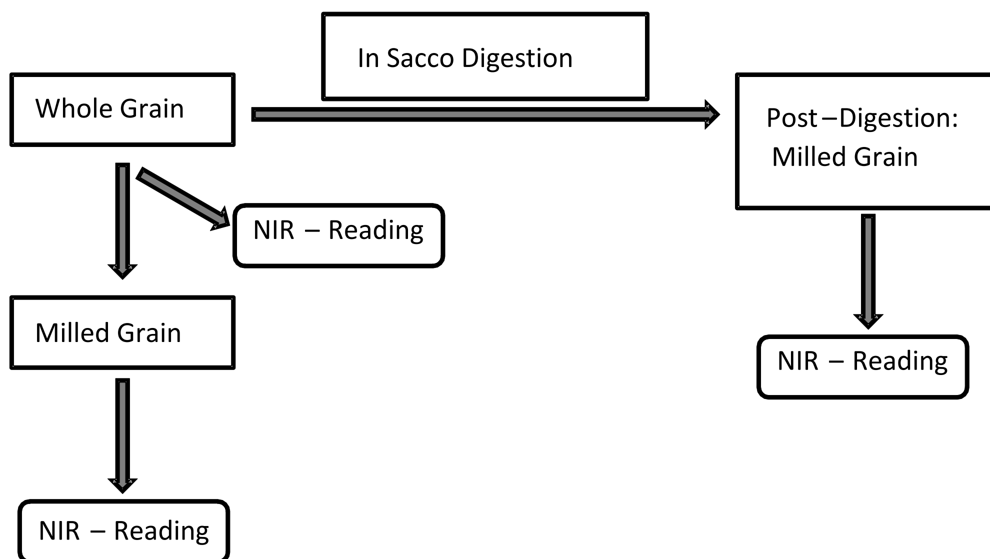

Figure 4.1: Flow diagram, of sample preparation and areas of NIR analysis and measurements.

NIR was performed on all grain samples with the use of the Foss NIR System 6500. The processed grain for analysis included the whole grain, milled grain (pre-digestion) and milled post-digestion grain residue. The calibrations and mathematical permutations for the NIR was setup and developed internally by the DEEDI for DMD and protein while starch and ADF calibrations were developed by the Premium Grains For Livestock Program (PGLP) (Black et al. 2005) using commercial winter and summer cereals. These calibrations were further refined and used by the Pork CRC (Cooperate Research Centre). Even though these calibration equations and mathematical permutations were developed for whole grain samples; processed and post-digestion samples were also analysed to provide indicative feed quality data. This allowed results within groups to be analysed and compared but not between groups. The samples were randomly loaded into a quarter cup cell and scanned. The predictions and replications were electronically recorded for further statistical analysis using ASReml (Gilmour, Thompson & Cullis 1995; Patterson & Thompson 1971), in similar manner as to the analysis performed in the previous two chapters. However, a sufficient number of experimental replications in NIR analysis data has allowed for the statistical manipulation of the data set. This allowed for the observation of the genotypic correlations and variance expressed across the Tallon x Scarlett population.

Once the data was recorded the samples were collected and stored in sealed containers for later analytical analysis as discussed in Chapter 3. The quarter cup cell was thoroughly cleaned with a brush to prevent any possible cross contamination between samples. The NIR readings had field level replication (n=168 of which the unique genotypes were n=117) that was of importance to the statistical analysis as this allowed for the identification of known and justified sources of variance. Statistical analysis was performed on the nutrient data (NIR predicted data) using ASReml-R. The data was analysed for the residual maximum likelihood using a linear

mixed model as describes in Chapter 3. The correlations between the NIR derived feed characteristics were calculated and expressed on a correlation matrix and bi-plot.

4.3 Results

A normal distribution across the sample population was observed for the pre-digestion and post-digestion ADF content of the sample grain, with both parents in their respective distribution curve falling on either side of the median (Figure 4.2). The data would suggest that NIR predicts an increase in ADF. As a result of digestion, NIR provides a higher evaluation of the ADF components within the fixed sample sizes measured.

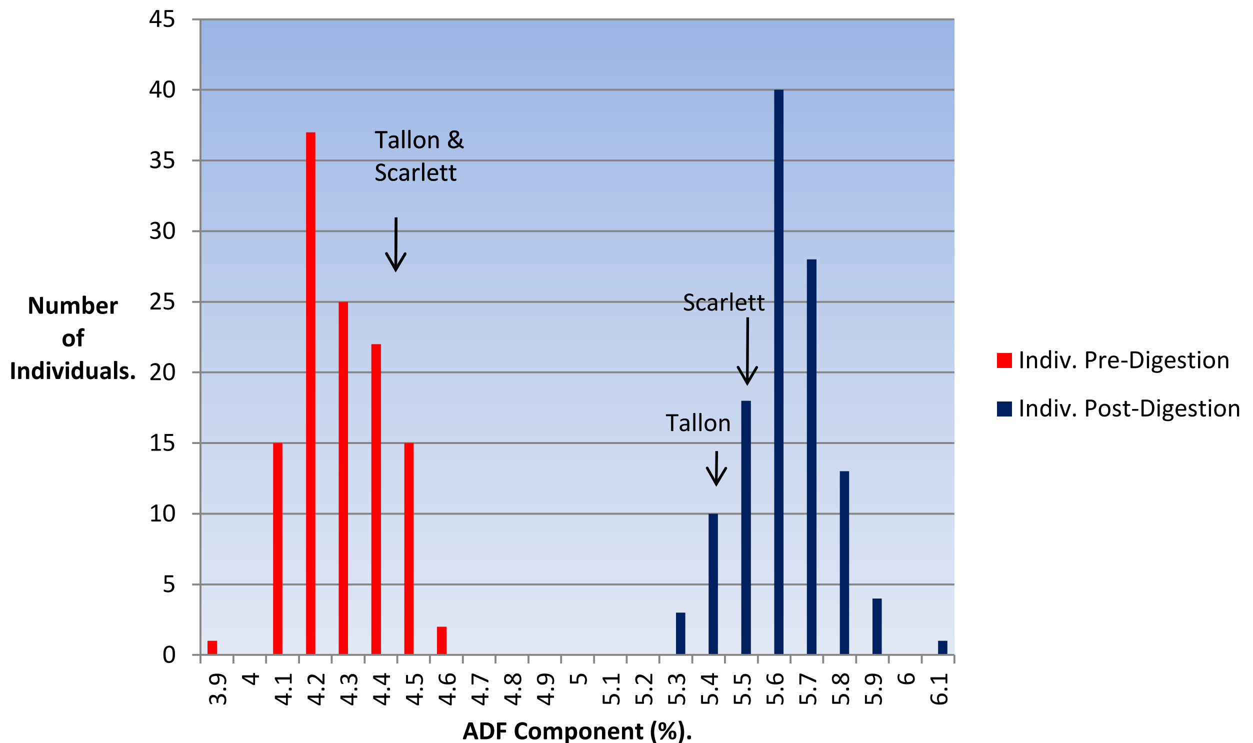

Figure 4.2: Pre- and Post-Digestion population distribution for the percentage ADF component across the lines of the Tallon x Scarlett population.

For protein content of the sample grain, a normal distribution across the sample population was observed for the pre-digestion and post-digestion, with both parents in their respective distribution curve falling on either side of the median (Figure 4.3).

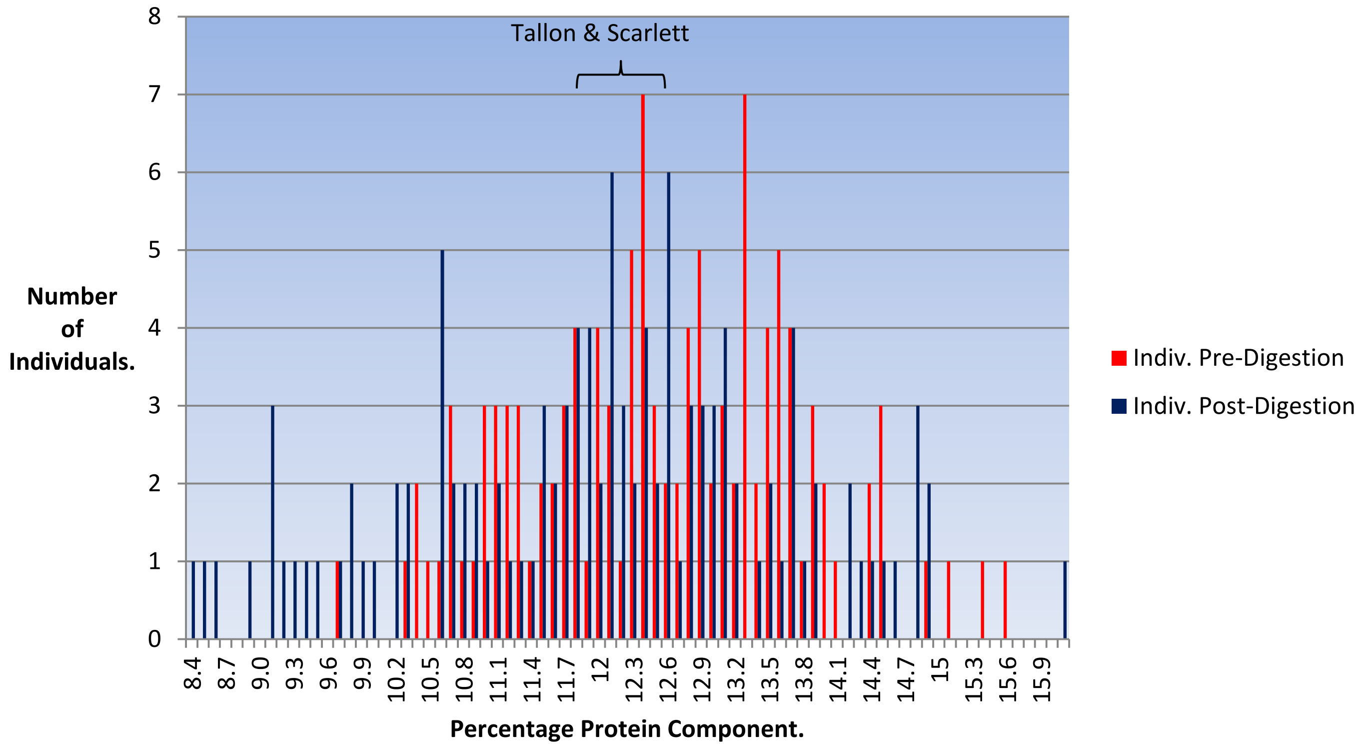

Figure 4.3: Pre- and Post-Digestion population distribution for the percentage Protein component across the lines of the Tallon x Scarlett population.

A negatively skewed population distribution was observed for the pre-digestion starch content of the sample grain, whilst a normal distribution was observed for the post-digestion sample population, with both parents in their respective distribution curve falling on either side of the median (Figure 4.4).

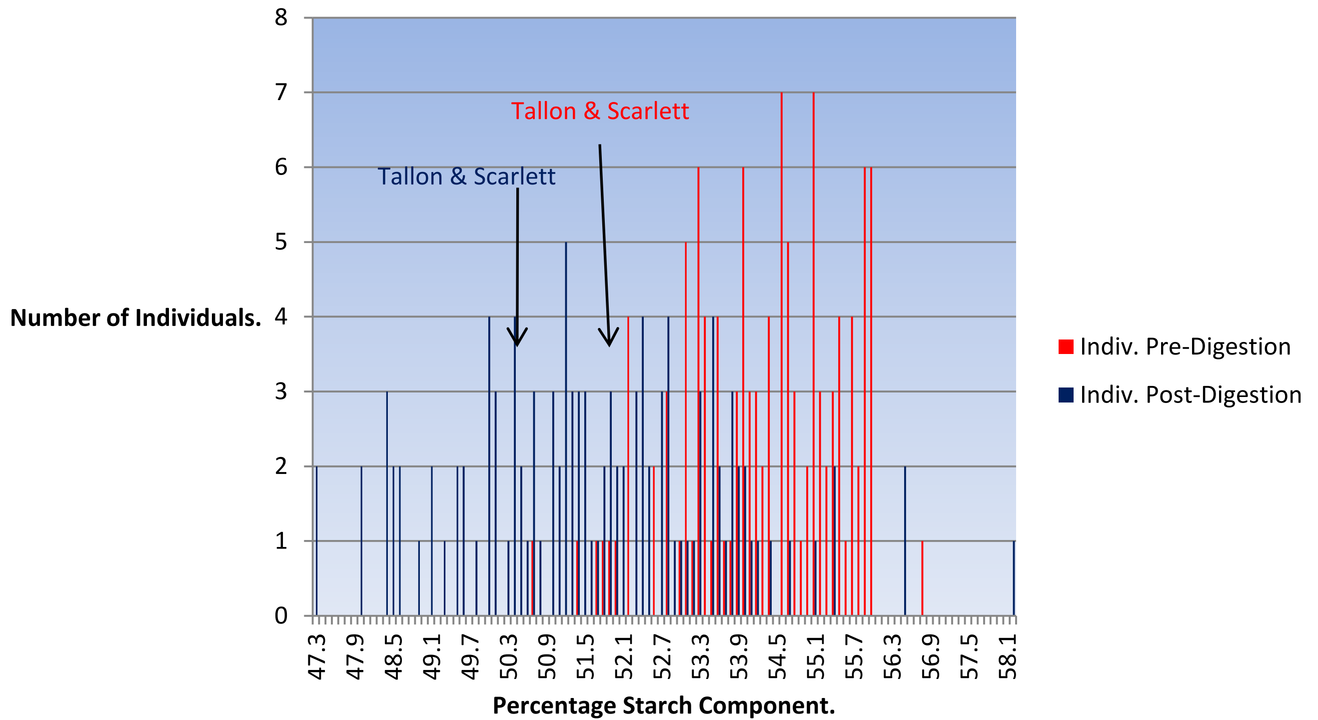

Figure 4.4: Pre- and Post-Digestion population distribution for the percentage Starch component across the lines of the Tallon x Scarlett population.

