INVESTIGATION OF LEPTIN GENOTYPES AND ECONOMICALLY IMPORTANT DAIRY TRAITS IN JERSEY COWS

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Submitted in fulfilment of the academic
requirements of the degree of
Master of Science
in the School of Biochemistry, Genetics, Microbiology and Plant Pathology,
University of KwaZulu-Natal
Pietermaritzburg 2005

ABSTRACT

Dairy farming is one of the most important agricultural industries in South Africa, and thus improving the performance of dairy cows, with respect to economically important dairy traits, would be beneficial. Selection of dairy cows has traditionally been phenotypic, but new molecular techniques have made it possible to evaluate phenotypic dairy traits at the DNA level, providing the possibility of more accurate selection. The economically important dairy traits, milk production and reproductive performance, are quantitative traits, and are therefore controlled by many genes and the environment. A number of genes have been identified that have been shown to influence economically important dairy traits, including the *lep* gene. This gene encodes the hormone leptin, which has been proven to regulate feed intake, energy balance, fertility and immune function. A polymorphism has been identified in the *lep* gene, which may be associated with economically important dairy traits. This study on a South African Jersey herd investigated the possible association of the polymorphism, RFLP-*Kpn* 2I, with milk production and reproductive performance. The lactation records of fifty Jersey cows that completed their first lactation between 1997 and 2004 were collected, and these cows were genotyped for the RFLP-*Kpn* 2I polymorphism, located at exon 2 of the *lep* gene. This involved the extraction of DNA from venous blood, using a salting out technique. The extracted DNA was amplified using PCR primers; the reverse primer included a purposeful mismatch. The role of the purposeful mismatch was to create a recognition site for a restriction enzyme (*Kpn* 2I), thus allowing the alleles of the polymorphism to be identified through a restriction digestion protocol. Two alleles were identified, the C- and the T-allele. The genotype of each cow was identified using PAGE. The significance of the genotype effects on the milk production traits and the reproductive performance traits were estimated using the F-statistic provided by a GLM Univariate analysis. In conclusion, no significant effect of the

RFLP-*Kpn* 2I polymorphism was found for milk yield, butterfat and protein percentage, ICP and SPC ($p > 0.05$), but a possible association with lactose percentage was suggested by the statistical analysis ($p < 0.05$). Further investigation of South African Jersey cows will be necessary in order for conclusive results to be obtained.

PREFACE

The experimental work described in this dissertation was conducted at the University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Annabel Fossey.

The results have not been submitted in any other form to another University and, except where the work of others is acknowledged in the text, are the results of my own investigation.

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Caryn Todd
December 2005

I certify the above statement is correct.

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Professor Annabel Fossey
Supervisor
December 2005

ACKNOWLEDGEMENTS

Kerry Etsebeth, THANK YOU! Thanks for all your support over the past two years in the lab and out of it. I would never have made it without you!

I would especially like to thank my supervisor, Professor Annabel Fossey, for all her guidance, and patience, during the practical side of my research, and particularly in terms of my write up. Thank you Annie!

Thank you to Dr Mervyn Beukes, your assistance with my research was much appreciated. Your knowledge and recommendations were always welcome, and I am so grateful that you were willing to help.

Megan Brunkhorst, thank you for all your help down in the lab, I am sure we must have driven you crazy sometimes with our stupid questions, but your patience and knowledge was much appreciated!

Carolyn Hancock, thank you so much for all your assistance and the opportunities you have given me over the past two years. I am very grateful.

In terms of finding a dairy herd, blood collection, and help with anything to do with animal science, I must thank Jon Tyler. It would have been a very frustrating two years without your valuable knowledge, and humour!

My greatest thanks must go to Mark Jonsson, owner of the Sarsden Jersey herd. It was wonderful of you to assist me in my research by allowing me access to your milk records and your precious cows. Thank you!

Finally to my family, thank you for all your support with my never ending studying! Mom and Dad, I promise I'll stop one day! Ashlea...we can do stuff now! I love you so much, thank you!

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LIST OF ABBREVIATIONS

[DNA] – DNA concentration

Δ BV / t - rate of genetic change per unit time

A – additive genetic component

AFC – age at first calving

AFLPs – amplified fragment length polymorphisms

AI – artificial insemination

ARC – Agricultural Research Council

BCL – blood cell lysis buffer

BCS – body composition score

BF – butterfat

BLUP – best linear unbiased prediction

BV – breeding value

CNS – central nervous system

D – dominance genetic component

DIM – days in milk

DNA – deoxyribose nucleic acid

dNTPs – deoxynucleotide triphosphates

DO – days open

E – environmental component

EBV – estimated breeding value

FCE – food conversion efficiency

Fwd – forward

FY – butterfat yield

G – genotypic value

GC % - percentage of guanine and cytosine residues

h^2 – narrow sense heritability

I – epistatic genetic component

i - intensity of selection

ICAR – International Committee for Animal Recording

ICP – inter-calving period

IL-2 – interleukin-2

ITS – internal transcribed spacer region

Kb – kilobases

kDa – kilo Dalton

L - generation interval

Lep gene – leptin gene

LH – luteinizing hormone

MAS – marker assisted selection

mRNA – messenger ribonucleic acid

MW – molecular weight

MY – milk yield

NEB – negative energy balance

NMR – nuclear magnetic resonance

NPY – neuropeptide Y

nrDNA – nuclear ribosomal DNA

Ob gene – obesity gene

OD – optical density

P – phenotypic value

PAG – polyacrylamide gel

PAGE – polyacrylamide gel electrophoresis

PCR – polymerase chain reaction

pol – polymerase

PY – protein yield

QTL – quantitative trait loci

RAPD – random amplified polymorphic DNA

$r_{BV, EBV}$ - accuracy of selection

Rev – reverse

RFLPs – restriction fragment length polymorphisms

rpm – revolutions per minute

RT – room temperature

sdH_2O – sterile distilled water

SNP – single nucleotide polymorphism

SPC – services per conception

STRPs – single tandem repeat polymorphisms

T_A – annealing temperature

T_m – melting temperature

UV – ultraviolet

σ_{BV} - genetic variation

CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

World agriculture is pivotal in providing food for the world's population. In particular, animal agriculture and livestock supply a large proportion of the world's food resources. Famine and food shortage have become an ever increasing problem in recent decades, as a result, it has become necessary for livestock agriculture to increase its outputs (FAO, 2004).

Cattle production accounts for the greatest fraction of livestock agriculture (FAO, 2004), and is grouped into the broad categories, dairy and beef (Ensminger, 1991). In both these industries, farmers and breeders select and breed animals that perform well in economically important traits (Taylor and Field, 1998). Improvement of such economically important traits contributes to economic growth, through an increased output, which could play a major role in combating world food shortages.

Dairy cattle are an important industry in the world; providing 91.4 % of the world's milk and dairy products (Ensminger, 1991), it is thus a very important industry. Milk yield, and protein and fat composition are the most important economic traits for dairy farmers and breeders; other traits considered by breeders and farmers include conformation, reproductive performance, food conversion efficiency (FCE), and disease resistance (Goddard and Wiggans, 1999). Due to the importance of this industry, efforts are ongoing to improve all aspects of milk production through the use of phenotypic and genotypic selection. Good progress has been made in improving dairy traits in the past half century (Taylor and Field, 1998; Philips, 2000).

In recent times dairy breeding has become one of the most advanced livestock

breeding programmes. This is due to extensive data recording practices, and the widespread use of artificial insemination (AI). With the development of molecular genetics and biotechnology in recent years, other avenues have been opened to facilitate the improvement of milk production. Knowledge of the genotypes linked to economically important traits of animals, together with information as to what influence these genotypes have on economically important traits, will allow farmers to make selection decisions before an animal has reached maturity, thus saving time and money (Kinghorn *et al.*, 2000).

1.2 DAIRY FARMING IN SOUTH AFRICA

The dairy industry is one of the largest agricultural industries in South Africa, although small in world terms, and dairy farms are spread across the country. At least six dairy breeds are used in the South African dairy industry; they include the Holstein-Friesian, Jersey, Guernsey, Ayrshire, Brown Swiss and the dairy Shorthorn. The Holstein-Friesian breed is the most predominately used breed, followed by the Jersey breed (U.S Library of Congress, 2005). The average production performances of the four main breeds used in South Africa for 1998 (Clover, 2000) are listed in Table 1-1.

Table 1-1 **Average milk yield, butterfat and protein percentages for four breeds of dairy cow used in South Africa, for the year 1998, according to Clover (2000).**

Breed	Average milk yield (kg)	Butterfat (%)	Protein (%)
Holstein-Friesian	7 926	3.53	3.15
Jersey	5 039	4.57	3.64
Ayrshire	6 538	3.79	3.32
Guernsey	5 170	4.41	3.46

1.2.1 **Jersey breed**

The Jersey cow originates from Jersey Island (Schmidt and Van Vleck, 1974). It is the smallest dairy breed; they are short horned and have a mainly fawn coat, sometimes including white areas of hair (Porter, 1991), and possess a number of unique traits that make them economically valuable, Figure 1-1 shows a typical Jersey cow. Their milk has high butterfat content (averaging 5.3 %), relative to other dairy breeds (Holstein-Friesians), although their milk yield is lower (approximately 5 000 kg per lactation) (Schmidt and Van Vleck, 1974; Buchanan and Dolezal, 1999). The Jersey is the earliest maturing breed, reaching peak milk production earlier than other breeds (Schmidt and Van Vleck, 1974). They possess a remarkable ability to adapt to tropical climates, despite their origin being temperate (Porter, 1991), and have thus become one of the important dairy breeds in South Africa.