The genotypic correlations between feed quality traits pre- and post-digestion were achieved by attributing sources of variance to the data set by using a mixed linear model (Table 4.1). Both the pre- and post-digestion NIR derived data set (Table 4.1) indicated that both the ADF and protein content for pre- and post digestion were perfectly correlated (r = 1.00). Pre-digestion starch content was also significantly correlated (r = 0.69) to its post-digestion content. Numerous other moderate and weak correlations were also determined and expressed in the correlation matrix (Table 4.1), some of which will be referred to in the discussion.

Significant negative correlations were detected between pre-digestion protein and both the pre- (r = -0.62) and post-digestion ADF concentrations (r = -0.63) and the pre- (r = -0.78) and post-digestion (r = -0.66) starch concentrations. Numerous other moderate and weak correlations were

also determined and expressed in the correlation matrix (Table 4.1), some of which will be referred to in the discussion.

Table 4.1: Correlations between feed quality traits both pre- and post digestion for Tallon x Scarlett, as measured with NIR.

		ADF:		**Starch:**		**Protein:**		**DMD:**
		Pre-	**Post-**	**Pre-**	**Post-**	**Pre-**	**Post-**	
ADF:	Pre-digestion	1						
	Post-digestion	1.00*	1					
Starch:	Pre-digestion	0.28	0.28	1				
	Post-digestion	0.28	0.27	0.69*	1			
Protein:	Pre-digestion	-0.62*	-0.63*	-0.78*	-0.66*	1		
	Post-digestion	-0.62*	-0.63*	-0.78*	-0.66*	1.00*	1	
DMD:		0.26	0.27	0.19	-0.28	-0.31	-0.31	1

*Indicates significant correlations.
**A significance level of 0.05 was used for the correlation analysis.

Statistical analysis on the NIR data set has identified sources of variance other than the genetic expression of the sample (Table 4.2). These sources of variance were identified and accounted for to determine the maximum genetic expression for each trait. The variance and correlations between the genetic expression and processing technique (whole and milled grain) was also examined. The genotypic variance expressed for different processed grain analysis found no statistical significance (P = 0.05) across the study and was thus not reported.

Table 4.2: Variance observed for the feed quality traits of interest in pre- and post-digestion samples across the Tallon x Scarlett population.

	Mean (%).	Genetic Variance.	Error Variance.
ADF:			
Pre-Digestion.	5.316	0.006	0.023
Post-Digestion.	5.621	0.005	0.012
Protein:			
Pre-Digestion.	8.676	0.982	0.523
Post-Digestion.	11.952	1.062	0.669
Starch:			
Pre-Digestion.	59.630	0.450	1.500
Post-Digestion.	51.870	1.910	3.000

With ADF, the main source of variance is experimental error (error variance), which is relatively small and of the same magnitude for both pre- (Var. = 0.023) and post-digestion (Var. = 0.012).

With protein, the main source of variance observed is contributed by genetic variance with the per-digestion (Var. = 0.98) and post-digestion variance (Var. = 1.062) being of the same magnitude when experimental error is taken into account. Furthermore the pre- and post-digestion protein content determined for different processing techniques, were significantly correlated (r = 1.00), when the nuisance variance was statistically manipulated within the data set.

The variance observed for starch is prominently expressed by both genetic and error variance, with post-digestion variance for both the genetic (Var. = 1.91) and error variance (Var. = 3.00) being the dominant contributing factor. Thus resulting in a moderate correlation (R^2 = 0.48) as seen in Figure 4.5, between pre- and post-digestion starch content.

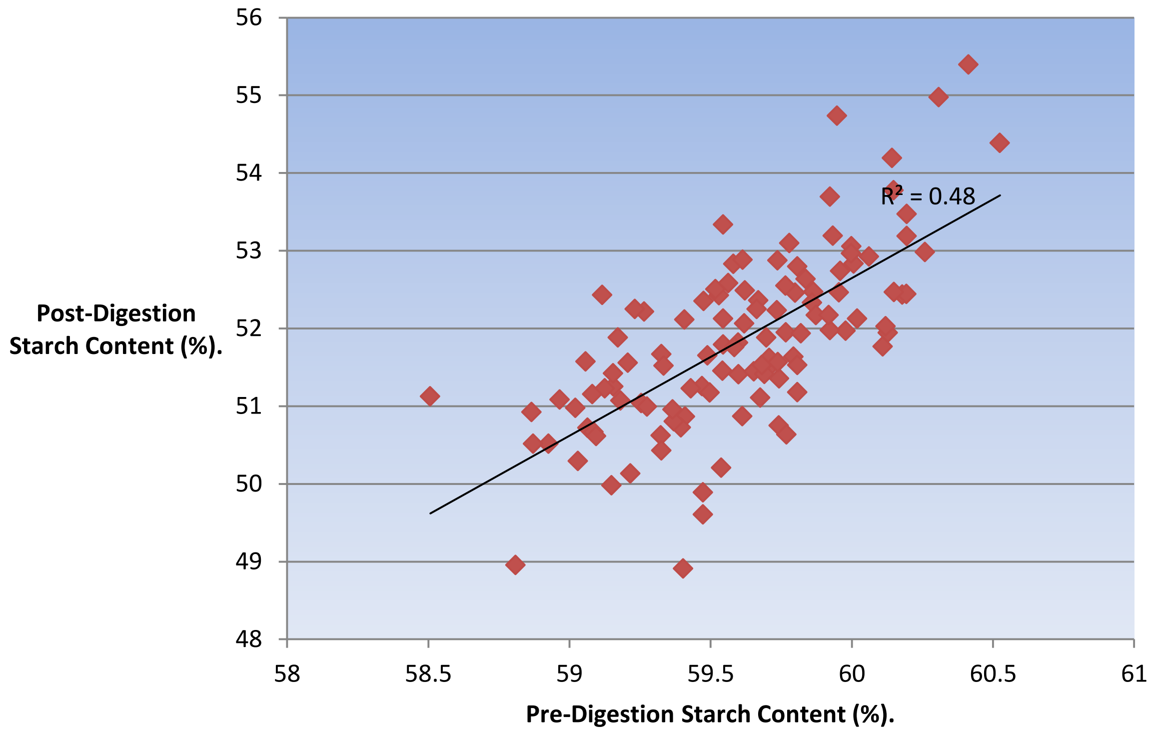

Figure 4.5: Correlation between pre- and post-digestion Starch content across the Tallon x Scarlett population, with the nuisance variables statistically manipulated within the NIR data set.

Bi-plots summarise the feed quality traits and exhibit similar correlations to that of the correlation matrix (Table 4.1). These correlations are presented two dimensionally on a graph explaining the observed variance in the data set (Figure 4.6). The variance observed in Figure 4.6 is 81% which can be broken down into two major components. Components one accounts for 59.7% and component two 21.3% respectively, and are represented on each axes of the graph. Bi-plots can also be three dimensional in their construction, with the y-axis as another component of observed variance. For a more detailed description of bi-plots refer to the results section of Chapter 3.

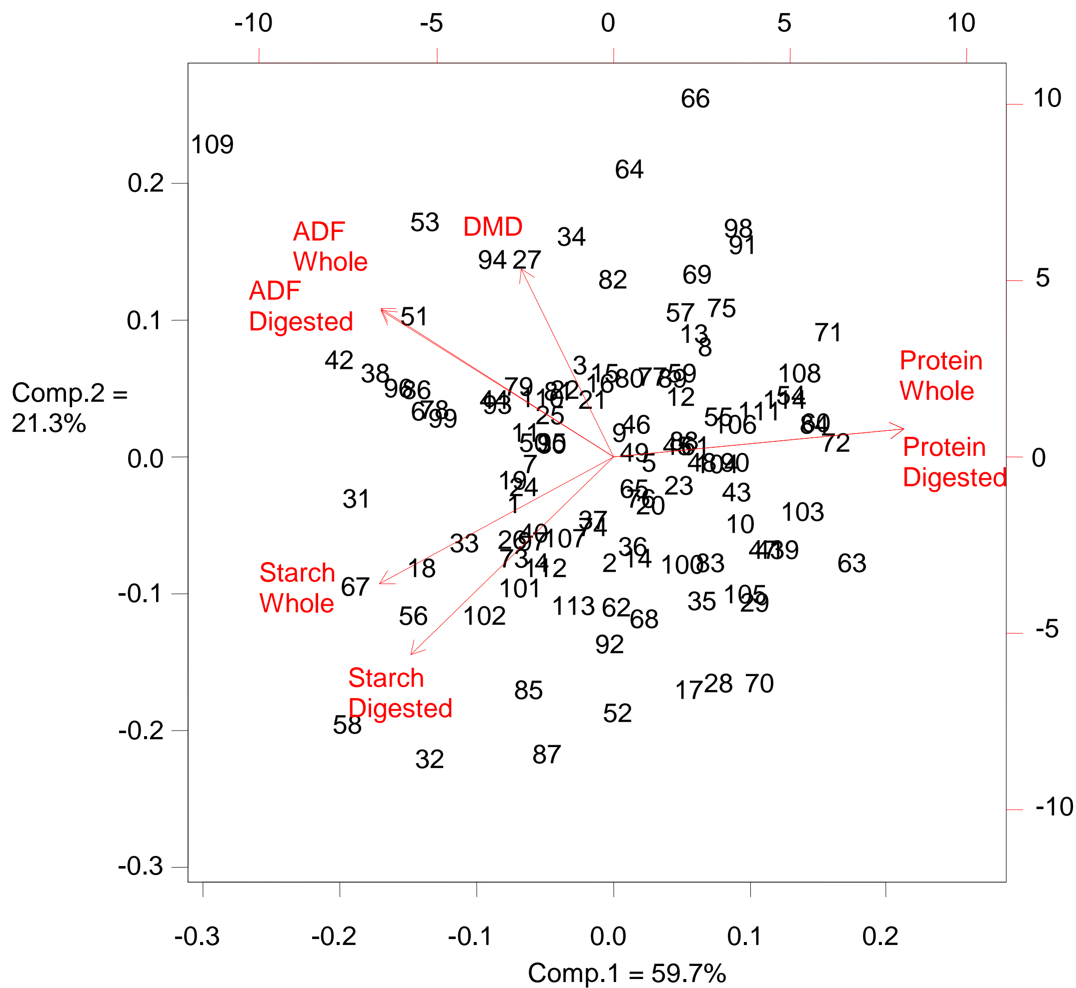

Figure 4.6: Bi-plot representing 81% variance of the data set explained. The variance of the bi-plot is broken down into two components found on the axis representing 59.7 % (x-axis) and 21.3% (y-axis). The correlation between two points observed is derived by the determination of the *cosine* of θ. The distance between the two points is interpreted as the variance between the points.

4.4 Discussion

Analysis on the NIR derived data showed a significant correlation between the pre- and post digestion feed traits ADF (r = 1.00), starch (r = 0.690) and protein (r = 1.00). These correlation values indicated that the genotypic expression of the Tallon x Scarlett lines did not influence the biochemical hydrolysis of substrate in the rumen. Of greater significance is the interaction between these feed traits.

The high number of field replications (n = 168 of which 117 lines where genotypically unique), made it possible to account for external sources of variance from the NIR data set. Thus the correlations and variance for each feed quality trait was individually examined.

4.4.1. ADF

Through analysis of variance (maximum likelihood) and fitting a mixed linear model to the data set no observable genotype x processing interaction was found as shown in Table 4.2. The greatest contributing factor to the observed variance in the data set was due to experimental error (Var. = 0.023). This low variance observed in the data set for the ADF content could be attributed to the fact that ADF is indigestible in the rumen. The fluctuations in the ADF content could be contributed to ADF particles escaping the rumen and passing through the intestine. More likely the fact that the experimental error is the greatest contributing factor to variance, would suggest that washing and preparation of the post ruminal digestion samples were responsible for the observed variance.

4.4.2. Protein

Correlation analysis found that the pre- and post-digestion grain protein content was significantly correlated to each other (r = 1.00). The extensive use of NIR to determine protein content may have been a contributing factor; as extensive use has allowed the development of accurate calibration equations across a number of barley grain varieties. The analysis of variance found that the genetic variance for pre- (Var. = 0.98) and post-digestion (Var. = 1.06) were the greatest contributing factors to the observed variance across the population with nuisance (that had no effect) parameters statistically manipulated. The use of protein for the determination of genetic variance of sample lines is thus ideal, and can partially be attributed to its relative structural stability.

4.4.3. Starch

The correlation analysis of pre- and post-digestion starch content found a strong correlation (r = 0.69) when sources of nuisance variance (external sources of variance that can be accounted for e.g.: human error, sample size etc.) was statistically manipulated in the data set. It was found that the genetic variance (Var. = 1.91) and error variance (Var. = 3.00) in the processing of pre- and post-digestion starch sample were the greatest contributing factor to the observed variance. This high degree of variance observed in the processing of the pre- and post-digestion samples translates into a moderate correlation ($R^2 = 0.48$) for processing (Figure 4.5). The variance and correlations observed in the different analysis suggests starch could be successfully used in the determination of genetic variance in the sample population. However caution needs to be taken due to the magnitude of error variance. This high error variance in the sample processing can be attributed to the relative instability of the starch structure and the ease with which it hydrolyses and denatures. Furthermore the error can also be attributed to the methodologies of grain processing prior to NIR analysis.

4.4.4. General

From the analysed data it is clear that the NIR calibration equations developed for wholegrain samples should not be used on processed nor post-digestion samples. For some feed traits where NIR has been extensively used and additional calibration equations have been developed, the analysed data seemed to be more uniform in nature. This indicated the need for the development of additional calibration equations and mathematical permutations for processed grain samples and digested residue. Since this research project was the first time that NIR has been extensively used in analysis of feed values including that of post-digestion samples, such calibration and scattering equations were not available.

Interaction between the different feed quality traits was also investigated. Significant; moderate and negative correlations were observed across the data set. A low correlation (r = 0.28) was found between pre-digestion ADF content and both pre- and post-digestion starch content. A similar correlation (r = 0.28 and r = 0.27) was to found between post-digestion ADF and pre- and post-digestion starch content. This anomaly of increasing ADF results and an increase in starch could possibly be explained by the high concentration of β-glucans and glucose molecules as structural components of the cell wall and fibre. It may also be possible that the diffraction of these bonds during NIR analysis may be interpreted as starch granules rather than fibre composition.

The greatest amount of starch digestion occurs in the rumen. By potentially increasing the fibre content, the amount of energy available to the animal increases as well as shifting the site of major starch and protein digestion (Hunt 1996; Kotarski, Waniska & Thurn. 1992). This could potentially increase the energy availability to the animal and maximise nutrient uptake in the small intestine. An additional bonus of this digestive site shift is the elimination of acidosis and bloat due to rapid starch digestion in the rumen (Hunt 1996).

Pre- and post-digestion protein content is negatively correlated to pre-digestion (r = -0.62) and post-digestion (r = -0.63) ADF. This could imply that when a significant increase in ADF content occurs, a decrease in protein content and subsequently protein digestion will follow. This is due to the fact that fibre prevents enzymes binding and microbial digestion. Fibre also provides microbes with energy essential for their biochemical functioning. This energy is used in protein synthesis from which the majority of the animals' protein requirements are satisfied (Swanson et al. 2002).

This protein synthesis reduces the need for protein digestion in the rumen. In animals with high protein demands such as steers and lactating cows the digestion of protein in the small intestine is essential, and achievable through the protein ADF interaction.

Pre- and post-digestion protein is negatively correlated to pre-digestion starch ($r = -0.78$) and post-digestion starch ($r = -0.66$). It has been documented that protein has an inhibitory effect on starch digestion. This inhibition by protein functions by actively reducing the binding sites for enzymes and microbes to the starch granule. The data suggests that increasing the protein content would alter the energy availability from either starch or fibre as primary sources. Even though protein is a major contributor to kernel weight, both the pre- and post-digestion protein content had a moderate negative correlation ($r = -0.31$) to DMD. The reason for this is that the fibrous husk prevents endosperm digestion. This hypothesis is substantiated by the fact that a moderate negative correlation is found between DMD and starch ($r = -0.28$). There was however a moderate correlation between DMD and pre-digestion ($r = 0.26$) ADF and also with post-digestion ($r = 0.27$) starch which further supports the hypothesis that the fibrous husk rather than the endosperm contributes to DMD. This contribution to DMD is purely in the rumen after a three hour incubation period.

Chapter 5.

5. Molecular mapping

5.1. Introduction

Marker-assisted selection (MAS) was proposed by Lande and Thompson (1990) as an effective method to screen for complex traits in the early generations of a breeding program. Barr et al. (2001) identified nine key steps in the identification of molecular marker(s) linked to traits for use in MAS. One of these steps includes the construction of a linkage map of the population to locate a quantitative trait locus (QTL) associated with the trait(s) of interest. A QTL is the location of a gene that affects a trait and is measured on a quantitative or linear scale (Lande & Thompson 1990; Tanksley 1993). QTL identification and analysis is comprised of four major components. Firstly the selection of a segregating population; secondly the presence of a segregating marker on the population of interest; thirdly the collection of phenotypic data on traits of interest for each individual in the population and lastly, the correlation of the phenotypic data to the genotypic data using an appropriate statistical approach.

The correlation of the genotypic and phenotypic data to specific regions of the genome at specified levels of statistical probability is used in the identification of QTLs. The mapping of a QTL is not as simple as mapping a major gene that affects a qualitative trait. Single marker analysis has traditionally been the tool used to detect a QTL in the vicinity of a marker studied individually. This approach was first described when the linkage between seed colour and bean weight was studied in *Phaseolus vulgaris* (Sax 1923). The differences in the phenotypic mean presents an approximation of the phenotypic effect of each allele at a specified QTL. The basis of this method is the classification of the offspring into one of two groups depending on the genotype at the marker location (Collard et al. 2005). Calculation of the mean trait value of each

offspring class and the subsequent comparison of the mean trait values are performed to obtain significant differences (Collard et al. 2005). Simple statistical tests such as t-tests, regression analysis and analysis of variance are used to determine whether an inferred phenotypic effect is significant (Liu 1998). Any significance obtained with these statistical tests indicates that a QTL is linked to the marker. The further a QTL is from the marker, the less likely it is to be detected statistically due to crossover events occurring between the marker and gene during meiotic events. Software programs are available for statistical analysis which is able to detect QTLs by identifying associations between the marker genotype and the quantitative trait phenotype.

Simple interval mapping (SIM) is another approach for QTL analysis. Interval mapping is based on the principle of testing a model for the presence of a QTL at various positions between two mapped maker loci. The model utilises regression and the maximum likelihood method involving the searching of QTL parameters that give the best estimate for quantitative trait distributions observed for each marker class (Brown 2006; Collard et al. 2005). An evaluation of the model is performed by computing the probability of observed distributions with and without fitting a QTL effect. The position of the QTL is derived by the maximum likelihood from the distribution of the likelihood values calculated for each locus (Collard et al. 2005). As the genotype of the QTL is unknown it is replaced by probabilities estimated from the nearest flanking markers. Regression mapping gives estimations of QTL positions and effects that are frequently identical to those given by the maximum likelihood method. The LOD scores are statistical measurements of linkage as revealed by analysis. These scores are the ratios of the probability that an effect occurs because of linkage versus the probability that the effect occurred by random chance. When multiple QTLs per linkage group are present, this method could fail to detect any effect or could detect “suggestive” QTLs (Martinez & Curnow 1992). These

"suggestive" QTLs are found not to be statistically significant as their LOD score falls below the chosen threshold. One of the factors that weakens interval mapping is fitting the model for a QTL at only one location. This approach is problematic in that the effects of additional QTLs contribute to sampling variance and the linkage of multiple QTLs will cause biased estimates. It also allows a QTL with large effects to mask other QTLs with smaller effects.

Composite interval mapping (CIM) was developed as a solution to the drawbacks of the simple interval mapping (Jansen & Stam 1994; Zeng 1994). QTL analysis in CIM is similar to interval mapping, except that variance from other QTLs is accounted for. This is achieved by the inclusion of partial regression coefficients from markers in other regions of the genome. CIM gives more power and precision than SIM because the effects of other QTLs are not present as residual variance. CIM is able to remove any bias caused by QTLs linked to the position being tested. Currently numerous software packages are available that can perform composite interval mapping (QTL Cartographer, Map Manager QTX, etc.). These programs are also able to perform simple interval mapping.

The aim of the research described in this Chapter was to identify significant QTLs associated with cattle feed quality traits within the Tallon x Scarlett population. The phenotypic data on pre- and post-digestion samples for the QTL analysis was collected through both conventional analytical chemistry techniques and NIRs. These data sets were then compared to determine whether the two phenotypic methods would link particular quality traits to similar sets of QTL. Post-digestion data was also subjected to QTL analysis in an attempt to identify additional QTL and thus increase our understanding of the ruminal digestion of barley grain.

5.2. Methodology

Phenotypic data for starch, protein and fibre content of pre- and post-digestion grain samples was collected as described in Chapters 2 & 3. Composite Interval Mapping was employed to detect QTLs and estimate the magnitude of their effects (Jansen and Stam, 1994; Zeng 1994). The genome was scanned at 2-cM intervals using stepwise regression analysis in QTL Cartographer 2.0 (Wang, Basten & Zeng 2002). The percentage of phenotypic variance (R2) explained by a specific QTL was taken at the peak of the QTL. A series of 1000 permutations were run to determine the experiment-wise significant level at P = 0.05 of Logarithm of Odds (LOD) for the trait (Churchill & Doerge 1994). Both the significant and suggestive QTLs were graphically drawn on an available map of the Tallon x Scarlett genetic map using MapChart 2.2 (Voorrips 2002).

The genetic map of the Tallon x Scarlett population was developed during the 1990s in Australia as part of the National Barley Molecular Marker Program. These parents were crossed by the Queensland Department of Employment, Economic Development and Innovation (DEEDI) in the attempt to identify malting quality QTLs. Tallon is a malting variety developed by the Queensland Breeding Program and Scarlett, a malting variety from Europe. .

Seeds of each line of the Tallon x Scarlett population were germinated in microplates on moist tissue paper. The microplates were incubated in a dark, temperature controlled Thermoline L+M incubator at 25°C for two weeks. The top seven centimetres of the leaf blades were harvested and immediately placed on ice to ensure no DNA hydrolysis was occurring. The harvested leaf material was stored in labelled 1.5 ml Eppendorf tubes and kept in a -20 °C freezer until the DNA was extracted.

DNA extractions were performed on the frozen samples using a Wizard® Genomic DNA Purification kit in accordance with the manufacturer's specifications (Appendix 17). After completion of the DNA extractions and subsequent re-suspension of the DNA in 100 µl of de-ionized water, the samples were kept at room temperature for 24 hours before being stored in a refrigerator (5°C). The quality of the DNA samples was determined by Agarose gel electrophoresis (Appendix 18). The gel was visualized using a Bio-Rad UV cabinet. After the quality of the extracted DNA was ascertained the individual DNA concentrations were determined with an Implen NanoPhotometer (Integrated Sciences). DNA samples were diluted to a concentration of 20 ng/µl of DNA.

Primer sequences for SSR markers were obtained from GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml) and a standard protocol was used to amplify the markers. The reaction mixture consisted of 20 ng DNA, 5 µM of each primer, 100 µM of each dNTP, 1.5 mM $MgCl_2$, 1x buffer (Bioline Pty Ltd., Australia) and 0.1 U Immolase™ DNA polymerase (Bioline Pty Ltd., Australia) in a total volume of 10 µl. The following polymerase chain reaction (PCR) cycle profile was used: 5 min at 98°C, followed by 35 cycles at 94°C for 30s, 50-65°C (depending on annealing temperature) for 30s and 72°C for 2 min and one cycle at 72°C for 7 min. The amplified products were visualized using a Gel-Scan 2000™ (Corbett Life Sciences, Sydney, Australia).