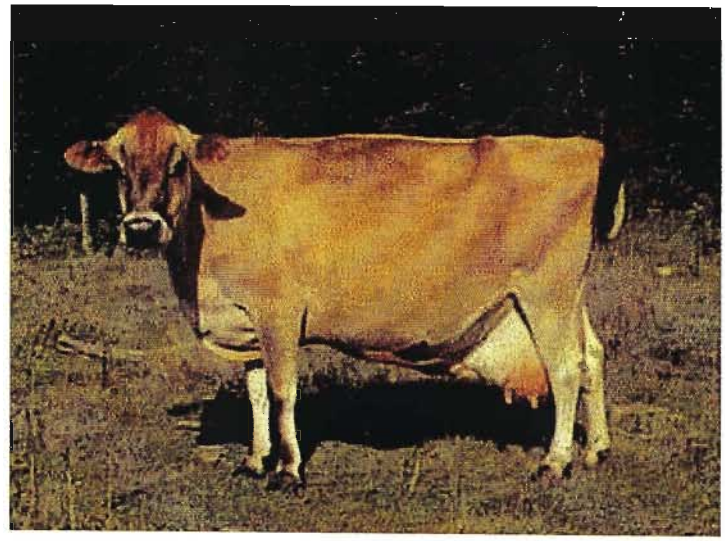


Figure 1-1 **Jersey cow with typical fawn colouring (Timber Trails Farm, 1999).**

Through many years of intensive breeding, a number of South African Jersey breeders have contributed to South Africa becoming one of the leading Jersey countries in the world. In 1998, the International Committee for Animal Recording (ICAR) produced a summary of Jersey breed statistics of the member countries, a

summary of these are reflected in Table 1-2.

Table 1-2 **Summary of world Jersey herd statistics for 1998, adopted from Van Niekerk (2001).**

Country	Number of registered cows	Standardized days in milk (DIM)	Milk yield (kg)	Increase[a] in milk yield (%)	Increase[a] in butterfat %	Increase[a] in protein %
United States of America	149 151	305	6 475	5.35	- 1.06	- 0.53
Denmark	72 615	365	5 389	10.91	- 0.98	1.48
South Africa	36 314	295	4 863	2.99	3.66	0.55
United Kingdom	20 475	305	4 611	6.27	- 1.08	1.84
Japan	2 157	305	5 623	- 0.64	- 1.21	0.26
New Zealand	419 709	215	2 705	- 5.88	- 0.69	- 2.43

[a] The increase in the production traits represents the improvement in the trait from 1996 to 1998.
Negative values refer to a decrease in production, while positive values refer to an increase in production.

Although the Jersey breed is high performing in the South African dairy industry, any improvement in the economically important production traits of the breed would be potentially beneficial. The production traits could be improved through altering those factors, both genetic and environmental, that influence these traits.

1.3 DAIRY TRAITS

1.3.1 Introduction

The most important traits in the dairy industry are those that have an impact on the economic performance of the industry. The primary product from the dairy industry is milk for human consumption, thus farmers aim to increase the milk yield and the components of the milk, to produce milk of a high quality (Theron *et al.*, 2000).

Linked to milk production is the fertility of the cows; in order for the cows to provide milk continuously it is essential for the cows to become pregnant annually, thus initiating the lactation cycle repeated. To facilitate annual calving, the cows are inseminated while they are still lactating. It is therefore clear that fertility is a vital factor in promoting an efficient dairy industry (Lucy, 2001), and is considered a very important economic trait.

This industry has, in recent years, become very sophisticated. Breeders and farmers are including many secondary traits that contribute to the primary traits in their production and selection criteria. Body conformation traits, including udder quality and the quality of the legs and feet, and traits associated with the longevity, health and welfare of the cows are becoming more valued with respect to their contribution to milk production (Moss, 1992).

1.3.2 Milk production traits

Milk production traits can be divided into those of quantity and quality. Milk quantity refers to the yield of milk produced by a cow, while milk quality concerns the butterfat, protein and lactose content in the milk.

Milk results from a series of reactions caused by complex physiological processes. The make-up of milk is a complex mix of components; these are displayed in Figure 1-2, and include water, fat, lactose, protein, and other minor components (Bawden and Nicholas, 1999).

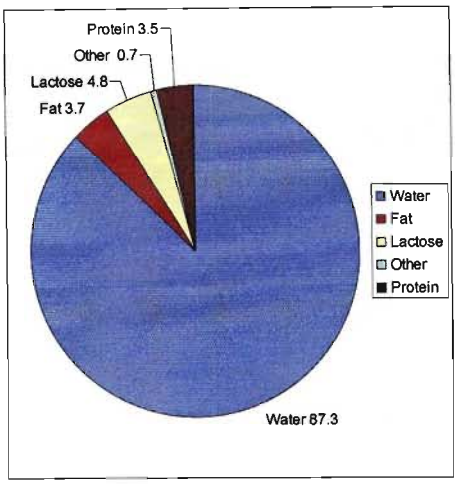


Figure 1-2 **Make up of milk and the percentage contribution of each component (adapted from (Bawden and Nicholas, 1999).**

Milk yield is one of the most important economic production traits in dairy cattle; a great deal of research has thus gone into improving milk yield (Goddard and Wiggans, 1999). It is a quantitative characteristic, which is affected by many genes, as well as the environment (Goddard and Wiggans, 1999; Kinghorn *et al.*, 2000). It is usually measured in terms of the volume of milk that a cow produces during the approximately 305 days after calving.

Milk quality refers to the components of milk that provide the nutritional value; these include butterfat (BF), protein and lactose. Milk quality is often described in terms of the percentage of BF, protein and lactose in the milk, although these components may also be expressed in kilograms per lactation. As in the case of milk quantity, it is a quantitative trait, and is affected by the genotype and the environment of a cow. In different areas of the world and in South Africa, there is a demand for different types of milk, with varying percentages of fat and protein.

These levels are adjustable through selection for animals that possess the desired characteristics. The protein component of milk is the most valuable, and any improvement in this component, without an accompanying increase in yield, would be beneficial (Bawden and Nicholas, 1999).

1.3.3 Reproductive performance traits

The fertility of a dairy cow is one of the most important reproductive performance traits, with others including the age at puberty and ease of calving of a cow. The fertility of a cow is described in numerous ways, including (Meyer *et al.*, 1990):

- Inter-calving period (ICP),
- Days open (DO),
- Services per conception (SPC) (AI's per conception), and
- Age at first calving (AFC).

Dairy cow fertility in the twenty-first century has been described as being at an all time low. This is causing inefficiency in dairy herds, since poor reproductive performance is the primary reason for the culling of dairy cows, which often results in the loss of valuable alleles in other traits (Ross, 1993; Lucy, 2001). Extensive research is thus required to improve the fertility of dairy cows.

1.4 GENETIC IMPROVEMENT OF DAIRY TRAITS

1.4.1 Introduction

Over the past fifty years great progress has been made in the genetic improvement of dairy cattle. Much of this has been due to the advanced nature of dairy breeding strategies; progeny testing is used throughout the commercial

industry, artificial insemination (AI) is also implemented, and computer-based analysis of available data is extensive. These practices provide farmers with a greater amount of information and resources on which to base their selection decisions (Bourdon, 1997). More recently, with the advancement of molecular genetics and biotechnology, a new avenue of genetic improvement is becoming available to farmers. This involves the identification of genetic markers, which are now effectively used in marker assisted selection (MAS) (Kinghorn *et al.*, 2000).

There are a number of important factors that need to be considered when breeding animals for agricultural production. These factors include (Kinghorn *et al.*, 2000):

- The breeding objective,
- The traits to be measured,
- The use of reproductive technology,
- The number of animals to be selected, and
- How these animals will be mated.

1.4.2 **Genetic model for quantitative traits**

The economically important dairy traits: milk yield, milk composition, and fertility are quantitative traits (Theron *et al.*, 2000); which are influenced by numerous genes and by many environmental factors (Snustad and Simmons, 2000).

An individual's phenotypic performance in these traits is therefore a combination of its genetic make up and the environment in which the individual lives (Taylor and Field, 1998). This is indicated by the well-known genetic model:

$$P = G + E$$

Where P – Phenotypic value
G – Genotypic value
E – Environmental component (Webster, 1993).

The genotypic value includes a number of factors such as (Kinghorn *et al.*, 2000):

- The additive genetic component (A),
- The dominance genetic component (D), and
- The epistatic genetic component (I).

The genetic model that reflects all these components is represented as follows:

$$P = A + D + I + E$$

Where P – Phenotypic value
A – Additive genetic component
D – Dominance genetic component
I – Epistatic genetic component
E – Environmental component

In a population or herd, phenotypic variation will occur because of variation in the genotypes between individuals, with respect to a quantitative trait. If it could be shown that the environment that a herd shares is the same, then it could be concluded that phenotypically superior individuals would be genotypically superior as well. A procedure that compares and evaluates this genetic superiority would greatly facilitate farmers and breeders in their decision making process when selecting (Kinghorn *et al.*, 2000).

1.4.3 **Genetic evaluation of dairy traits**

When making selection decisions it is necessary to have some method of evaluating the traits under selection; information available to farmers and breeders include the breeding value (BV) of a particular individual, the narrow sense heritability (h^2) of a particular trait in a specific herd or population, and the correlation between two traits.

Breeding value

When making selection decisions farmers and breeders wish to choose as parents those individuals with the best set of alleles. These animals would have the best breeding value (BV), which represents that part of an individual's genotypic value, which is transmitted to its offspring (Bourdon, 1997). Breeding value is an additive value and is due to the independent effect of the alleles of the genes; it represents the additive genetic component in the genetic model for quantitative traits. The gene combination effects that result at the formation of the genotypes are excluded.

The practical use of the breeding value is to estimate the value of an individual as a genetic parent; it is determined from the performance of a large number of an individual's progeny. It is essentially impossible to verify the exact breeding value of an individual, because quantitative traits are influenced by more than one gene, only a sample half of the parent's genes are transmitted to its offspring, there are a large number of possible gene combinations in the offspring, and finally, the performance of the offspring is influenced by its environment (Falconer, 1989; Bourdon, 1997). While it is not possible to determine the accurate breeding value, an estimate can be calculated, the estimated breeding value (EBV), which is used to select individuals as parents for the next generation (Bourdon, 1997).

Heritability

Heritability is an indication of the strength of the relationship between the phenotypic performance of a population or herd, and the breeding values for a trait in a population (Bourdon, 1997). Heritability indicates whether the phenotypic value is on average a good indication of the breeding value. It shows the degree of the genetic influence in the performance of a trait.