5.3. Results

Attempts were made to add markers (Appendix 18) to the Tallon x Scarlett map through the screening of SSR markers in regions of low marker density. This potential SSR marker list for filling the map was provided by DEEDI. None of the markers tested were polymorphic in the population and markers could therefore not be added to the existing Tallon x Scarlett map. The

QTL analysis of the phenotypic feed quality data was performed on the existing version of the Tallon x Scarlett molecular map. This map consists of 129 DArTs, 21 SSRs and 83 AFLPs markers with a total map distance of 1224.5 cM. The average distance between markers was 5.3 cM.

Composite interval mapping was used to determine whether any of the markers on the Tallon x Scarlett map are linked to the phenotypic feed quality data obtained through analytical chemistry. The permutation tests indicated that a LOD score of 2.7 or greater was significant. Three regions on the Tallon x Scarlett genome were identified which showed a significant linkage to a feed quality trait (Table 5.1 and Figure 5.1a & 5.1b). A QTL for total digested protein was identified on chromosome 2H between markers bPb-1611 and bPb-6296, and explained 13% of the phenotypic variance. QTLs for DMD were identified on chromosomes 5H and 7H. The QTL on chromosome 5H was the most significant (LOD = 5.0) and explained 19% of the phenotypic variance. It was located between the flanking DArT markers bPb-8072 and bPb-1820. The QTL on 7H was located between flanking markers bPb-1767 and bPb-0758 and explained 9.9% of the phenotypic variance. The QTLs on 2H and 5H were contributed by Tallon whereas the QTL on 7H was contributed by Scarlett.

A number of suggestive QTL for pre-, post-digestion protein and digested protein were also identified (Table 5.1) with LOD scores ranging from 1.6 to 2.4. These QTLs explained between 5.7 and 14.4% of the phenotypic variance. Suggestive QTL for DMD were identified on chromosomes 1H, 3H, 5H, 6H and 7H and explained between 5.9 and 9.1% of the phenotypic variance.

Table 5.1: Summary of QTL associated with feed quality traits in a Tallon x Scarlett population. The phenotypic data was derived through analytical chemistry as described in Chapters 2 and 3.

Trait		Chromosome	Flanking Markers	LOD	Phenotypic Variance (%) Explained	Parent
Significant QTLs						
Protein:	Digested	2H	bPb-1611 - bPb-6296	3.6	13.0	Tallon
DMD		5H	bPb-8072 - bPb-1820	5.0	19.0	Tallon
		7H	bPb-1767 - bPb-0758	3.3	9.9	Scarlett
Suggestive QTL						
Protein:	Pre-Digestion	1H	bPb-3660 - CTC 206	1.8	5.7	Tallon
		5H	nAG/CAC 348 - bPb-4621	1.7	10.0	Scarlett
		7H	bPb-0259 - hAC/CTG122	2.1	7.0	Scarlett
	Post-Digestion	1H	bPb-3756 - CTC 206	1.8	5.9	Tallon
		5H	nAG/CAC348 - bPb-4621	1.6	9.6	Scarlett
		7H	bPb-0259 - hAC/CTG122	2.1	7.2	Scarlett
	Digested	1H	fGA/CCC 137 – bPb-3984	1.6	14.4	Scarlett
		2H	bPb-1611 - bPb-0299	2.4	8.2	Tallon
DMD:		1H	fAT/CTC 122 - fAT/CTG 88	2.1	5.9	Scarlett
		3H	hAA/CGT107 - fAA/CCG 148	2.4	9.1	Tallon
		5H	fAT/CTG 267 - bPb-8072	1.9	6.5	Tallon
		6H	bPb-3919 - bPb-2410	1.6	7.9	Scarlett
		7H	bPb-3484 - fAC/CAC 245	2.6	7.4	Scarlett

QTL analysis of phenotypic data derived through NIR analysis (Chapter 4) identified nine QTLs for both pre- and post-digestion feed quality traits (Table 5.2), with one of which between bPb-3278 - fAA/CCG148 on chromosome 3H linked to several related characteristics. These feed quality QTLs are associated with the four feed quality attributes, dry matter digestibility (DMD), acid detergent fibre (ADF), protein and starch content.

The most significant NIR derived QTLs (LOD = 6.0) were for digested protein and pre-digestion starch content. These QTLs were located in the same region on chromosome 3H between flanking markers bPb-3278 and fAA/CCG 148, and explained 26.4% and 24.5%, respectively of the phenotypic variance. QTLs for pre- and post-digestion protein and post-digestion and digested starch were also identified in the same region of chromosome 3H (Figure 5.1)

Table 5.2: Summary of QTL associated with feed quality traits in a Tallon x Scarlett population. The phenotypic data was derived through NIR analysis as described in Chapter 4.

Trait		Chromosome	Flanking Marker	LOD	Phenotypic Variance (%) Explained	Parent
Significant QTLs						
Protein:	Pre-Digestion	3H	bPb-3278 - fAA/CCG 148	4.1	19.0	Scarlett
	Post-Digestion	3H	bPb-3278 - fAA/CCG 148	4.1	19.0	Scarlett
	Digested	3H	bPb-3278 - fAA/CCG 148	6.0	26.4	Scarlett
Starch:	Pre-Digestion	3H	hAA/CGT 331 - hAA/CGT144	2.8	9.2	Scarlett
		3H	bPb-3278 - fAA/CCG 148	6.0	24.5	Scarlett
		3H	bPb-9599 - fGA/CCC262	3.4	10.3	Scarlett
	Post-Digestion	3H	bPb-3278 - fAA/CCG148	4.5	19.1	Scarlett
	Digested	7H	hAC/CGT 144 - fAG/CAT63	3.3	14.1	Scarlett
ADF	Digested	5H	fAA/CAC 178 - nAG/CCA125	3.1	10.0	Tallon
Suggestive QTLs						
Protein:	Pre-Digestion	3H	bPb-3332 - hAA/CGT331	2.5	8.6	Tallon
		3H	bPb-9111 - bPb-9599	2.3	7.4	Scarlett
		7H	nGA/CGG 97 - Bmag321	1.9	5.8	Tallon
		7H	bPb-8833 - HVM 49	1.8	9.5	Tallon
	Post-Digestion	3H	bPb-3332 - hAA/CGT331	2.5	8.6	Tallon
		3H	bPb-9111 - fGA/CCC262	2.3	7.4	Scarlett

		7H	nGA/CGG 97- Bmag 321	1.9	5.8	Tallon
		7H	hAA/CGT142 - bPb-8644	1.8	9.5	Tallon
	Digested	3H	hAA/CGT331 - hAA/CGT144	2.5	8.9	Tallon
		3H	bPb-9111 - fGA/CCC262	2.4	8.2	Scarlett
		7H	HVM49 - bPb-2854	1.6	7.7	Tallon
Starch:	Pre-Digestion	3H	bPb-7448 - fAA/CCG234	1.6	6.3	Scarlett
		3H	hAA/CGT311 - hAA/CGT144	2.7	9.6	Scarlett
		3H	bPb-4616 - fAA/CCG 148	2.1	6.8	Tallon
		3H	bPb-9111 - bPb-9599	2.2	7.4	Tallon
		7H	nGA/CGG 97 - Bmag 321	2.3	6.9	Scarlett
	Post-Digestion	3H	bPb-4616 fAA/CCG 148	2.5	10.0	Scarlett
		3H	bPb-4616 - fAA/CCG 148	1.6	5.5	Scarlett
		3H	bPb-9111 - fGA/CCC 262	1.6	5.5	Scarlett
		4H	fAA/CCT145 - bPb-1325	2.4	11.8	Scarlett
		7H	bPb-8833 - HVM 49	2.2	15.5	Tallon
	Digested	7H	fAT/CTC173 – bPb-3484	2.7	15.0	Tallon
		7H	hAC/CGT144 – bPb-3484	1.9	6.6	Tallon
ADF:	Pre-Digestion	4H	bPb-3468 – HVM67	2.5	16.6	Tallon
		5H	fAA/CAC178 - nAG/CCA125	1.6	6.3	Tallon
		7H	HVM49 - bPb-2854	2.2	10.0	Tallon
	Post-Digestion	4H	bPb-3468 - HVM67	2.7	19.0	Tallon
		6H	bPb-6385 - fAA/CCA 227	1.8	7.4	Tallon
	Digested	3H	bPb-3278 - hAA/CGT 107	1.7	8.0	Scarlett
		4H	bPb-1325 - bPb-3468	2.6	19.8	Tallon
		5H	bPb-7407 - bPb-1820	2.5	10.4	Tallon
		5H	fAA/CCG 279 - nAT/CAT79	1.9	6.8	Scarlett
		6H	bPb-6385 - fAA/CCA 227	1.6	6.7	Tallon

Suggestive QTLs for a number of traits were also detected on chromosome 1H (Protein, DMD), 2H (Protein), 3H(Protein, ADF, Starch, Fibre), 4H (ADF, Starch), 5H(Protein, ADF, DMD), 6H (ADF, DMD) and on 7H (Protein, ADF, Starch, DMD) (Table 5.1 and Table 5.2). The phenotypic variance explained ranged between 5.5% and 24.5% across the genome for these traits. Some of these suggestive traits form part of the base of the significant QTLs but still fell below the LOD threshold point.

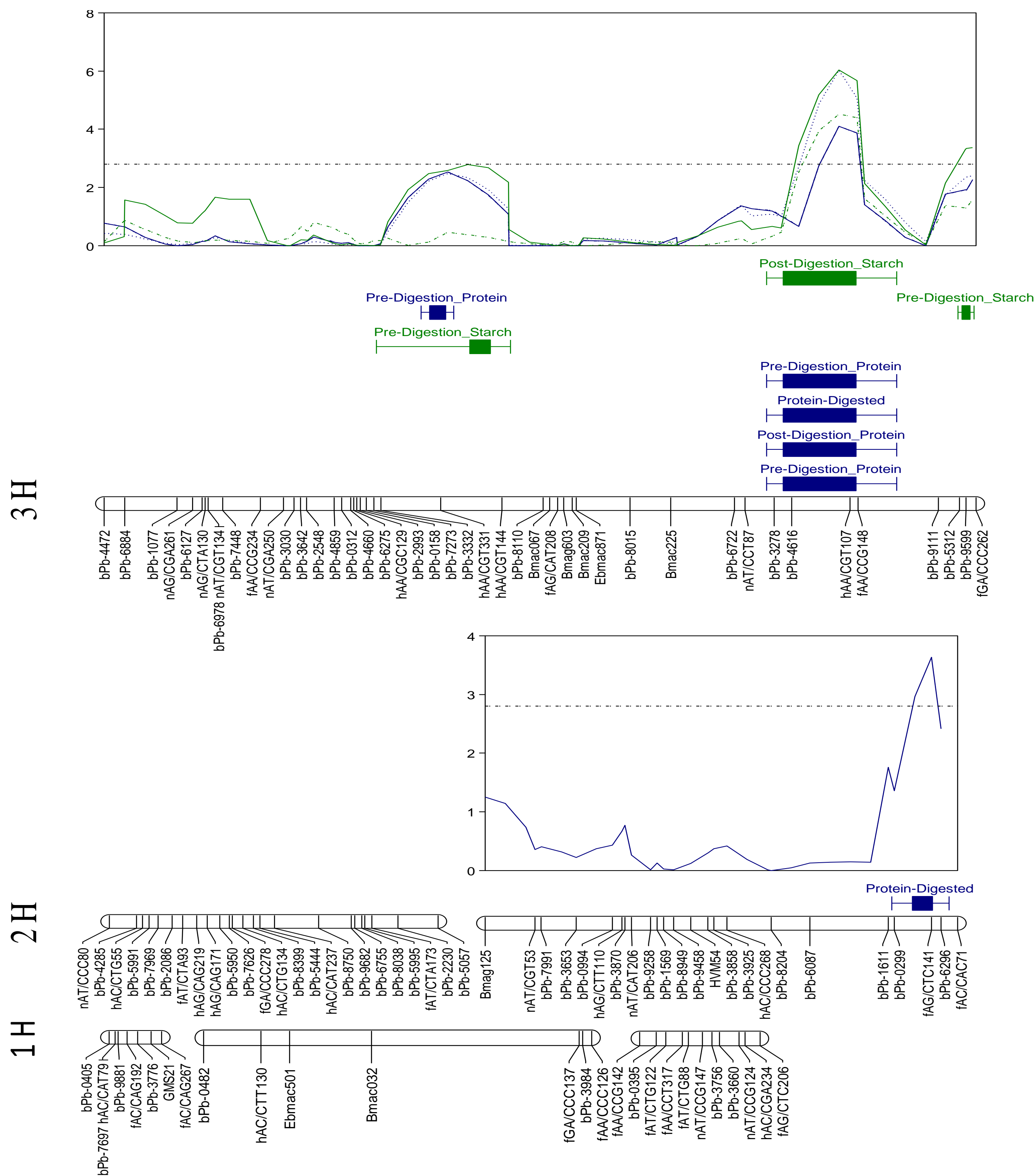

Figure 5.1a: QTL analysis for feed quality traits in Tallon x Scarlett F6-derived RIL population (Chromosome 1H – 3H).

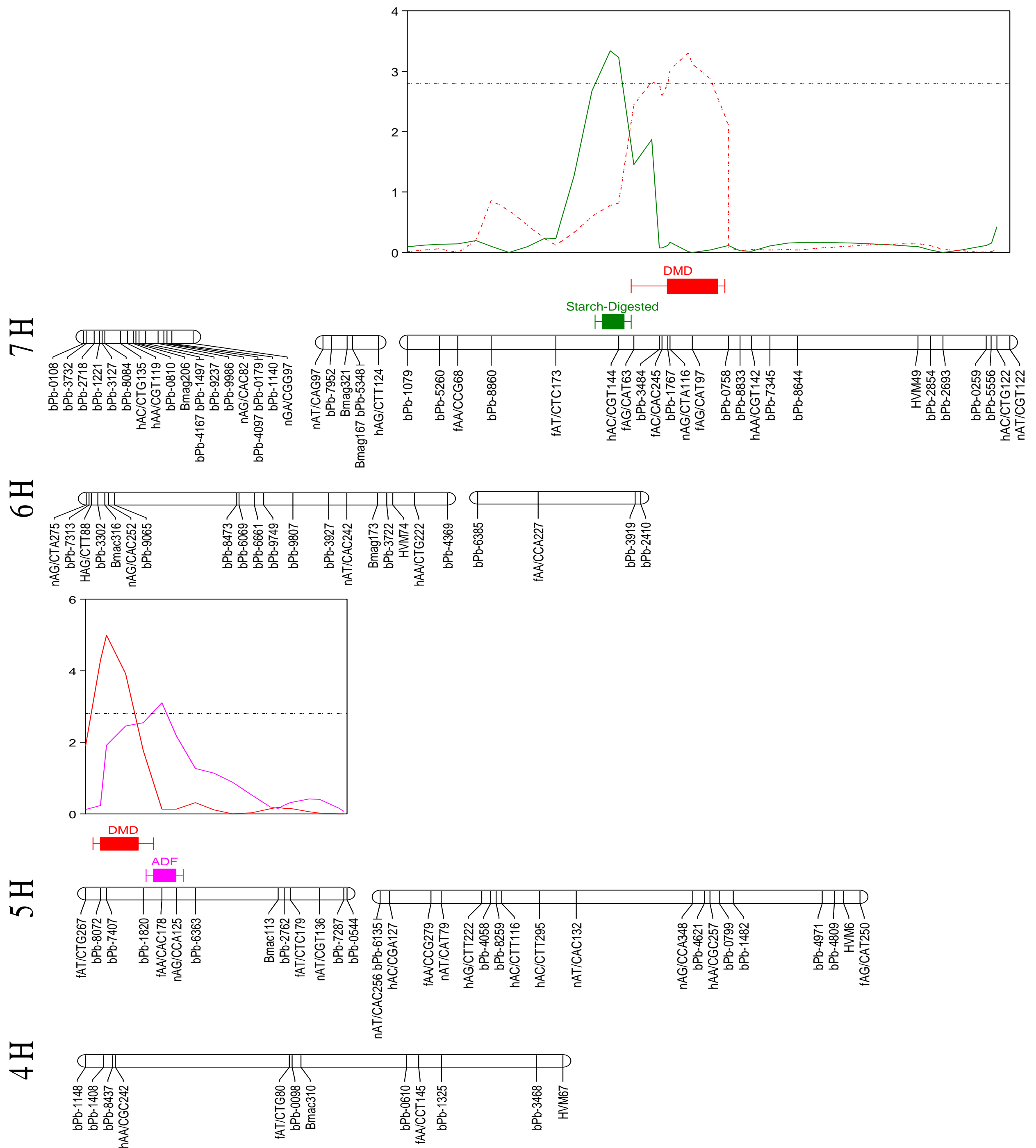

Figure 5.1b: QTL analysis for feed quality traits in Tallon x Scarlett F6-derived RIL population (Chromosome 4H – 7H).

5.4. Discussion

QTL analysis using a F6-derived RIL barley population, Tallon x Scarlett has identified specific genomic regions associated with the quantitative genetic control of feed quality traits. Numerous QTL were located across all seven barley chromosomes, with a limited number of significant QTLs (LOD > 2.7) identified on chromosome 2H, 3H, 5H and 7H of the barley genome.

The identification of QTLs using NIR calibrations on whole grain samples is a well established method (Lawson et al. 2009). However the identification of QTLs for traits at the post-digestion, for which no accurate NIR calibrations are available, is of interest. QTLs determined for pre- and post-digestion were located in the same genomic regions. Since the post-digestion NIR calibrations are limited, the clustering effect observed may be attributed to this. It may however also be possible that the same QTL does control the different aspects of the trait (e.g. pre-digestion content, post-digestion content, amount digested). Thus it is of great interest and importance that the NIR spectra be explored further and studied in detail for pre- and post-digestion data collection for QTL analysis.

This QTL study is the first to focus on pre-digestion, post-digestion and digested feed quality traits of barley in detail. Previous research has mapped significant QTLs for the barley genome of traits similar to those of interest to the feed industry. Thus the identification of QTLs associated with protein, starch, ADF and DMD is of importance. The QTL analysis for these feed traits within the Tallon x Scarlett population did not associate with that of previous findings, such as those identified for protein in malt quality (Fox et al. 2003) and in feed quality research (Abdel-Haleem 2004). In this study putative QTLs for protein were identified on chromosomes

2H and 3H, with a QTL for ADF on chromosome 5H. QTL for starch digestion were located on chromosomes 3H and 7H, while DMD was located on 5H and 7H. These putative QTL were derived from phenotypic data obtained by two different analytical means, and did not yield the same QTLs for similar traits. The suggestive QTL identified in this study primarily increase the chromosomal range across which the putative QTL are found, with the exception of pre-digestion starch content. The latter is found only on chromosome 3H with flanking markers bPb-7448 - fAA/CCG234.

Within the QTL data set obtained, the integration of multiple traits, in a single selection should be possible. A "suggestive" QTL for protein content contributed by Tallon was found on chromosome 1H, while another associated with protein digestion was contributed by Scarlett. These QTLs are not similar to those identified in similar studies on the same population (Lawson et al. 2009). The selection for one of these traits may pose restrictions in multiple trait integration due to the parental allele clustering.

In research conducted by Abdel-Haleem (2004), QTLs for starch and ADF were tightly clustered on both chromosome 2H and 7H for each trait. When viewed on the barley consensus map these tight clusters were closely linked to a protein QTL on these same chromosomes. However in this study starch QTL were not located in the same region as the ADF QTL as described by Abdel-Haleem (2004). The only tightly linked traits were protein and starch on chromosome 3H with flanking markers: bPB-2993 and hAA/CGT144 and also bPb-4616 and fAACCG148.

Though this study utilised NIR as rapid non-destructive method for phenotypic data collection for QTL analysis; the QTL analysis did not yield the same QTLs as derived through

conventional analytical techniques. These dissimilar QTL could be attributed to the possible lack of NIR calibration equations and mathematical permutations for processed grain and post-digestion residue samples. Additionally the insufficient number of replications during the analytical chemistry could have resulted in unintentional bias in the nutritional data set, due to the fact that genetic variance was attributed to only half the data set.

The present research supports previous studies that the genetic improvement of feed quality based on protein, starch, ADF and digestibility (DMD) is possible through the appropriate selection of alleles (Bowman et al. 1996; Surber et al. 2000). Though clustering can be problematic, it does allow for the selection of a specific phenotypic trait in a single chromosomal cluster. Current breeding programs can benefit from the selection of these clusters containing alleles for multiple traits rather than developing and expanding individual sets of alleles.

The development of feed barley and the selection of appropriate quality attributes are complex and identification of feed quality QTLs are infrequent (Bowman & Blake 1996; Fox et al. 2008). The present study has identified some important genetic regions controlling feed traits such as dry matter digestibility, acid detergent fibre, protein and starch content. The availability of molecular markers that define these regions may be an effective tool for future feed grain improvement through breeding. The breeding of feed barley through marker assisted selection could be highly beneficial by reducing cost and increasing productivity.

Chapter 6.

6.1. General discussion, Future directions and Conclusions.

6.1.1. General Discussion

Animal feed is the largest domestic market for Australian grain, consuming 28% of cereal grain production. The feedlot, dairy and chicken industries have driven feed grain demand from 5.7 million tonnes in 1993 to 11.9 million tonnes in 2007, an average increase of 4.1% each year (Martin et al. 2009; Roberts, Haseltine & Maliyasena 2009). Within eastern Australia (Queensland, New South Wales and Victoria) the livestock feed industry uses 43% of the cereal grain produced, while flour milling and malting consume 14% and 2% respectively. Even with the cutbacks due to drought in 2007-08, the feedlot industry is the sector with greatest growth over the past 14 years (Martin et al. 2009; Roberts, Haseltine & Maliyasena 2009).

At present barley is the second most widely produced cereal crop in Australia, with an average production of approximately 4.1 million metric tonnes of feed barley, and a domestic consumption of about 2 million tonnes per annum (Martin et al. 2009; Rebetzke 2007). The domestic demand for barley is driven mainly by the livestock industry (Smith 1995) which use cereal grain as a primary energy source. Relative price and availability of barley determines its competitiveness against other grains in the market place.

Research in Australia on barley has traditionally focused on agronomic traits and disease resistance with little input in genetic factors affecting feed quality (Anderson & Reinbergs 1985). With recent research identifying chemical and physical factors such as starch, protein, fibre (ADF) and dry matter digestibility (DMD) as important feed quality traits (Surber et al. 2000), greater emphasis is now placed on breeding for these characteristics. In this current study a mapped F6 – derived recombinant inbred line (RIL) population of Tallon x Scarlett has been

employed to determine ADF, protein, starch and DMD through *in sacco* trials conducted in *Bos taurus* cattle.

Phenotypic results from *in sacco* digestion trials on the Tallon x Scarlett population of interest showed transgressive segregation for feed quality traits. This segregation indicated that feed quality trait expression is controlled by multiple genes derived from both parents (Poehlman & Sleper 1995). The phenotypic data showed distinct correlations between feed traits whether derived through analytical chemistry techniques or NIR measurements. These correlations were performed on a data basis where know sources of variance was statistically manipulated. The correlations with the analytical chemistry results may be slightly weighted as the variance was statistically manipulated within the data of only half the feed traits. This limited removal of variance was due to the lack of replication in fibre (ADF) and starch analysis caused by financial constraints. These correlations, however, provide us a fundamental understanding of feed quality interactions during ruminal digestion.

Correlation analysis on NIR derived feed quality trait measurements was not the same as those derived through analytical chemistry. The high degree of correlation observed between feed quality traits in NIR values is ascribed to the statistical manipulation of known variance from the entire data set and the high degree of repeatability of this technique.

Analysis of the genetic map of the Tallon x Scarlett population allowed the detection of 12 putative QTLs linked to feed quality traits. These QTLs were predominantly found on chromosome 3H, 5H and 7H. However a major QTL for protein digestion was identified on chromosome 2H. Scarlett had the greatest contribution to feed quality traits in the population tested. Similar studies conducted abroad (Abdel-Haleem 2004) found similar QTLs on the same chromosomes as the present study. Epistasis (gene interaction) was not examined extensively in

the present study, however increasing the map saturation, may assist in the clear identification of any such interactions.However, experimental and environmental factors contribute to the variation of QTL location. This re-enforced the need for studies of this nature to be conducted under Australian conditions with local cattle and feed grains. The aim of the experiment was not just to identify QTLs associated with feed quality, but also to help establish efficient and reliable protocols for future research of this nature in Australia.

6.1.2. Future Directions

Greater consensus in the feed industry as to what defines feed quality is essential. Once a clear definition of feed quality is agreed upon amongst plant breeders and other industry players, the individual traits and the intricate interactions between these traits can be studied in detail. Such data would encourage more detailed mapping studies. From the present research, the putative and suggestive QTLs derived from Scarlett, indicated it may be necessary to run molecular markers across its pedigree to potentially identify additional QTLs linked to feed quality traits. The greater number of QTLs so mapped might lead to the breeding and growing of an industry specific barley crop with a higher energy and nutrient content.