Heritability, a population measure, describes a specific trait, at a particular time in a population or herd (Meyer *et al.*, 1990). Narrow sense heritability is the ratio of breeding value variance to the phenotypic variance, or $h^2 = \sigma^2_A / \sigma^2_P$. In contrast the broad sense heritability (H^2) is the ratio of genotypic variance to the phenotypic variance, or $H^2 = \sigma^2_G / \sigma^2_P$. Broad sense heritability is of little value since it reflects the effect of the gene combination value (GCV) in addition to the genotypic value; GCV is not transmitted from parent to offspring, and thus has no value when selecting individuals as parents of the next generation. Narrow sense heritability on the other hand represents only the genotypic value, that which is transmittable from parent to offspring (Bourdon, 1997).

The value of heritability ranges from 0 to 1, where estimates of traits greater than 0.4 are regarded as highly heritable. On the other hand, traits with heritability values between 0.2 and 0.39 have medium heritability, and traits with values less than 0.2 have low heritability (Taylor and Field, 1998). Therefore, when heritability for a trait is high, phenotypic selection will be a useful breeding tool for improving the trait. On the other hand, if heritability is low, then phenotypic selection will not be a reliable tool to apply in a breeding programme (Taylor and Field, 1998).

Correlation

Genetic correlation is described as the strength of the relationship between the breeding values of two different traits. It is useful in estimating the correlated

response for correlated traits. Correlations between traits are caused mainly by pleiotropy (a single gene affecting more than one trait) and linkage (when two genes affecting two different traits are closely linked, they usually segregate together) (Bourdon, 1997).

Correlations, which are population measures, are either positive or negative (ranging from -1 to +1), and may be favourable or unfavourable. It should, however, be pointed out that a negative correlation is not necessarily unfavourable. Both positive and negative correlations can be favourable or unfavourable. The larger the magnitude of the correlation (farther from 0), the stronger the relationship between the traits will be (Bourdon, 1997). A value of 0 indicates no relationship between the two traits.

Knowledge of correlations is useful in developing selection strategies, based on a correlated response to selection, that is, a genetic change in one or more traits of interest, resulting from selection for another. Selection for a trait is either direct or indirect. Selection is viewed as direct when the trait of interest is used in the selection criteria, while indirect selection is based on the selection of one trait, but includes an improvement in the trait of interest, due to the two traits being correlated. Indirect selection is of particular use when the trait of interest is difficult to measure, or is only expressed in one sex, or is a carcass trait.

1.4.4 **Factors affecting genetic improvement**

There are a number of important factors that influence the genetic progress that may be achieved when breeding for a particular trait, these include:

- Accuracy of selection,
- Selection intensity,
- Genetic variation, and
- Generation interval.

Accuracy of selection refers to the strength of the relationship between the actual BV of an individual, and the estimated BV. If the accuracy were perfect, it would imply that the actual BV was known, thus a high accuracy of selection is preferential since this would provide the best estimate of an individual's BV. The selection intensity is the proportion of the population that is selected for breeding; the smaller the proportion selected, the higher the intensity, and the faster the response to selection. The variability of the BV for a specific trait under selection in a population is referred to as the genetic variation. A larger genetic variation is favourable because it allows animals that are much better than average to be selected. A shorter generation interval, the time needed for one generation to be replaced by the next, leads to faster genetic response.

Considering these factors together they can be used to calculate the response to selection or the rate of genetic change per unit time:

$$\Delta \mathbf{BV} / t = (r_{BV,\,EBV})\,(i)\,(\sigma_{BV}) / L$$

Where Δ BV / t = rate of genetic change per unit time

$r_{BV,\,EBV}$ - Accuracy of selection

i - Intensity of selection

σ_{BV} - Genetic variation

L - Generation interval

Maximum genetic progress is achieved by maximizing the accuracy of selection, the intensity of selection and the genetic variation. The fastest progress will be made when the generation interval is shortened (Bourdon, 1997).

1.4.5 Genetic improvement of milk production and reproductive performance traits

Genetic variation is necessary for progress to be made through selection, if only a

small amount of variation exists in a population then the superior animals selected as parents and their progeny will be only a little better than average, and the rate of progress will be slow. If variation is high, and only the very best animals are selected, based on accurate milk production measurements, then the selected individuals and their progeny will produce higher milk yields and more preferable milk component percentages than average, and the rate of change will be fast (Bourdon, 1997). The same can be said for fertility traits.

Evidence that genetic variation exists for milk production comes from the fact that across dairy breeds variation in milk yields and compositions occur (Ensminger, 1991; Taylor and Field, 1998). For example, as was indicated in Table 1-1, Holstein cows produce the highest average milk yield per lactation (7 926 l), and Jersey cows the lowest (5 039 l), whereas for butterfat, Jersey cows produce a higher percentage (4.57 %) than the other dairy breeds. These differences are due to the different genetic makeup of the breeds (Ensminger, 1991).

Within dairy breeds variation in production traits also exists, different animals of the same breed have different performance levels with respect to milk yield and quality, as well as fertility; these differences can be present within a particular herd, which is further evidence for genetic variation, if one assumes that the cows have a common environment (Ensminger, 1991).

Due to the variation in milk yield and composition between different dairy breeds and within dairy breeds, it has been possible to improve milk production through selection, by selecting animals with superior production performances to become parents (Taylor and Field, 1998).

Accuracy of selection is related to the heritability of a particular trait in a population. Accuracy of selection needs to be high to obtain a faster response to selection. Heritability is the ratio of the genetic variation to the phenotypic variation, and tells one how much of the variation in phenotype is due to the

genotype. Heritability estimates provide useful information regarding the accuracy of selection, since it represents the strength of the relationship between true breeding value and the estimated breeding value. High heritability estimates therefore represent a greater degree of accuracy, which increases the rate of genetic change (Bourdon, 1997).

Examples of the heritability estimates for milk yield and the various components of milk are displayed in Table 1-3. The heritability for milk yield is moderate, allowing reasonable genetic progress to be made through the use of phenotypic selection (Taylor and Field, 1998). The heritability of butterfat percentage is classed as high, phenotypic selection for this trait would result in fast genetic change. The heritability for protein percentages ranges from moderate to high.

Table 1-3 Heritability estimates for milk yield, butterfat and protein percentage, and butterfat and protein yield for different dairy breeds.

Trait	Heritability	Reference
Milk yield (MY)	0.25 – 0.56	Meredith (1995); Taylor and Field (1998); Olori *et al.* (2002); Berry *et al.* (2003)
Butterfat %	0.55 – 0.72	Meredith (1995); Bourdon (1997)
Protein %	0.50 – 0.64	Meredith (1995); Bourdon (1997)
Butterfat yield (FY)	0.44 – 0.52	Meredith (1995); Berry *et al.* (2003)
Protein yield (PY)	0.32 – 0.45	Meredith (1995); Berry *et al.* (2003)

While great improvements have been achieved for milk production traits over the past two decades, the opposite is true for reproductive performance traits, specifically fertility (Lucy, 2001). This drastic decline in fertility is believed to have occurred mainly due to increased phenotypic selection for milk production, which is unfavourably correlated to fertility, although other factors may have influenced

this decline as well, such as poor animal health, poor living conditions, and increased temperatures (Dematawewa and Berger, 1998; Lucy, 2001; Olori *et al.*, 2002; Berry *et al.*, 2003; Wall *et al.*, 2003).

Estimates of heritability, reflected in Table 1-4, for reproductive performance traits are typically low, usually less than 0.05, which means that phenotypic selection for fertility is unreliable (Kirkpatrick, 1999; Wall *et al.*, 2003). This implies that the environment and management factors have a large influence on the fertility of a cow (Weigel and Rekaya, 2000; Olori *et al.*, 2002).

Table 1-4 **Heritability of the reproductive fertility traits: inter-calving period (ICP), days open (DO) and services per conception (SCP).**

Trait	Heritability	Reference
ICP	0.03 – 0.10	Bourdon (1997); Olori *et al.* (2002); Wall *et al.* (2003)
DO	0.03 – 0.04	Walker *et al.* (1994); Dematawewa and Berger (1998)
SPC	0.02 – 0.03	Walker *et al.* (1994); Wall *et al.* (2003)

Farmers have typically selected for milk production traits over the past fifty years due to the high heritability estimates making phenotypic selection reliable, as a result fertility has been neglected, and so a drastic decline in dairy herd fertility has occurred (Lucy, 2001).

The existence of possible correlations between traits has been mentioned. One of the most documented correlations in dairy cattle is that between milk yield and fertility, this correlation is positive value, but unfavourable. Many of the genes that influence milk production also affect other reproductive performance. Table 1-5 shows the genetic correlations between important dairy production and reproduction traits.

Table 1-5 Genetic correlations between various dairy cattle traits.

Correlated traits	Correlation	Reference
MY and butterfat %	- 0.25 – - 0.50	Bath *et al.* (1978); Bourdon (1997)
MY and protein %	- 0.30	Bath *et al.* (1978)
MY and PY	0.80 – 0.90	Bath *et al.* (1978); Bourdon (1997); Dematawewa and Berger (1998)
MY and FY	0.45 – 0.61	Bourdon (1997); Dematawewa and Berger (1998)
FY and PY	0.60 – 0.77	Bourdon (1997); Dematawewa and Berger,(1998)
FY and butterfat %	0.55	Bourdon (1997)
PY and butterfat %	- 0.15	Bourdon (1997)
MY and ICP	0.27 – 0.67	Olori *et al.* (2002); Pryce *et al.* (2002); Wall *et al.* (2003)
MY and DO	0.63	Dematawewa and Berger (1998)
MY and SCP	0.06	Wall *et al.* (2003)
FY and DO	0.57	Dematawewa and Berger (1998)
PY and DO	0.59	Dematawewa and Berger (1998)
ICP and SCP	0.61	Wall *et al.* (2003)
ICP and body composition score (BCS)	- 0.14 – -0.48	Pryce *et al.* (2002); Wall *et al.* (2003)

The correlation between milk yield and ICP and DO is strong, but, it is an unfavourable correlation. An increase in milk yield, results in a decrease in the fertility or an increase in the ICP and DO. This demonstrates why fertility has declined with selection for milk production, as milk production levels have increased, the ICP and DO have also increased, thus decreasing fertility. Fertility traits have been shown to be correlated to traits other than yield, one important such trait is body composition score (BCS). A favourable genetic correlation exists between fertility and BCS; cows with a high BCS during lactation will become fertile sooner after calving, than one with a low BCS (Dechow *et al.*, 2002; Wall *et al.*, 2003). BCS could therefore be used to indirectly select for improvements in fertility (Pryce *et al.*, 2002).