Additional markers need to be found in genomic regions where feed quality QTLs have been reported. The advent of these potential new markers would facilitate and promote marker assisted selection in breeding programs targeted to feed quality. The refinement, discovery and development of new genetic techniques may help validate some of the suggestive markers found in this study.

The use of existing technology applied in new ways is also important in accurate and inexpensive data collection. The use of NIR in phenotypic data collection is indicative of this. Additional research needs to be done in the accurate formulation of calibration equations for the

reading of post-digestion nutrient content. This would allow for rapid, non-destructive and accurate measurement of both pre- and post-digestion feed nutrient content. This rapid measuring would lower the cost of phenotypic data collection in the construction of linkage maps associated with feed quality traits and thus the identification of corresponding QTLs.

6.1.3. Conclusions

- The genetic components in studies centred on the use of fistulated cattle can be dissected by appropriate experimental designs.
- The genetic improvement of feed quality, based on digestibility and starch content, can be achieved through selection of appropriate molecular markers.
- The availability of molecular markers that define critical QTL will be an effective tool for selection and genetic improvement of feed barley in the future.
- The development of NIR calibrations appropriate for the detection of post-digestion nutrient measurements is essential for its establishment as a rapid feed quality measurement technique.
- It is possible to perform complex feed quality trait analysis, under Australian conditions and cattle breeds, with established protocols used in this study.

7. References.

Abdel-Haleem, HA 2004, 'Genetics and mapping of quantitative trait loci of feed Quality-related traits in barley (H*ordeum vulgare l.*)', Montana State University.

Ahn, S & Tanksley, SD 1993, 'Comparative linkage maps of the rice and maize genomes', Proceedings of the National Academy of Science, no. 90, pp. 7980-4.

Anderson, MK & Reinbergs, E 1985, Barley breeding, Barley Agronomy Monograph, 26, Madison, WI.

Annison, EF & Bryden, WL 1998, 'Perspectives on ruminant nutrition and metabolism', Nutrition Research Reviews, vol. 11, pp. 173 - 98.

Barnes, RJ, Dhanoa, MS & Lister, SJ 1989, 'Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra', Applied Spectroscopy, vol. 43, pp. 772-7.

Barr, A, Eglinton, J, Langridge, P, Warner, P & Chalmers, K 2001, 'Marker assisted selection - where to now?', paper presented to 10th Australian Barley Technical Symposium.

Beames, RM, Helm, JH, Eggum, BO, Boisen, S, Knudsen, KEB & Swift, ML 1996, 'A comparison of methods for measuring the nutritive value for pigs of a range of hulled and hulless barley cultivars', Animal Feed Science Technology, no. 62, pp. 189-201.

Beauchemin, KA, Jones, SDM, Rode, LM & Sewalt, VJH 1997, 'Effects of barley fibrolytic enzymes in corn or barley diets on performance and carcass characteristics of feedlot cattle', Canadian Jouranl of Animal Science, no. 77, pp. 645-53.

Becker, JP, Vos, P, Kuiper, M, Salamini, F & Heun, M 1995, 'Combined mapping of AFLP and RFLP markers in barley', Molecular General Genetics, no. 249, pp. 65-73.

Bhatty, RS 1993, Nonmalting uses of barley, Barley: Chemistry and Technology, AACC., St. Paul, MN

Bhatty, RS, Berdahl, JD & Christison, GI 1975, 'Chemical composition and digestible energy of barley', Canadian Jouranl of Animal Science, no. 55, pp. 759-64.

Bjørnstad, Å, Skinnes, H & Thoresen, K 1993, 'Comparison between doubled haploid lines produced by anther culture, the *Hordeum* bulbosum-method and lines produced by single seed descent in barley crosses', Euphytica, no. 66, pp. 135-44.

Black, JL, Tredrea, AM, Nielsen, SG, Flinn, PC, Kaiser, AG & Barneveld, RJv 2005, 'Feed Uses for Barley', paper presented to Proceedings 12th Australian Barley Technical Symposium.

Blake, T, Bowman, JGP, Hensleigh, P, Kushnak, G, Carlson, G, Welty, L, Eckhoff, J, Kephert, K, Wichman, D & Hayes, PM 2002, 'Registration of Valier barley', Crop Science, no. 42, pp. 1748-9.

Blake, TK, Kadyrzhanova, D, Shepard, KW, Islam, AKMR, P.L Langridge, McDonald, CL, Erpelding, J, Larson, S, Blake, NK & Talbert, LE 1996, 'STS-PCR markers appropriate for wheat-barley introgression', Theorey Applied Genetics, no. 93, pp. 826-32.

Blanco, M, Coello, J, Iturriaga, S, Maspoch, S & Pezuela, Cdl 1997, 'Calibration in near infrared diffuse reflectance spectroscopy. A comparative study of various methods', Journal of Near Infrared Spectroscopy, vol. 5, pp. 67-75.

Boss, DL & Bowman, JGP 1996, 'Barley varieties for finishing steers: Feedlot performance, in vivo diet digestion, and carcass characteristics', Journal of Animal Science, no. 741967-1972.

Bothmer, R 1992, The wild species of *Hordeum*: Relationships and potential use for improvement of cultivated barley, Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology, CAB International.

---- 2003, Diversity in barley: (Hordeum vulgare). ELSEVIER Science, Amsterdam.

Bothmer, R, Jacobsen, CB & Jorgensen, RB 1992, 'An ecogeographical study of the genus *Hordeum*, systematic and ecogeographical studies on crop gene pools', International Board for Plant Genetic Resources.

Botstein, D, White, RL, Skolinck, M & Davis, RW 1980, 'Construction of a genetic linkage map in man using restriction fragment length polymprphisms', American Journal of Human Genetics, no. 32, pp. 314-31.

Bowman, J & Blake, T 1996, 'Barley feed quality for beef cattle', paper presented to International Oat Confonference VII & International Barley Genetics Symposium.V, Saskatoon, Canada,.

Bowman, J & Sowell, B 2002, Feeding the beef cow herd, Livestock Feeds and Feeding, Prentice Hall, New Jersey.

Bowman, JGP, Blake, TK, Surber, LMM, Habernicht, DK & Bockelman, H 2001, 'Feed quality variation in the barley core collection of the USDA national small grains collection', Crop Science, no. 41, pp. 863-70.

Bowman, JGP, Blake, TK, Surber, LMM, Habernicht, DK, Daniels, TK & Daniels, JT 1996, 'Genetic Factors Controlling Digestibility Of Barley For Ruminants', Proceedings, Western Section, American Society of Animal Science, vol. 47.

Bradshaw, WL, Hinman, DD, Bull, RC, Everson, DO & Sorensen, SJ 1996, 'Effects of barley variety and processing methods on feedlot steer performance and carcass characteristics', Journal of Animal Science, no. 74, pp. 18-24.

Briggs, DE 1978, Barley, John Wiley & Sons Inc., New York.

Brown, TA 2006, Genome 3, 3rd edn, Wiley-Liss, New York.

Burger, WC & LaBerge, DE 1985, 'Malting and brewing quality', Barley Agronomy Monograph, no. 2-17.

Burow, MD & Blake, TK 1997, 'Molecular tools for the study of complex traits', in A Paterson (ed.), *Molecular Dissection of Complex Traits*, CRC Press, pp. 17-30.

Burr, B, Burr, FA, Thompson, KH, Albertson, MC & Stuber, CW 1988, 'Gene mapping with recombinant inbreds in maize', Genetics, no. 188, pp. 519-26.

Castiglioni, P, Ajmone-Marsan, P & Wijk, Rv 1999, 'AFLP markers in a molecular linkage map of maize: codominant scoring and linkage group distribution ', Theorey Applied Genetics, no. 99, pp. 425-31.

Cecava, MJ 1995a, Protein requirements of beef cattle, Beef Cattle Feeding and Nutrition. 2nd ed, Academic Press, New York.

---- 1995b, Rumen physiology and energy requirements, Beef Cattle Feeding and Nutrition. 2nd ed, Academic Press, New York.

Chalmers, MI, Cuthbertson, DP & Synge, RLM 1954, 'Ruminal ammonia formation in the relation to protein requirement of sheep. I. Duodenal administration and heat processing as factors influencing fate of casein supplements', Journal of Agricultural Science, no. 44, pp. 354-262.

Churchill, GA & Doerge, RW 1994, 'Empirical threshold values for quantitative trait mapping', Genetics, no. 138.

Collard, BCY, Jahufer, MZZ, Brouwer, JB & Pang, ECK 2005, 'An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts', Euphytica, vol. 142, pp. 169 - 96.

Condit, R & Hubbel, S 1991, 'Abundance and DNA sequence of two-base repeat regions in tropical tree genomes', Genome Research, no. 34, pp. 66-71.

Costa, JM, Corey, A, Hsyes, PM, Jobet, C, Kleinhofs, A, Kopisch-Obush, A, Kramer, SF, Kudrna, D, Li, M, Riera-Lizarazu, O, Sato, K, Szucs, P, Toojinda, T, Vales, MI & Wolfe, RI 2001, 'Molecular mapping of the Oregon wolfe barleys: a phenotypically polymorphic doubled-haploid population', Theorey Applied Genetics, no. 103, pp. 415-24.

Deaville, ER & Flinn, PC 2000, Near-infrared (NIR) spectroscopy: an alternative approach for the estimation of forage quality and voluntary intake, Forage evaluation in ruminant nutrition, CABI Publishing, Wallingford.

Devaux, P & Kasha, KJ 2008, Overview of Barley Doubled Haploid Production, Advances in Haploid Production in Higher Plants, Springer Netherlands.

DFID 2006, *Digestive Systems and Digestion of Feeds* NR International managers of the Livestock Production Programme (LPP) 2009.

Dickson, AD 1968, 'Barley for malting and food', In Barley: Origin, Botany, Cultivars, Winter Hardiness, Genetics, Utilization and Pests, pp. 112-20.

Elementar 2009, *Vario Max Elementar Analyticalsystems*, Hanau.

Ellis, RP, McNicol, JW, Baird, E, Booth, A & Lawrence, P 1997, 'The use of AFLPs to examine genetic relatedness in barley', Molecular Breed, no. 3, pp. 359-69.

Faichney, GJ 1996, 'Rumen physiology: the key to understanding the conversion of plants into animal products', Australian Journal of Agricultural Research, vol. 47, no. 2, pp. 163 - 74.

Farm, Sot 2005, *The ruminant digestive tract*, Waikato University.

Flint, HJ & Forsberg, CW 1995, Polysaccharide degradation in the rumen: biochemistry and genetics, Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction, Ferdinand Enke Verlag, Stuttgart.

Foster, AE 1987, Barley, vol. 2, Principles of Cultivar Development, Macmillan Inc., New York.

Fox, G, Bowman, J, Kelly, A, Inkerman, A, Poulsen, D & Henry, R 2008, 'Assessing for genetic and environmental effects on ruminant feed quality in barley (*Hordeum vulgare*)', Euphytica, vol. 163, pp. 249-57.

Fox, GP, Panozzo, JF, Li, CD, Lance, RCM, Inkerman, PA & Henry, RJ 2003, 'Molecular basis of barley quality', Australian Journal of Agricultural Research, vol. 54, pp. 1081-101.

Franckowiak, J 1997, 'Revised linkage maps for morphological markers in barley (*Hordeum vulgare*)', Barley Genet. Newsletter no. 26, pp. 9-21.

Frègeau-Ried, J, Choo, TM, Ho, KM, Martin, RC & Konishi, T 2001, 'Comparisons of two-row and six-row barley for chemical composition using doubled-haploid lines', Journal of Crop Science, no. 41, pp. 1737-43.

Gilmour, AR, Thompson, R & Cullis, BR 1995, 'Average Information REML, an efficient algorithm for variance parameter estimation in linear mixed models', Biometrics, vol. 51, pp. 1440 - 50.

Givens, DI & Deaville, ER 1999, 'The current and future role of near infrared reflectance spectroscopy in animal nutrition: a review', Australian Journal of Agricultural Research, vol. 50, pp. 1131-45.

Haard, NF & Chism, G 1996, Characteristics of edible plant tissues, Food Chemistry, Marcel Dekker Inc, New York.

Haley, CS & Knott, SA 1992, 'A simple regression method for mapping quantitative trait loci in line crosses using flanking markers', Heredity, no. 69, pp. 315-24.

Harlan, RJ 1968, ' On the origin of barley', In Barley: Origin, Botany, Cultivars, Winter Hardiness, Genetics, Utilization and Pests. USDA, pp. 9-31.

Hayes, PM, Cerono, J, Witsenboer, H, Kuiper, M, Zabeau, M, Sato, K, Kleinhofes, A, Kudrna, D, Kilian, A, Saghai-Maroof, M & Hoffman, D 1997, *Characterization and exploiting genetic diversity and quantitative traits in barley (Hordeum vulgare) using AFLP markers*.