Knowledge of these genetic correlations provides farmers with more information to make selection decisions with, since the correlated response to selection can be

taken advantage of, whereby selection in one trait results in a response in a genetically correlated trait (Bourdon, 1997).

Milk production and reproductive performance traits, both quantitative traits, are improved through manipulation of the genetic components of the phenotype, as well as control of the environment. Management of the dairy herd accounts for a large proportion of the environmental component. In dairy farming, management practices are very stringent, and kept at the highest standards to ensure optimum production and performance. For the purposes of this review, the environment will not be covered, since the focus of the research is on the effect of the genotypic contribution.

1.4.6 **Breeding practices**

The main breeding objective of any dairy breeding programme is to produce cows with the best possible genetic ability to be profitable (Theron *et al.*, 2000). Initially, when farmers began selecting for important dairy traits, selection was subjective. In recent years, however, selection has become far more accurate with the introduction of AI, the extensive recording of herd data, and the development of computer programmes for analyzing the data, and estimating the genetic value of individuals (Kinghorn *et al.*, 2000).

Artificial insemination has essentially opened the world's resources, and made superior genetic bulls available to farmers, anywhere in the world. These AI bulls, in addition to being available worldwide, are also able to sire thousands of progeny in many different countries. AI allows the accurate prediction of EBV's of males, due to the large number of progeny records available (Kinghorn *et al.*, 2000).

Milk recording schemes provide accurate information to dairy farmers, which allows them to make informed selection decisions to improve the performance of their cows. Through the use of computer programmes, for example Best Linear

Unbiased Prediction (BLUP), the information can be used to estimate the breeding values of individual animals, and it can be applied to make management decisions. BLUP is useful because it adjusts for certain environmental effects and takes information from all relatives into account when estimating EBV's; this improves accuracy and reliability (Hallowell, 2000; Loubser, 2000).

In order to implement a successful breeding programme it is essential to establish a breeding goal, this is a set of characteristics of the cow that the farmer is intending to advance through selection. The fewer traits included in the goal, the greater the possibility for genetic progress in each of those traits; although, selecting for a large number of traits can lead to a better overall economic improvement, provided that the additional traits are of economic significance (Bourdon, 1997).

Parents are chosen if they rank highly according to the breeding goal, for example they may be high milk producers or have sired high milk producers. Most selection focus is on the bull selected; this is because the selection intensity for the bull is much higher than for the cows, because they are able to produce many progeny in a breeding season, due to the implementation of AI. This increase in selection intensity results in a faster response to selection (Bourdon, 1997).

Both inbreeding and out-breeding are practiced in the dairy industry. Out-breeding or crossbreeding is utilized to exploit heterosis, due to non-additive gene action, and complementarity, due to additive gene action; while inbreeding is used to maintain superior genotypes or to increase hybrid vigour when two inbred lines are crossed, by mating closely related individuals (Cunningham, 1981; Lasley, 1987; Bourdon, 1997).

In recent years, much development has occurred in the field of molecular markers, and their use in dairy breeding programmes. The utilization of DNA markers in marker assisted selection may be the future of the dairy breeding industry,

permitting more accurate selection decisions to be made.

1.5 MOLECULAR MARKERS AND MARKER ASSISTED SELECTION

Markers are reference points on biomolecules, these markers are polymorphic. There are two types of markers: phenotypic and genotypic. Phenotypic markers represent physical differences caused by differences in the DNA sequence, for example different blood group or histocompatability antigens, and protein allozymes. Genotypic markers represent and identify differences in the DNA molecule itself.

Phenotypic markers, particularly allozymes, were predominately used to determine the genetic variation between individuals prior to the development of molecular DNA techniques. However, with the advent of molecular technology and the first identification of polymorphisms at the DNA level in 1985, DNA markers have begun to replace phenotypic markers. A DNA marker can be any sequence of DNA, including both coding and non-coding DNA. DNA markers represent constant 'landmarks' in the genome of organisms, which display a large amount of polymorphism, thus making markers useful in a large variety of fields, including DNA fingerprinting, genotyping, individual identification, paternity testing, inbreeding assessment, genetic diversity and population structure analysis.

DNA markers are categorized into two major groups: single-locus markers and multi-locus markers. Single-locus markers focus on only one locus and are thus locus specific, while multi-locus markers analyze numerous loci simultaneously. Single-locus markers include:

- Simple tandem repeat polymorphisms (STRPs), and
- Restriction fragment length polymorphisms (RFLPs).

Multi-locus markers include:

- Random amplified polymorphic DNA (RAPDs), and
- Amplified fragment length polymorphism (AFLPs).

DNA markers are utilized in marker assisted selection (MAS) as tools to assess the DNA of individuals; a genotypic analysis. The information provided by the DNA markers provides an accurate description of the genes that affect the phenotype under investigation. MAS is thus used to make accurate selection decisions about which individuals will be chosen as the parents of the next generation.

1.5.1 **DNA markers**

DNA markers are used extensively today to identify the genotypic characteristics of individuals, by detecting variation in a DNA sequence, and can be followed through generations. There are three main advantages of DNA markers; they are not influenced by changes in the environment, they are potentially infinite in number and cover the entire genome, and they provide an objective measure of variation. The major disadvantage of many of these markers is that more technically complex equipment is required, and development may be very expensive.

A number of DNA marker options are available to analyze the genetic variation between individuals. It is necessary to choose a DNA marker that meets the requirements of the investigation being undertaken, the DNA marker must consistently reveal adequate genetic variation to answer a particular question, with a minimum amount of effort and expense (Parker, 1998).

A number of criteria exist to select an appropriate DNA marker for analyzing genetic variation, including (Bourdon, 1997):

- DNA markers should be highly polymorphic,
- DNA markers should be neutral; have no effect on the trait of interest or the reproductive fitness of the organism,
- DNA markers should preferably be co-dominant, allowing all possible genotypes to be identified,
- DNA markers should be located at known chromosomal positions and distributed evenly throughout the genome,
- DNA markers should produce the same results every time the marker is used; reproducible,
- DNA markers should be able to be scored objectively, and
- DNA markers should be inexpensive and easy to employ.

Single-locus markers

Single-locus DNA markers investigate only one specific locus per marker. Prior knowledge of the DNA sequence is required in order to develop the DNA markers, specific primers must be designed that will bind to desired locus (STRPs), or enzyme recognition sequences identified within the area of interest (RFLPs). This is time-consuming and expensive, a drawback of single-locus markers. Once this initial development step is achieved, however, then single-locus markers are relatively inexpensive and easy to utilize. In addition, these DNA markers are found abundantly throughout the entire genome, thus representing a large amount of genetic variation, this means that, despite their disadvantages, they have become very popular in a variety of different studies, including comparisons of genetic variation between populations and genotyping of individuals in populations (Bruford *et al.*, 1992). Single-locus DNA markers are co-dominant, which is a further advantage since it allows both the homozygotes and the heterozygotes to be identified. The two main markers included in this category are: RFLPs and STRPs.

An RFLP is a single nucleotide polymorphism that eliminates a restriction site in a strand of DNA. Today, most RFLPs make use of PCR amplification and PCR primers, although many are still identified through the use of a hybridization probe. PCR amplification amplifies a specific sequence of DNA from the genome, to produce a fragment of specific length, which either includes or excludes a specific known restriction site. A restriction enzyme is used to digest the fragment; if a restriction site is present, the DNA strand is cleaved, resulting in the strand decreasing in size and thus showing up as two different bands on a gel. If the restriction site is not present, then the restriction enzyme does not digest the fragment, and a single band appears on the gel. Differences in the length of the fragments generated occur as a result of mutations, insertions, deletions and base substitutions. Due to the nature of RFLP markers there is a limit on the number of alleles possible at a specific locus; only two or a few alleles are possible at each locus since the restriction enzyme either cuts or it does not.

STRPs occur due to differences in the number of repeats of a specific, short DNA sequence in the genome; this sequence may be repeated many times in tandem, at a locus in the genome (Avise, 1994). Genetic variation or polymorphisms are evident as the number of core sequences present in an individual. The number of repeats of the core sequence is termed the alleles (Parker *et al.,* 1998). STRPs may differ in the sequence and length of the repeating unit, as well as the minimum and maximum number of tandem copies that occur in DNA molecules in the population. Based on the number of nucleotides making up the core sequence STRPs are grouped into two types: microsatellites or minisatellites. Microsatellites (or simple sequence length polymorphisms – SSLPs) are STRPs with repeating units of 2 – 9 bp, minisatellites (or a variable number of tandem repeats – VNTR) are STRPs with repeating units of 10 – 60 bp. STRPs have a high polymorphic content, brought about through the varying number of repeats between individuals, thus resulting in a large number of possible alleles at one locus (Avise, 1994).

Multiple-locus markers

Multiple-locus markers evaluate a number of different loci concurrently, utilizing one or a few arbitrary primers. As a result, these markers are not locus specific; they could amplify any area of the genome. The use of arbitrary primers means that no prior information is required regarding the DNA sequence, thus making the multiple-locus approach much easier, less expensive and less time-consuming. Multiple-locus DNA markers are found abundantly in the genome, and display a moderate amount of variation at the loci. The results produced through the use of multiple-locus DNA markers, multiple-locus fingerprints, have high information content and can be useful for individual identification (Fowler *et al.*, 1998) and parentage analyses (Questiau *et al.*, 1999). This is one of the major advantages of multi-locus DNA markers; many loci can be assessed at once, although within a locus only two alleles are possible. These alleles are represented by either the presence or absence of a band on a gel. One drawback of multiple-locus markers is that they are dominant, thus it is not possible to discern between homozygous and heterozygous genotypes. As mentioned above there are two major multiple-locus DNA markers, RAPDs and AFLPs.

RAPD analysis utilizes a single arbitrary primer of approximately 10 to 11 nucleotide bases in length. The primer anneals to multiple sites on the template DNA due to their short nature. When primers anneal in opposite orientation at a distance within the limits of the PCR reaction, then the sequence is amplified. This produces bands on an agarose gel, which is used to identify the alleles (Welsh & McClelland, 1990). The different alleles are due to polymorphisms, often SNPs, that are present in the annealing region of the primer; these mutations may be caused by insertions, substitutions, or deletions in the primer annealing region or in the intervening sequence, thus changing its length and removing the annealing site from the expected position. The presence of these polymorphisms is the basis of RAPD markers, since their presence alters the ability of the primers to anneal, and thus amplify the DNA. In RAPD analysis, polymorphisms are

identified as the presence or absence of a band on a gel.

AFLP analysis is based on repeated amplification using PCR on a subset of restriction fragments from a total digest of genomic DNA, and are a modification of RAPDs that increases specificity (Desmarais *et al.*, 1998). AFLPs are created by attaching double stranded oligonucleotide sequences, which match the primer sequences perfectly, to genomic restriction fragments enzymatically, prior to amplification. This allows more specific polymorphisms to be detected than with RAPD analysis.

1.5.2 **Marker assisted selection**

Marker assisted selection (MAS) is a technique that uses of DNA markers to provide information on the genotype of an animal, which is then used in making more accurate selection decisions (Kinghorn *et al.*, 2000). Once a marker is identified that is linked to genes affecting economically important production traits, the marker can be used to identify which animals should be used to breed for the next generation. The most accurate markers are those that identify a section of the gene of interest, thus making it completely accurate, or as close to the gene of interest as possible, so as not to be disrupted by recombination. Direct markers are the most accurate; they lie within the gene of interest, while indirect markers are found near the gene of interest, but where recombination is possible.

Marker assisted selection has a number of major advantages. These include the fact that the marker genotypes of the animals can be determined using collected samples, taken from animals at birth, thus allowing the animals' genotypes to be assessed before the animals reach maturity or before sufficient records are available. In addition MAS allows animals to be objectively genotyped; the results are not influenced by any external biases. The DNA marker is measurable in both sexes, regardless of whether the trait is only observable in one sex or not. This provides a great advantage with respect to dairy farming, because it allows bulls to

be selected based on their DNA marker analysis, instead of having to wait for progeny performance records from their daughters. MAS is especially useful in traits that are lowly heritable, difficult and expensive to measure, expressed in only one sex, or measured late in life, or only at death.

The limitation of MAS at present is the identification of markers that are linked to advantageous or disadvantageous alleles. However, this information is continually being discovered and reported, thus making the use of MAS more feasible as time continues.

1.5.3 **Identification of quantitative trait loci for milk production and reproductive performance for use in MAS**

Quantitative trait loci are genes or areas on genes that affect a single quantitative trait. Knowledge of these traits is important since many economically important dairy traits are quantitative in nature, for example, milk yield and fertility. Identifying the loci responsible for these traits will provide an avenue for further research into how these traits can be improved. Quantitative trait loci are identified through the use of genetic markers. For many years the identification of QTL's in livestock species has been inhibited by the lack of genetic markers available. Recently, several polymorphic DNA markers have been identified, which has allowed searches for QTL's affecting milk production and reproductive performance to be carried out (Ashwell *et al.*, 1998).

The identification of associations between QTL's and molecular markers will provide a method for determining the possible use of certain markers as selection tools in the process of improving the performance of dairy cows. Strong associations have been identified between microsatellite markers and QTL's for protein and fat percentage on chromosome 3 and 6; fat percentage and milk yield on chromosome 14; and protein percentage on chromosome 20 (Ashwell *et al.*, 1998; Ashwell *et al.*, 2004). The *DGAT1* gene on bovine chromosome 14 has

been identified as a possible causal gene for the QTL for milk production. The *DGAT1* gene was shown to have a significant influence on butterfat yield, protein yield, and milk yield; however it would be difficult to use as a marker because a polymorphism in the gene results in increased milk fat yield, but decreased milk protein percentage and total volume (Spelman *et al.*, 2002). Another QTL for milk production traits has been identified on chromosome 4, in the region of the serum amylase-1 gene and the leptin gene (Lindersson *et al.*, 1998).

The κ-casein (chromosome 6) and β-lactoglobulin genes are linked to economically important traits, milk protein yield and butterfat yield respectively. Many of the alleles of these genes are known, and genetic markers are available for use in MAS. Use of these markers is becoming popular in Europe and America, while South Africa is lagging behind (Harris and van Zyl, 2000).

The leptin (*lep*) gene on bovine chromosome 4 (previously known as the *ob* gene) is a potential QTL with an influence on milk production traits (Silva *et al.*, 2002; Buchanan *et al.*, 2003), meat production traits (Buchanan *et al.*, 2002), and reproductive performance traits (Gonzalez *et al.*, 2000; Liefers *et al.*, 2002; Buchanan *et al.*, 2003). Several markers in this gene have been shown to be polymorphic, and alleles in the *lep* gene may have an influence on milk yield, butterfat and protein percentage, as well as fertility traits (Buchanan *et al.*, 2003). This gene may be a potential candidate gene for a marker for the genetic selection of various traits in cattle (Hossner, 1998).

1.6 LEPTIN METABOLISM AND GENETICS

1.6.1 Introduction

The *lep* gene produces the 16 kDa hormone leptin, which is secreted by adipocytes (Houseknecht *et al.*, 1998). Leptin has been shown to have roles in

energy balance, the regulation of food intake, fertility, immune functions and fat mobilization of mammals (Liefers *et al.*, 2002). Based on this knowledge it has been suggested that leptin may also influence lactation processes, since body fat reserves play an important role in sustaining high milk production during early lactation (Buchanan *et al.*, 2003). Due to this influence on lactation and fertility, leptin has become a focal point of dairy research.

The concept of a specific hormone that influences appetite and energy balance was first suggested by Kennedy (1953). This led to the eventual discovery of the *obese* (*ob*) mutation in *ob* / *ob* mice and rats, which can be seen in Figure 1-3 (Zhang *et al.*, 1994; Fruhbeck *et al.*, 1998; Houseknecht *et al.*, 1998; Houseknecht and Portocarrero, 1998; Williams *et al.*, 2002). The symptoms displayed by ob / ob mice included severe obesity and type II diabetes, as well as infertility (Zhang *et al.*, 1994). Although the metabolism and physiology of the *ob* / *ob* mice was known prior to 1994, it was not until much later that the actual mutation was investigated, when biotechnology tools became more advanced (Houseknecht *et al.*, 1998).

Figure 1-3 **A wild-type mouse (right) shown with an *ob* / *ob* mouse (left), indicating the severe obesity experienced by *ob* / *ob* mice (Zimmerman, 2005).**

In 1994, Friedman's research group at Rockefeller University cloned the *ob* gene in mice. Their results indicated that the product of the *ob* gene might be involved in signalling between adipose tissue and the central nervous system (CNS) (Zhang *et al.*, 1994). Once the *ob* gene had been characterised it was shown to encode the 16 kDa hormone leptin, which is secreted by adipocytes into the

bloodstream (Friedman and Halaas, 1998; Fruhbeck *et al.*, 1998).

Since the cloning of the *ob* gene in mice, the gene has been cloned in a number of other species: humans (Zhang *et al.*, 1994), pigs (Bidwell *et al.*, 1997; Ramsay *et al.*, 1998), cattle (Lien *et al.*, 1997; Ji *et al.*, 1998), sheep (Dyer *et al.*, 1997) and chickens (Taouis *et al.*, 1998). Comparative studies have shown that the *ob* gene is highly conserved among vertebrates. The pig *ob* gene shares 95 %, 92 % and 89 % sequence homology to the cattle, human and mouse *ob* genes, respectively. The cattle leptin gene has approximately 87 % sequence homology to the mouse and human leptin gene. Chicken leptin is 97 %, 96 % and 83 % homologous to mouse, rat and human leptin, respectively (Houseknecht and Portocarrero, 1998).

1.6.2 **Leptin genetics**

The *ob* gene, known more commonly, and referred to from this point on, as the *lep* gene in livestock genetics, consists of three exons, two of which are coding, and two introns, shown in Figure 1-4 (Houseknecht and Portocarrero, 1998). The *lep* gene is approximately 18.9 kb in size and encodes a 4.5 kb messenger ribonucleic acid (mRNA) that is expressed in the adipose tissue. The mRNA contains a highly conserved 167-amino-acid open reading frame (Zhang *et al.*, 1994), and a 21-amino-acid signal sequence common to secretory proteins (Houseknecht and Portocarrero, 1998).

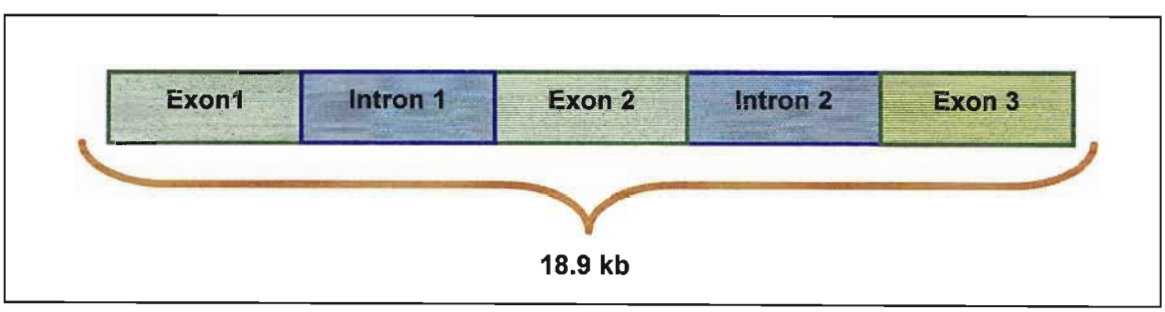


Figure 1-4 **Structure of the *lep* gene, showing the location of the introns and exons.**

The lep gene is expressed in the adipose tissue and placenta of mammals, and is subject to nutritional regulation, as well as regulation by adipose tissue mass and hormones such as insulin and glucocorticoids (Fruhbeck *et al.*, 1998; Houseknecht and Portocarrero, 1998).

Expression of both leptin mRNA and the actual hormone is highly correlated with fat mass, body mass index, and adipocyte size. The larger the size of the adipocyte, the greater the level of leptin mRNA expression and leptin secretion (Hossner, 1998; Houseknecht and Portocarrero, 1998).