Heald, PJ 1952, 'The assessment of glucose-containing substances in rumen micro-organisms during a digestion cycle in sheep', British Journal of Nutrition, no. 5, pp. 84-93.
.

Hernández, P, Dorado, G, Prieto, P, Gimenez, MJ, Ramírez, MC, Laurie, DA, Snape, JW & Martín, A 2001, 'A core genetic map of *Hordeum chilens*e and comparisons with maps of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*)', Theorey of Applied Genetics, no. 102, pp. 1259-64.

Herrera-Saldana, RE, Huber, JT & Poore., MH 1990, 'Dry matter, crude protein and starch degradability of five cereal grains', Journal of Dairy Science, no. 73, pp. 2386-93.

.

Hockett, EA & Nilan, RA 1985, Genetics, Barley Agronomy Monograph, ASA, CSSA and SSSA, Madison, WI.

Hunt, CW 1996, 'Factors affecting feeding quality of barley for ruminants', Animal Feed Science Technology, no. 62, pp. 37 - 48.

Huntington, GB 1994, 'Ruminant starch utilization progress has been extensive', Feedstuffs, no. 66, p. 16.

---- 1997, 'Starch utilization by ruminants: from basics to the bunk', Journal of Animal Science, no. 75, pp. 852-67.

Ipharraguerre, IR, Clark, JH & Freeman, DE 2005, 'Varying Protein and Starch in the Diet of Dairy Cows. I. Effects on Ruminal Fermentation and Intestinal supply of Nutrients', Journal of Dairy Science, no. 88, pp. 2537-55.

Jakob, SS, Meister, A & Blattner, FR 2004, 'The Considerable Genome Size Variation of *Hordeum* Species (Poaceae) Is Linked to Phylogeny, Life Form, Ecology, and Speciation Rates', Molecular Biology and Evolution, vol. 21, no. 5.

Jansen, RC & Stam, P 1994, 'High resolution of quantitative traits into multiple loci via interval mapping', Genetics, no. 136, pp. 1447-55.

Kakeda, K, Ibuki, T, Suzuki, J, Tadano, H, Kurita, Y, Hanai, Y & Kowyama, Y 2008, 'Molecular and genetic characterization of the *S* locus in *Hordeum bulbosum L.*, a wild self-incompatible species related to cultivated barley', Molecular Genetics Genomics, vol. 280, no. 509 -519.

Kanazin, V, Talbert, H, See, D, DeCamp, P, Nevo, E & Blake, T 2001, 'Discovery and assay of single nucleotide polymorphisms in barley (*Hordeum vulgare*)', Plant Molecular Biology, no. 48, pp. 529-37.

Kang, MS 2002, Quantitative genetics, genomics, and plant breeding, CABI Publishers, Oxon, UK.

Kellems, RO & Church, DC 2002a, Feedstuffs, Livestock Feeds and Feeding, Prentice Hall, New Jersey.

---- 2002b, Nutrients: their metabolism and feeding standards, Livestock Feeds and Feeding. 5th ed, Prentice Hall, New Jersey.

Kemalyan, RE, Clark, CK, Peterson, MK & Newman, CW 1989, 'In vitro dry matter disappearance rate and purine accumulation of proanthocyanidin-free barley cultivars', Proceedings, Western Section, American Society of Animal Science, no. 40, p. 414.

Khorasani, GR, Helm, J & Kennelly, JJ 2000, 'In situ rumen degradation characteristics of sixty cultivars barley grain', Canadian Jouranl of Animal Science, no. 80, pp. 691-701.

Kim, M, Hyun, J, Kim, J, Park, J, Kim, M, Kim, J, Lee, S, Chun, S & Chung, I 2007, 'Relationship between phenolic compounds, anthocyanins content and antioxidant activity in colored barley germplasm', Journal of Agricultural Food Chemistry, vol. 5, no. 12, pp. 4802 - 9.

Kleinhofs, A & Graner, A 2001, 'An integrated map of the barley genome', in RL Philips & IK Vasil (eds), *DNA-Based Markers in Plants*, 2 edn, Kluwer Academic Publications, pp. 187-99.

Kleinhofs, A, Kilian, A, MMaroof, MAS, Biyashev, RM, Hayes, P, Chen, FQ, Lspitan, N, Fenwick, A, Blake, TK, Kanazin, V, Ananiev, E, Dahleen, L, Kurdna, D, Bollinger, J, Knapp, SJ, Liu, B, Sorrells, M, Heun, M, Franckowiak, JD, Hoffman, D, Skadsen, R & Stefffenson, BJ 1993, 'A molecular, isozymes, and morphological map of the barley (*Hordeum vulgare*) genome', Theorey Applied Genetics, no. 86, pp. 705-12.

Kong, D, Choo, TM, Jui, P, Ferguson, T, Therrien, MC, Ho, KM, May, KW & Narasimhalu, P 1995, 'Variation in starch, protein, and fiber of Canadian barley cultivars', Canadian Jouranl of Plant Science, no. 75, pp. 865-70.

Körnicke, F 1885, Die Arten und Varietäten des Getreibes, vol. 1, 2 vols., Handbuch des Getreidebaues, Emil Strauss, Bonn.

---- 1985, Die Arten und Varietäten des Getreides.

Kotarski, SF, Waniska, RD & Thurn., KK 1992, 'Starch hydrolysis by the animal microflora', Nutrure, no. 122, pp. 178-90.

Kowk, P & Gu, Z 1999, 'Single nucleotide polymorphism libraries: why and how are we building them?', Molecular Medicine Today, no. 2, pp. 538-43.

Lande, R & Thompson, R 1990, 'Efficiency of marker-assisted selection in the improvement of quantitative traits', Genetics, no. 124, pp. 743-56.

Lander, ES & Botstein, D 1989, 'Mapping mendelian factors underlying quantitative traits using RFLP linkage maps', Genetics, no. 121, pp. 185-99.

Larson, SR, Kadyrzhanova, D, McDonald, C, Sorrells, M & Blake, TK 1996, 'Evaluation of barley chromosome-3 yield QTL in a backcross F2 population using STS-PCR', Theorey of Applied Genetics, no. 93, pp. 618-25.

Lawson, W, Mace, E, Collard, BCY, Fox, G, Kelly, A, Sutherland, M, Franckowiak, J & Bowman, J 2009, *Investigating the molecular dynamics of feed quality traits in two barley F6-derived RIL populations*, Cairns, Australia.

Lehman, KB, Okine, EK, Mathison, GW & Helm, J 1995, ' In situ degradabilities of barley grain cultivars ', Canadian Jouranl of Animal Science, no. 75, pp. 485 - 7.

Li, JH, Vasanthan, T, Rossnagel, B & Hoover, R 2001, 'Starch from hullless barley. I. Granule morphology, composition and amylopectin structure', Food Chemistry, no. 74, pp. 395-405.

Lincoln, SE, Daly, MJ & Lander, ES 1993, Constructing genetic linkage maps with MAPMAKER/EXP version 3.0: A tutorial and reference manual, Whitehead Institute for Biomedical Research, Cambridge.

Liu, BH 1998, 'Statistical genomics: linkage, mapping, and QTL analysis', CRC Press.

Liu, ZW, Biyashev, RM & Maroof, MAS 1996, 'Development of simple sequence repeat DNA markers and their integration into a barley linkage map', Theorey of Applied Genetics, no. 93, pp. 869-76.

Macaulay, M, Ramsay, L, Powell, W & Waugh, R 2001, 'A representative, highly informative ‘genotyping set’ of barley SSR's', Theorey of Applied Genetics, no. 102, pp. 801-9.

MacGergor, AW & Fincher, GB 1993, Carbohydrates of the barley grain, Barley: Chemistry and Technology, AACC, St. Paul, MI.

Mano, Y, Sayed-Tabatabaei, BE, Graner, A, Blake, T, Takaiwa, F, Oka, S & Komatsuda, T 1999, 'Map construction of sequence-tagged sites (STSs) in barley (*Hordeum vulgare L*)', Theorey of Applied Genetics, no. 98, pp. 937-46.

Martin, JM, Blake, TK & Hockett, EA 1991, 'Diversity among American spring barley cultivars based on coefficients of parentage', Crop Science, no. 31, pp. 1131-7.

Martin, P, Mackinnon, D, Dharma, S, Liao, B, Phillips, P, Crooks, S & Penm, J 2009, 'Australian farm survey results', Australian Bureau of Agricultural and Resource Economics.

Martinez, O & Curnow, RN 1992, 'Estimating the locations and the sizes of the effects of quantitative trait loci using flanking markers', Theorey of Applied Genetics, no. 85, pp. 480-8.

Matsushima, JK 1979, Feeding beef cattle, Advances Series in Agriculture Sciences, Springer-Verlag, New York.

McDonald, IW 1954, 'The extent of conversion of food protein to microbial protein in the rumen of the sheep', Journal of Biochemistry, no. 56, pp. 120-5.

McDonald, P, Edwards, RA, Greenhalgh, JFD & Morgan, CA 1995, Animal Nutrition. 5th ed, Longman Scientific & Technical, New York.

McLean, RA, Sanders, WL & Stroup, WW 1991, 'A Unified Approach to Mixed Linear Models', The American Statistician, vol. 45, no. 1, pp. 54 - 64.

McNiven, MA, PrestlØkken, E, Mydland, LT & Mitchell, AW 2002, 'Laboratory procedure to determine protein digestibility of heattreated feedstuffs for dairy cattle', Animal Feed Science Technology, no. 96, pp. 1-13.

Megazyme 2009, *Total Starch Assay Procedure (Amyloglucosidase/ α-Amylase Method)*, Megazyme International Limited, Ireland.

Meissner, HH & Preez, HPFd 1996, 'Ruminal and postruminal digestion of dietary protein and starch in steers: 1 Effects of protein concentration, degradation of protein and energy content of the diet', South African Journal of Animal Science, vol. 26, no. 3/4.

---- 2006, 'Ruminal and postruminal digestion of dietary protein and starch in steers: Effects of protein concentration, degradation of protein and energy content of the diet', South African Journal of Animal Science, vol. 26, no. 314.

Molina-Cano, JL, Francesch, M, Perez-Vendrell, AM, Ramo, T, Voltas, J & Brufau, J 1997, 'Genetic and Environmental Variation in Malting and Feed Quality of Barley', Journal of Cereal Science., vol. 25, pp. 37-47.

Naik, S, Gill, KS, Rao, VSP, Gupta, VS, Tamhankar, SA, Pujar, S, Gill, BS & Ranjekar, PK 1998, 'Identification of a STS marker linked to the Aegilops speltoides-derived leaf rust resistance gene *Lr*28 in wheat', Theorey of Applied Genetics, no. 97, pp. 535-40.

NationalResearchCouncil 1996, *Nutritional requirements of beef cattle. 7th ed*, National Academy Press, Washington, D.C.

Newman, CW & Newman, RK 1992, Nutritional aspect of barley seed structure and composition, Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology, CAB International, Wallingford England.

Nielsen, G & Scandalios, JG 1974, 'Chromosomal location by use trisomics and new alleles of an endopeptidase in *Zea mays*', Genetics, no. 77, pp. 679-86.

Nilan, RA 1964, The cytology and genetics of barley, vol. 32, Monogr. Suppl. 3.

Nilan, RA & Ullrich, SE 1993, 'Barley: Taxonomy, origin, distribution, production, genetics, and breeding', Barley: Chemistry and Technology, pp. 1-29.

Noon, CD, Seoane, JR & Scott, SL 1998, 'The use of corn and barley in diets for veal calves: effects on performance, diet digestibility and carcass quality', Canadian Jouranl of Animal Science, no. 78, pp. 351-8.

Norris, KH, Barnes, RF, Moore, JE & Shenk, JS 1976, ' Predicting forage quality by infrared reflectance spectroscopy', Journal of Animal Science, vol. 43, pp. 889-97.

Obara, Y, Dellow, DW & Nolan, JV 1991, The influence of energy-rich supplements on nitrogen kinetics in ruminants, Physiological Aspects of Digestion and Metabolism in Ruminants, Academic Press, New York.

Olson, M, Hood, L, Cantor, C & Botstein, D 1989, 'A common language for physical mapping of the human genome', Science no. 254, pp. 1434-5.

Ørskov, ER 1986, 'Starch digestion and utilization in ruminants', Journal of Animal Science, no. 63, pp. 1624-33.

Osborne, BG 2006, 'Application of near infrared spectroscopy in quality screening of early-generation material in cereal breeding progammes', Journal of Near Infrared Spectroscopy, vol. 14, pp. 93 - 101.