1.6.3 **Leptin protein structure**

Leptin has been classified as a member of the haemopoietic cytokine family, based upon its structural characteristics; this family of long-chain helical cytokines includes interleukin-2 (IL-2) and growth hormone (Fruhbeck *et al.*, 1998; Houseknecht *et al.*, 1998). These common structural characteristics include a three-dimensional fold and a four-α-helix bundle structure, which are evident in Figure 1-5 (Rock *et al.*, 1996).

Nuclear magnetic resonance (NMR) has indicated a 4-α-helix bundle structure for leptin (Kline *et al.*, 1997). The four anti-parallel α-helices are connected by two long crossover links and one short loop, which is arranged in a left-handed twisted helical bundle (Zhang *et al.*, 1997). The NMR analysis also indicated a single disulfide bond, between cysteine 96 and cysteine 146, which is critical for structure folding and receptor binding. A mutation in either of these cysteines causes the protein to become biologically inactive (Rock *et al.*, 1996; Zhang *et al.*, 1997; Fruhbeck *et al.*, 1998; Houseknecht and Portocarrero, 1998).

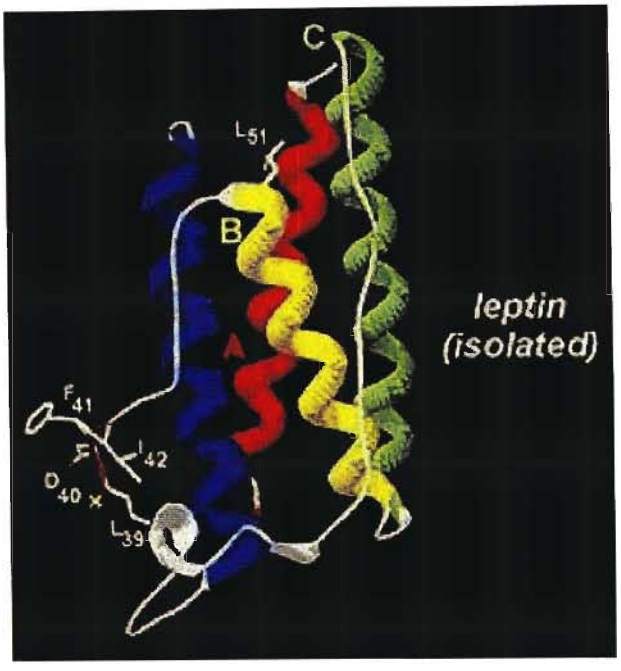


Figure 1-5 **Structure of the 16 kDa leptin protein, with the 4-α-helix bundle structure (Hebrew University of Jerusalem, 2005).**

1.6.4 Leptin function in milk production and reproductive performance

Certain proteins are able to stimulate biological processes in a variety of cell types. A number of cytokines, including leptin, have been shown to display this functional pleiotropy (Fruhbeck *et al.*, 1998). Leptin has been implicated in a wide range of biological functions, including appetite control, energy expenditure, and regulation of neuroendocrine axes, including the reproductive axis (Fruhbeck *et al.*, 1998; Hossner, 1998; Houseknecht *et al.*, 1998; Taouis *et al.*, 1998; Williams *et al.*, 2002). Leptin's control of feed intake has been shown to be mediated primarily by the regulation of neuropeptide Y (NPY), a protein found in the hypothalamus (Stephens *et al.*, 1995). The regulation of the reproductive axis comes about from leptin's control of the release of gonadotropins, possibly in the pituitary gland or hypothalamus (Brann *et al.*, 2002).

Leptin is a factor in the lipostatic theory of weight regulation, which says that weight is maintained by a fat-secreted "factor" (leptin) that reports the body's

energy reserve status to the brain, resulting in the regulation of feeding behaviour, energy expenditure and nutrient partitioning (Houseknecht *et al.*, 1998; Houseknecht and Portocarrero, 1998).

Increased levels of leptin inhibit the anabolic pathways that stimulate food intake and decrease energy expenditure. Catabolic pathways, which inhibit food intake, and promote energy expenditure and weight loss, are stimulated by the presence of leptin (Schwartz *et al.*, 1999).

The body condition and nutrition level of cattle are important factors determining an animal's reproductive potential (Asdell, 1949; Short and Bellows, 1971). A relationship between adiposity and the regulation of the central reproductive axis was suggested over 20 years ago; however the factor that allowed for communication between the systems was unclear. This changed with the discovery of leptin; many believe that this hormone could be the factor that links nutritional status to reproduction (Barash *et al.*, 1996; Houseknecht *et al.*, 1998; Williams *et al.*, 2002).

As mentioned previously, leptin is believed to play vital roles in energy and fat metabolism, as well as in reproduction, in mammals (Liefers *et al.*, 2002). Based on this knowledge it has been suggested that leptin may influence lactation processes, and thus milk production, and fertility in cattle (Liefers *et al.*, 2002; Buchanan *et al.*, 2003). In order to optimise reproduction and lactation in mammals, it is essential that food intake and energy metabolism are carefully regulated, and it is here that leptin plays a role (Houseknecht *et al.*, 1998).

During early lactation cows rely on their body fat reserves to sustain high milk production (Buchanan *et al.*, 2003), which results in the cows having a negative energy balance (NEB) (Buchanan *et al.*, 2003; Jorritsma *et al.*, 2003). This NEB results because the energy required to sustain high milk production, and to maintain the cow's condition, far exceeds the energy available from food intake

(Jorritsma *et al.*, 2003). Since leptin significantly influences energy balance and fat metabolism, it also has an influence on milk production (Buchanan *et al.*, 2003). Evidence for this relationship comes from the fact that leptin concentrations indicate the cow's energy balance during lactation (Liefers *et al.*, 2003b).

Before a cow calves it has a good body condition, which then provides the fat stores to allow milk production to occur after calving (Buchanan *et al.*, 2003). Due to this high body condition the cow has a higher fat percentage, which results in increased serum leptin concentration, because leptin expression and secretion increase with the size of the adipocytes (Hossner, 1998). In response to the increased leptin concentration, there is an increase in metabolic rate, which leads to an increase in milk production (Houseknecht and Portocarrero, 1998), but a decrease in food intake (Liefers *et al.*, 2003b), which is why the NEB results after calving.

However, once the cow has calved and lactation begins, the body condition of the cow drops dramatically, due to the increased fat metabolism associated with an increased metabolic rate. Consequently, the serum leptin concentration falls, reflecting the NEB, this prompts an increase in food intake. Eventually energy balance is restored, which allows the cow to become fertile again (Liefers *et al.*, 2002).

A problem arises if the energy balance of the cow is not restored quickly enough after calving, the fertility of the cow (seen as the length of the postpartum anoestrus period) can be compromised (Liefers *et al.*, 2002). If the cow remains in a state of NEB, then fat stores are used to sustain milk production and maintenance, with reproductive processes having the least priority (Liefers *et al.*, 2003b). This occurs because the NEB, and accompanying low leptin concentration, suppresses the luteinizing hormone (LH) pulse frequency, causing a delay in the first ovulation (Hossner, 1998; Williams *et al.*, 2002; Liefers *et al.*, 2003b). The severity of this infertility depends on the extent and length of the NEB

(Liefers *et al.*, 2003b).

This relationship between leptin and milk production and fertility can explain the unfavourable genetic correlation between fertility and high milk yield. Over the years, dairy farmers have selected phenotypically for high milk yield, which has been achieved, but not without a severe consequence, a drastic decline in fertility levels (Royal *et al.*, 2002). This is because, when milk yield is higher, cows will experience a more severe NEB, which is in turn associated with lower leptin concentrations, which, as previously mentioned, suppresses the LH pulse frequency, thus delaying the first ovulation after calving (Hossner, 1998; Williams *et al.*, 2002; Liefers *et al.*, 2003b).

1.7 *LEP* GENE AND ITS RELATIONSHIP TO MILK PRODUCTION AND FERTILITY

1.7.1 Introduction

Research has provided support for the presence of a link between the hormone leptin and various biochemical pathways; this evidence has prompted researchers to study the gene coding for leptin, the *lep* gene, and its possible relationship to numerous traits, which have a critical influence on efficient and successful dairy cattle production.

1.7.2 *Lep* gene in cattle

In the bovine species numerous polymorphisms have been reported at a number of sites in the *lep* gene, the presence of these highly polymorphic markers has allowed researchers to study the role of leptin in cattle in greater detail (Liefers *et al.*, 2002). Descriptions of the possible markers identified thus far, as well as, their exact location in the *lep* gene, and their possible relationship or association with

various valuable traits are indicated in Table 1-6.

Table 1-6 Markers identified in the lep gene and their locations and associations.

Polymorphism	Association	Beef / Dairy	No. of animals	Reference
103	No	Beef / Dairy	246	Lagonigro *et al.* (2003)
126	No	Beef / Dairy	246	Lagonigro *et al.* (2003)
252	Feed intake	Beef / Dairy	246	Lagonigro *et al.* (2003)
RFLP – *Kpn* 2I (R4C)	Carcass traits	Beef	154	Buchanan *et al.* (2002)
	No	Dairy	623	Liefers *et al.* (2003a)
	No	Beef / Dairy	246	Lagonigro *et al.* (2003)
	Milk yield Protein yield	Dairy	416	Buchanan *et al.* (2003)
	No	Dairy	117	Madeja *et al.* (2004)
	No	Beef	3129	Barendse *et al.* (2005)
RFLP – *Sau*3 AI	Milk yield Protein yield	Dairy	623	Liefers *et al.* (2002)
	Calving interval Weight at first calving	Beef	149	Almeida *et al.* (2003)
	No	Dairy	117	Madeja *et al.* (2004)
RFLP – *Bsa* AI	No	Beef	96	Almeida *et al.* (2003)
RFLP – *Hph* I	No	Dairy	623	Liefers *et al.* (2002)
	No	Beef	100	Almeida *et al.* (2003)

Polymorphism	Association	Beef / Dairy	No. of animals	Reference
BM1500	Milk yield Protein yield Fat yield	Dairy	117	Madeja *et al.* (2004)
	No	Dairy	623	Liefers *et al.* (2002)
	Fat deposition	Beef	158	Fitzsimmons *et al.* (1998)
	No	Beef	102	Almeida *et al.* (2003)

Much of the current research is focusing on the RFLP-*Kpn* 2I polymorphism; it is believed that this may be the functional mutation in the *lep* gene, due to the conformational alteration it causes in the leptin protein (Buchanan *et al.*, 2002). Though, to date only one group have researchers have identified any significant associations (Buchanan *et al.*, 2002; Buchanan *et al.*, 2003).

Knowledge on the allelic variation of the *lep* gene can play a role in the dairy industry, since, if it were possible to identify genetic markers in this gene they could be used to select those animals that have preferable milk production and fertility traits, allowing time and money to be saved. This would be achieved by establishing the relationship between the polymorphisms and milk production and fertility, allowing a farmer to know the expected performance of a mature cow when that cow is still a heifer. The farmer would then be able to select heifers for breeding, based on this information.

1.8 AIM

Currently, the RFLP-*Kpn* 2I polymorphism is the most researched area of the *lep* gene, due to the effect of the mutation. The C to T transition that occurs causes an amino acid change from arginine to cysteine; this alteration results in a

structural change in the leptin protein. From this it is believed that this structural change may cause the protein to bind to its receptor less stringently, resulting in a difference in the efficiency of the protein (Buchanan *et al.*, 2002). However, although much research has been conducted in dairy breeds, very little is known about the effects of the RFLP-*Kpn* 2I polymorphism with respect to the Jersey breed and nothing is known about its effect on the South African Jersey population. If it were possible to gather a greater understanding of this polymorphism in the Jersey breed, it would facilitate molecular marker development, and eventually, selection for superior parents in terms of economically important dairy traits.

An investigation was undertaken to investigate the RFLP-*Kpn* 2I alleles in a South African commercial Jersey dairy herd. The aim of this investigation was to:

- Assess the genotypic variation of the *lep* gene of a selected commercial Jersey herd,
- Assess the phenotypic performance of a selected commercial Jersey herd, with respect to economically important dairy traits, namely milk production and fertility, and
- Assess whether an association exists between the phenotypic performance of the cows in the selected Jersey herd for economically important dairy traits and the genotypes of the *lep* gene.

CHAPTER 2 MATERIALS AND METHODS

2.1 INTRODUCTION

Leptin is a hormone believed to affect milk production and fertility in cattle. This hormone is encoded by the *lep* gene, which is found on bovine chromosome 4 (Friedman and Halaas, 1998). Knowledge of the genotypes of the *lep* gene in different cattle breeds, at specific loci, may be of advantage in developing breeding programmes and strategies for dairy farmers. It is possible that particular genotypes may be associated with improved performance in the economically important milk production and fertility traits (Liefers *et al.*, 2002; Buchanan *et al.*, 2003).

This investigation was undertaken to determine the relationship between the *lep* gene genotypes and economically important dairy traits, namely milk yield and quality and fertility, in Jersey cows. The different components of this investigation of the *lep* genotypes and phenotypic performance of a Jersey herd involved:

1. Assessment of the genotypic variation of the *lep* locus in a selected Jersey herd,
2. Assessment of the phenotypic performance of milk production traits in a selected Jersey herd,
3. Assessment of the phenotypic performance of fertility traits in a selected Jersey herd,
4. Assessment of the genotypic and phenotypic relationship between the *lep* locus and milk production traits in a selected Jersey herd, and
5. Assessment of the genotypic and phenotypic relationship between the *lep* locus and fertility traits in a selected Jersey herd.

All recipes of solutions and buffers have been taken up in Appendix A.

2.2 MATERIALS

Jersey cows were selected for this investigation because they represent a well-known dairy breed in South Africa, with a reputation for a high butterfat component in their milk (Loubser *et al.*, 2000). In South Africa, especially in KwaZulu-Natal, there are a number of major commercial Jersey dairy herds supporting the extensive dairy industry. Therefore, an appropriate Jersey herd had to be identified for use in the investigation, before a sample of cows could be selected.

2.2.1 Identification of a suitable Jersey herd

The identification of a suitable Jersey herd was the first step in this investigation. The selected herd was required to display genetic variation at the *lep* locus and to have extensive records, so that genotypic and phenotypic relationships of the cows could be evaluated. Thus, in consultation with Jersey SA, the South African Jersey breed association, a suitable Jersey herd was identified that met the following criteria:

- Relatively constant management system over the investigation period;
- Presence of some phenotypic variation, suggesting inherent genetic variation within the herd; and
- Registration with the National Dairy Animal Recording Scheme, a service of the Agricultural Research Council (ARC).

A herd owned by the Jonsson family in Balgowan, in the KwaZulu-Natal Midlands, the Sarsden Jersey herd, was thus suggested by Jersey SA. Through consultation with Mark Jonsson it was established that this herd had been subjected to relatively constant management conditions over the investigation period. All cows in the herd had been fed the same diet, and exposed to the same housing and environmental conditions. Genetic variation in the herd was

expected, due to the observation of phenotypic variation in milk production and fertility traits, suggesting the potential for inherent genetic variation. In addition, the use of artificial insemination, and bulls from outside the herd would also have introduced new genetic material to the herd, thus adding genetic variation. The registration of the cows was important since this allowed for the phenotypic data to be easily obtained. It was concluded that this herd met the required criteria and was therefore suitable for the investigation. However, an initial assessment of the phenotypic variation in the herd was performed to confirm the presence of phenotypic variation in the milk production and fertility traits. The mean and standard deviation, and the range for six milk production and fertility traits were determined.

2.2.2 **Selection of Jersey cows from the Sarsden Jersey herd**

Once the phenotypic variation in the selected Sarsden Jersey herd had been established, fifty Jersey cows from the herd were selected for further molecular analysis, required for genotyping of the leptin locus. Only 50 cows were selected due to a number of constraints, including money, access and time limitations, in addition the aim of the research was to carry out a pilot study investigating whether this locus was worthy of further investigation.

50 cows were randomly selected during a midday milking session. As the cows arrived at the dairy the first 50, at the farmer's convenience, were included in the sample, and were assigned a unique sample number. The identification number of each cow was recorded and a blood sample collected. The identification, birth date and lactation, at the time of blood collection, of the 50 cows are shown in Table 2-1.

Table 2-1 **Description of the Jersey cow herd selected for the genotypic investigation.**

Sample no.	Identification no.	Name	Birth date	Lactation
J1	41013863	LETLANDS SNOWGIRL	31/10/1998	4
J2	42362962	ALFREDS HYDRANGEA	12/7/1999	4
J3	42000307	ALFREDS QUEEN MARY	02/04/1999	3
J4	38696860	LINCOLNS STARLET	24/08/1997	6
J5	42862730	ALRIGHT UP THE MILK	31/10/1999	3
J6	44584084	HOTSHOTS KATHERINE	11/05/2001	2
J7	43014794	APEX STARBRIGHT	20/01/2000	3
J8	44407682	LASSOS STARGAZER	15/01/2001	2
J9	45614674	GROOTMANS LOOK SHARP	28/09/2001	1
J10	47266424	XF LASSO QUESTION ME	06/11/2000	2
J11	43014364	APEX FLAB IT DOWN	24/12/1999	3
J12	44582781	ABSAS CHRISSIE	20/03/2001	1
J13	43391846	BARRYS DELICATE	24/03/2000	3
J14	41014168	VIEWS PEANUTS	12/11/1998	4
J15	40328668	JASHOTS CAMMOMILE	24/05/1998	3
J16	40595779	NOGMELKS PRIMROSE	23/08/1998	4
J17	41012212	BARRYS JUNTA	18/08/1998	3
J18	39090121	LINCOLNS LOOK AHEAD	01/10/1997	5
J19	44288504	LASSO PRESS ON	15/11/2000	2
J20	43536580	DUNKERS CANDY FLOSS	04/07/2000	2
J21	43536507	GENERATE BLONDIE	29/06/2000	2
J22	35566850	SOLARS GRACIOUS	27/02/1996	6
J23	36842722	LINCOLNS BERNINA	06/09/1996	7
J24	44289304	GOLIATH BETTER BE	05/12/2000	2
J25	44291334	LASSO STARRY SKY	04/01/2001	2
J26	33615006	MALCOLMS LAST PARTY	21/03/1995	7
J27	43391655	KENTS MONEY BAGS	19/03/2000	3
J28	44348233	GENERATE KERRY	26/11/2000	2
J29	42863027	ALRIGHTS THEA	04/11/1999	3
J30	43191394	MR WORLDS MOONBEAM	15/02/2000	3
J31	45617172	TSUNAMI DARLING LASS	16/11/2001	1
J32	44409274	FUTURE IN THE FRAME	28/02/2001	2
J33	37284122	SOLARS CHRISTINE	03/12/1996	6

Sample no.	Identification no.	Name	Birth date	Lactation
J34	45737830	HENRIS FLY THE FLAG	23/12/2001	1
J35	44407807	FUTURE SHOW WINNER	22/01/2001	2
J36	44775435	CENTURIONS THEA	22/06/2001	2
J37	38913943	SNOWLORD ELF	19/09/1997	4
J38	42362020	PITINOS HONEY JAR	20/06/1999	3
J39	42865568	ALERTS GEMINI	01/12/1999	3
J40	45185592	BRUTOS FRANCHISE	29/08/2001	1
J41	42628073	BARRYS CREAMLINE	10/10/1999	3
J42	38379426	LUKES GRETTA	20/05/1997	5
J43	39658349	JUPITERS FANTASIA	31/10/1997	5
J44	46343802	KINGS RICH REWARD	26/10/2000	2
J45	42627984	DIAMONDS DARKLING	09/10/1999	3
J46	42771717	ALFREDS MARY	30/09/1999	3
J47	44348555	GENERATE EVENESS	10/12/2000	2
J48	43683218	BEACONS PRIKKELPOP	14/08/2000	3
J49	38886826	SBL QUIZICAL	26/01/1997	5
J50	43669845	MARKS SNOWPARTY	21/09/2000	2

2.2.3 **Collection and classification of phenotypic data**

Phenotypic performance records of the selected Jersey cows were obtained to determine the relationship between the economically important dairy traits under investigation and the genotypes of the selected cows. Milk production and fertility records of the cows from the Sarsden Jersey herd were obtained from Graham Hallowell, Programme Manager of the National Dairy Animal Improvement Scheme (ARC). These data were supplied in a Microsoft Office Excel spreadsheet (2003) and were grouped into three different categories, which included the following records:

- **Cow identification data:**
    - Animal name, animal number, farm animal number, birth date, sire name, sire animal number, dam name, dam animal number.