Osborne, BG, Fearn, T & Hindle, PH 1993, Practical NIR Spectroscopy with applications in Food and Beverage Analysis, Second edn, Longmans Scientific and Technical, Harlow.

Owens, FN, Secrist, DS, Hill, WJ & Gill, DR 1998, 'Acidosis in cattle: a review', Journal of Animal Science, no. 76, pp. 275-86.

Pathak, AK 2008, 'Various factors affecting microbial protein synthesis in the rumen', Veterinary World, vol. 1, no. 6, pp. 186 - 9.

Patterson, HD & Thompson, R 1971, 'Recovery of inter-block information when block sizes are unequal', Biometrika, vol. 58, pp. 545 - 54.

Pauly, T, Spörndly, R & Udén, P 2006, 'Rumen degradability In sacco of physically and chemically treated oat and barley grain.', Journal of the Science of Food and Agriculture, vol. 58, no. 4, pp. 465 - 73.

Perry, TW, Cullison, AE & Lowrey, RS 1999, Feeds and Feeding, Prentice Hall, New Jersey.

Pillen, K, Binder, A, Kreuzkam, B, Ramsay, L, Waugh, R, Förster, J & Léon, J 2000, 'Mapping new *EMBL*-derived barley microsatellites and their use in differentiating German barley cultivars', Theorey of Applied Genetics, no. 1011, pp. 652-60.

Poehlman, J & Sleper, D 1995, Breeding Field Crops, Iowa State University Press, Ames

Qi, X, Stam, P & Lindhout, P 1998, 'Use of locus-specific AFLP markers to construct a high density molecular map in barley', Theorey of Applied Genetics, no. 96, pp. 379-84.

Qureshi, AA, Burger, WC, Peterson, MD & Elson, CE 1996, 'The structure of an inhibitor of cholesterol biosynthesis isolated from barley', Journal of Biological Chemistry, vol. 261, pp. 10544-50.

Ramsay, L, Macaulay, M, Ivanissevich, S, MacLen, K, Cardle, L, Fuller, J, Edwards, KJ, Tuvesson, S, Morgante, M, Massari, A, Maestri, E, Marmiroli, N, Sjakste, T, Ganal, M, Powell, W & Waugh, R 2000, 'A simple sequence repeat-based linkage map of barley', Genetics, no. 156, pp. 1997-2005.

Ranhotra, GS, Gelorth, JA, Astroth, K & Bhatty, RS 1991, 'Relative lipidemic responses in rats fed barley and oat meals and their fractions', Cereal Chemistry, vol. 68, no. 548-551.

Rebetzke, G 2007, *CSIRO: Barley Overview*, CSIRO, 2009.

Ried, D 1985, 'Morphology and anatomy of the barley plant', Barley Agronomy Monograph, pp. 75-102.

Ried, DA & Wiebe, GA 1968, Taxonomy, botany, classification and world collection, Barley: Origin, Botany, Cultivars, Winter Hardiness, Genetics, Utilization and Pests, USDA.

Roberts, I, Haseltine, C & Maliyasena, A 2009, 'Factors affecting Australian agricultural exports', Australian Bureau of Agricultural and Resource Economics.

Russell, J, Fuller, J, Young, G, Thomas, B, Taramino, G, Macaulay, M, Waugh, R & Powell, W 1997, 'Discriminating between barley genotypes using microsatellite markers', Genome Research, no. 40, pp. 442-50.

Russell, JB & Rychlik, JL 2001, 'Factors that alter rumen microbial ecology', Science, no. 292, pp. 1119-22.

Sax, K 1923, 'The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*', Genetics, no. 8, pp. 552-60.

Schwarz, G, Michalek, W, Mohler, V, Wenzel, G & Jahoor, A 1999, 'Chromosome landing at the Mla locus in barley (*Hordeum vulgare L.*) by means of high-resolution mapping with AFLP markers', Theorey of Applied Genetics, no. 98, pp. 521-30.

See, D, Kanazin, V, Talbert, H & Blake, T 2000, 'Electrophoretic detection of single nucleotide polymorphisms', Biotechniques, no. 28, pp. 710-6.

Shan, X, Blake, TK & Talbert, LE 1999, 'Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat', Theorey of Applied Genetics, no. 98, pp. 1072-8.

Shenk, JS, Westerhaus, MO & Abrams, SA 1989, *Protocol for NIRS calibration: monitoring analysis results and recalibration. In: 'Near infrared reflectance spectroscopy (NIRS): analysis of forage quality*.

Shewry, PR 1993, Barley seed proteins, Barley: Chemistry and Technology, Amer. AACC. , St. Paul, MI.

Shewry, PR & Morell, M 2001, 'Manipulating cereal endosperm structure, development and composition to improve end-use properties', Advances in Botanical Research, no. 34, pp. 165-236.

Shin, JS, Chao, S, Corpuz, L & Blake, T 1990, 'A partial map of the barley genome incorporating restriction fragment length polymorphism, polymerase chain reaction, isozyme, and morphological marker loci', Genome Research, no. 33, pp. 803-10.

Smith, W 1995, Crop Production: Evolution, History, and Technology Wiley New York.

Soest, PJV 1994, Nutritional Ecology of the Ruminant, Second Edition edn, P.J. Van Soest, Cornell University Press, New York.

Soest, PJV, Robertson, JB & Lewis, BA 1991, 'Methods for dietary fibe, neutral detergent fiber, and nonstarch polysaccharides in relation to aimal production.', J. Dairy Sci, vol. 74, pp. 3583 - 97.

Spooner, S, Treuren, Rv & Vicente, MCd 2005, *Molecular markers for genebank management* USDA.

Stern, MD, Bach, A & Calsamiglia, S 2006, *New concepts in Protein Nutrition of Ruminants.*, Tempe, AZ.

Struss, D & Plieske, J 1998, 'The use of microsatellite markers for detection of genetic diversity in barley populations', Theory of Applied Genetics, no. 97, pp. 308-15.

Subrahmanyam, NC & Bothmer, Rv 1987, ' Interspecific hybridization with Hordeum bulbosum and development of hybrids and haploids', Hereditas no. 106, pp. 199-27.

Surber, LMM, Bowman, JGP, Blake, TK, Hinman, DD, Boss, DL & Blackhurst, TC 2000, 'Prediction of barley feed quality for beef cattle from laboratory analyses', Proceedings, Western Section, American Society of Animal Science, no. 51, pp. 454-7.

Surber, LMM, Bowman, JGP, Daniels, TK, Milner, TJ, Lewis, AL, Coulson, DM & Blake, TK 1998, 'Feeding value of barley varieties for finishing cattle', Proceedings, Western Section, American Society of Animal Science, no. 49, pp. 268-71.

Sutton, JD 1985, 'Digestion and absorption of energy substrates in the lactating cow', Journal of Dairy Science, no. 68, pp. 3376-93.

Swanson, KC, Matthews, JC, Woods, CA & Harmon, DL 2002, 'Postruminal Administration of Partially Hydrolyzed Starch and Casein Influences Pancreatic α-Amylase Expression in Calves', American Society for Nutritional Sciences, vol. 3166, no. 2.

Szalay, A, Antal, I, Zsigmond, Z, Marton, S, Eros, I, Regdon, G & Pintye-Hodi, K 2005, 'Study on the Relationship between Particle size and Near Infrared Diffuse Reflectance Spectroscopic Data', Particle and Particle System Characterization, no. 22, pp. 219-22.

Tacconi, G, Cattivelli, L, Faccini, N, Pecchioni, N, Stanca, AM & Valé, G 2001, ' Identification and mapping of a new leaf strip resistance gene in barley (*Hordeum vulgare L.*)', Theory of Applied Genetics, no. 102, pp. 1286-91.

Takahashi, R 1955, 'The origin and evolution of cultivated barley', Advanced Genetics, vol. 7, pp. 226-7.

Tanksley, SD 1993, 'Mapping polygenes', Nature Reviews Genetics, no. 27, pp. 205-33.

Tanno, K, Taketa, S, Takeda, K & Komatsuda, T 2002, 'A DNA marker closely linked to the *vrs1* locus (row-type gene) indicates multiple origins of six-rowed cultivated barley (*Hordeum vulgare L.*)', Theory of Applied Genetics, no. 104, pp. 54 - 60.

Tinker, NA & Mather, DE 1998, *MQTL: software for simplified composite interval mapping of QTL in multiple environments*, <http://probe.nalusda.gov:8000/otherdocs/
jqtl/ jqtl1995-02/jqtl16r2 html
Tinker NA, Fortin MG & Mather>.

Tragoonrung, S, Kanazin, V, Hayes, PM & Blake, TK 1992, 'Sequencetagged-site-facilited PCR for barley genome mapping', Theory of Applied Genetics, no. 84, pp. 1002-8.

USDA 2008, *World Agricultural Production*, USDA.

Vanzant, ES, Robert, RC & Titgemeyer, EC 1998, 'Standardization of *in situ* techniques for ruminant feedstuff evaluation', Journal of Animal Science, no. 76, pp. 2717-29.

Voorrips, RE 2002, 'MapChart: Software for the graphical presentation of linkage maps and QTLs', The Journal of Heredity, vol. 93, pp. 77 - 8.

Vos, P, Hogers, R, Bleeker, M, Reijans, M, Lee, TVD, Hornes, M, Frijters, A, Pot, J, Peleman, J, Kuiper, M & Zabeau, M 1995, 'AFLP: a new technique for DNA fingerprinting', Nucleic Acids Research, no. 23, pp. 4407-14.

Wang, S, Basten, CJ & Zeng, Z-B 2002, 'Windows QTL Cartographer: WinQtlCart V2.0'.

Waugh, R, Bonar, N, Baird, E, Thomas, B, Graner, TA, Hayes, P & Powell, W 1997, 'Homology of AFLP products in three mapping populations of barley', Molecular and General Genetics, no. 255, pp. 311-21.

Weber, D & Helentjaris, T 1989, 'Mapping RFLP loci in maize using B-A translocations', Genetics, no. 121, pp. 583-90.

Weeden, NF, Timmerman, GM, Hermmat, M, Kneen, BE & Lodhi, MA 1992, Inheritance and reliability of RAPD markers, Applications of RAPD technology to plant breeding. Joint Plant Breeding Symposia Series. , CSSA, ASHS and AGA. .

Weising, K, Nybom, H, Wolff, K & Meyer, W 1995, DNA fingerprinting in plants and fungi, CRC Press.

Wendorf, F, R.Schild, Hadidi, NE, Close, AE, Kobusiewicz, M, Wieckowska, H & Issawi., B 1979, 'Use of barley in the Egyptian late Paleolithic', Science, no. 205, pp. 1341-7.

Wettstein-Knowles, Pv 1992, 'Cloned and mapped genes: current status', in PR Shewry (ed.), *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology.* , CAB International., pp. 73-98.

Williams, JGK, Kubelik, AR, Livak, KJ, Rafalski, JA & Tingey, SV 1990, 'DNA polymorphism amplified by arbitrary primers are useful as genetic markers', Nucleic Acids Research, no. 18, pp. 6531-5.

Wrigley, CW 1999, 'Potential methodologies and strategies for the rapid assessment of feed-grain quality', Australian Journal of Agricultural Research, no. 50, pp. 789-805.

Wrolstad, RE, Decker, EA, Schwartz, SJ & Sporns, P 2004, Handbook of Food Analytical Chemistry, Water, Proteins, Enzymes, Lipids, and Carbohydrates., 3 vols., Handbook of Food Analytical Chemistry, Wiley and Sons, New Jersey.

Yang, WZ, Beauchemin, KA, Farr, BI & Rode, LM 1997, 'Comparison of barley, hull-less barley, and corn in the concentrate of dairy cows', Journal of Animal Science, no. 80, pp. 2885-95.

Young, B 2008, *Barley; The Versatile Crop*, Southern Illinois University, College of Science.

Zeng, ZB 1994, 'Precision mapping of quantitative trait loci', Genetics, no. 136, pp. 1457-68.

Zinn, RA & Owens, FN 1983, 'Influence of feed intake level on site of digestion in steers fed a high concentrate diet', Journal of Animal Science, vol. 56, pp. 471 - 5.

Zinn, RA, Montano, M & Shen, Y 1996, 'Comparative feeding value of hulless vs. covered barley for feedlot cattle', Journal of Animal Science, no. 74, pp. 1187 - 93.

Zohary, D & Hopf, M 1988, 'Domestication of plants in the old world: The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley', Oxford University Press, pp. 52-63.

---- 2000, *Domestication of Plants in the Old World: The Origin and Spread of Cultivated Plants in West Asia, Europe, and the Nile Valley* 3rd edn, Oxford University Press, 0198503571.