- **Production data:** All production data was standardized for a 305 day lactation length, for comparative purposes
    - Days in milk, milk yield, milk yield (305 days), butterfat yield, butterfat yield (305 days), butterfat percentage (BF %), protein yield, protein yield (305 days), protein percentage, lactose yield, lactose yield (305 days), lactose percentage.

- **Reproduction data:**
    - Inter-calving period (ICP), services per conception (SCP) (calculated as artificial inseminations (AI) required for a cow to conceive during a single breeding period), and age at first calving (AFC).

The dataset obtained was then assessed for completeness. All records that did not contain a full set of information were deleted from the spreadsheet and excluded from all calculations and assessments.

2.2.4 **Collection of genotypic data**

The *lep* gene of Jersey cows; more specifically exon 2 of the *lep* gene, was investigated due to the known functions of its protein product, leptin, and previously reported links to economically important dairy traits (Buchanan *et al.*, 2003). Primers were designed by Buchanan *et al.* (2002) that amplified a section of exon 2 of the *lep* gene. These primers were able to, together with a restriction event; distinguish between two different alleles, thereby allowing the genotypes to be identified.

Lep gene and the RFLP-Kpn 2I primers

A portion of exon 2 of the *lep* gene was amplified using the primer pair RFLP-*Kpn* 2I designed by Buchanan *et al.* (2002). These primers are used, together with a restriction enzyme, to identify the two alleles of a particular SNP in exon 2, namely

the C- and the T-alleles. This SNP results in a change of a single nucleotide from cytosine (C) to thymine (T), and an alteration in the amino acid sequence, with arginine being replaced by cysteine (Buchanan *et al.*, 2002). These primers introduce a purposeful mismatch into the sequences produced through the PCR, which creates a possible recognition site for the restriction enzyme (*Kpn* 2I), which was not present in the template DNA. However, in order for the recognition sequence to be recognised by the restriction enzyme, the sample must contain the cytosine (C) nucleotide at the SNP site (the first base position of the 25th codon in exon 2), thus providing the correct recognition sequence. Thus the C-allele of the SNP will be restricted by the restriction enzyme, while the T-allele will not. The binding of the primers to exon 2 of the *lep* gene and the SNP are shown in Figure 2-2. The DNA sequence of exon 2 is indicated in both the 5' to 3' and the 3' to 5' orientation, for both a C- and a T-allele. The area on exon 2 where the forward and reverse primers bind is shown in blue, and the red sequences in exon 2 represent the restriction enzyme recognition sites, where the restriction enzyme *Kpn* 2I will bind and thus restrict the amplified DNA fragment. The letters indicated in black, within the restriction site, indicate the SNP, while the uppercase letters within the primer sequences represent the purposeful mismatch.

Once the 94 bp RFLP-*Kpn* 2I amplification products had been successfully amplified, a restriction enzyme was employed to digest the 94 bp amplification products to elucidate the genotypes of the cows. This allowed the two forms of the SNP to be distinguished. The SNP in exon 2 can be distinguished using the *Kpn* 2I restriction enzyme (Buchanan *et al.*, 2002); however due to the unavailability of this enzyme in South Africa, *Mro* I, an isoschizomer of *Kpn* 2I, was used. The amplification products of 94 bp were thus subjected to restriction by Mro I to identify the alleles present. Cleavage of the amplification product indicated the presence of the recognition site, and thus the C-allele, while the T-allele did not have the recognition site and thus remained undigested (Figure 2-2). When the enzyme cleaves the C allele, the 94 base pair amplification product (a portion of exon 2 of the *lep* gene) is cut to produce two fragments, a 75 base pair fragment

and a 19 base pair fragment. The T allele is not cleaved by the enzyme (Buchanan *et al.*, 2002). The possible expected results of the digestion of the amplification product by *Mro* I are shown in Figure 2-1.

Figure 2-1 **Expected results from the restriction digestion of the amplification product, showing the three possible genotypes (CC, CT, TT).**

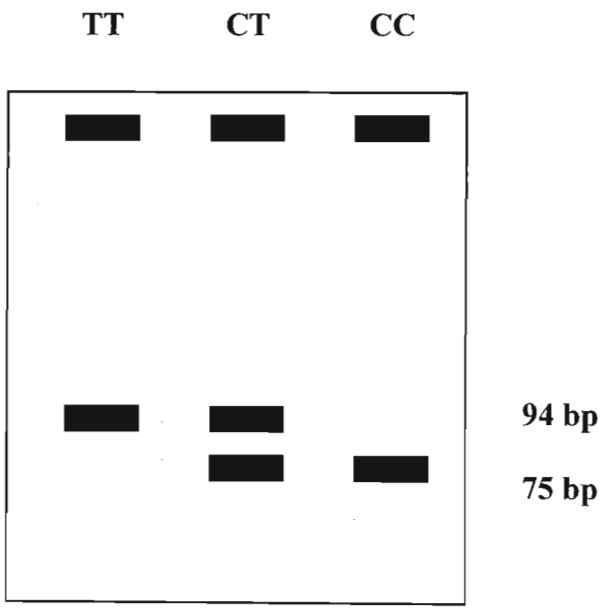
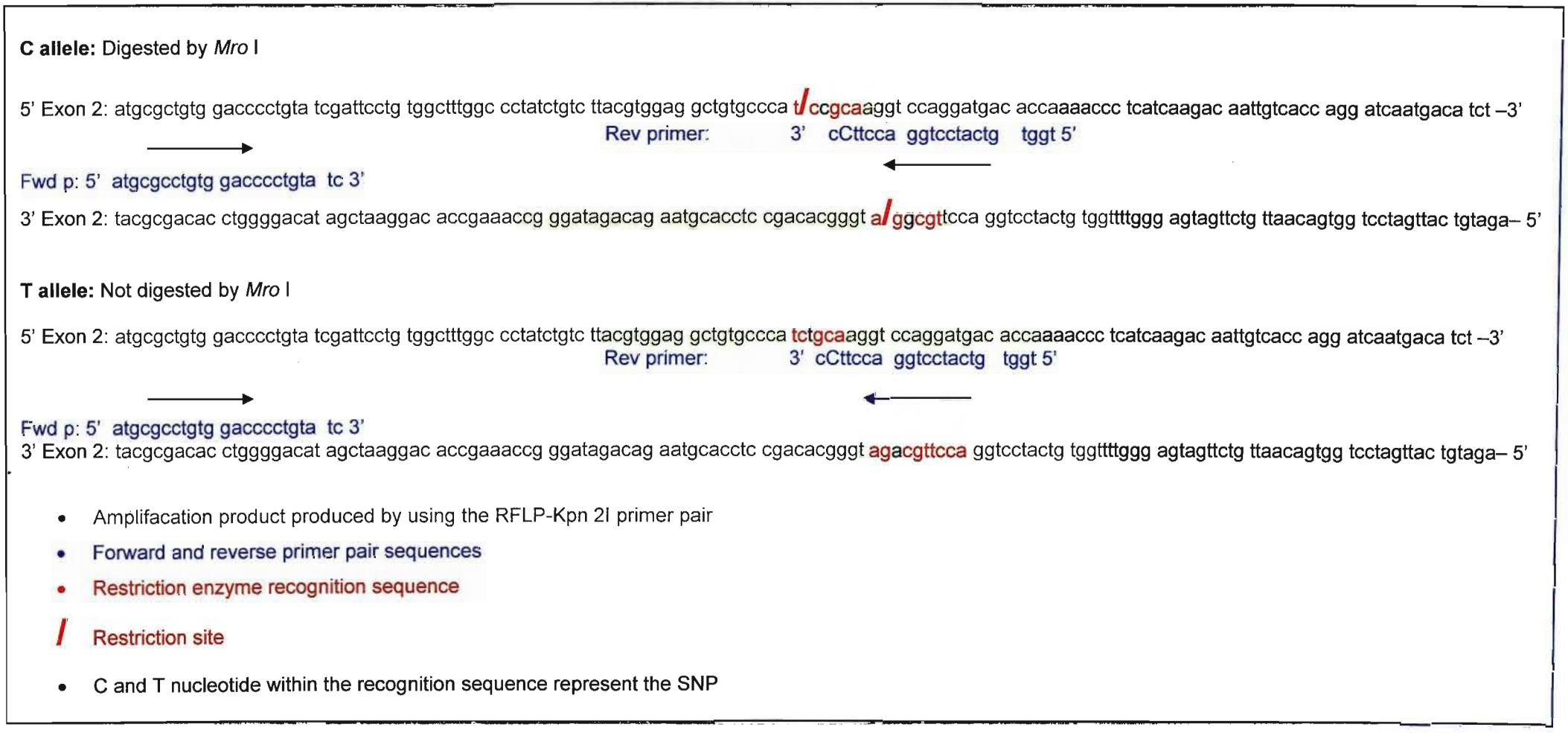


Figure 2-2 **Binding of the RFLP-*Kpn* 2I primers to exon 2 of the bovine *lep* gene, showing the sequence of both the C- and the T-allele, and the restriction site of *Kpn* 2I and *Mro* I.**



DNA source

Venous blood was used as a source of DNA. A qualified animal scientist (Jonathon Tyler) collected the blood from the animals. Blood was collected from the cows immediately after milking using sterile technique; by firstly, swabbing the area with alcohol to sterilize it, and then piercing the caudal vein, between the vertebrae of the tail, with the sterile Precision Glide needle (1.2 x 38 mm) contained in a needle holder. The blood was then collected into a 4 ml VacutainerTM tube, containing EDTA to prevent coagulation of the blood. Each tube was gently inverted a number of times to ensure that the blood mixed with the EDTA to prevent the formation of blood clots.

The blood containing Vacutainer™ tubes were then placed in an empty ice box and transferred to the laboratory within four hours, at which time the blood was transferred to two 1.5 ml eppendorf tubes (1 ml into each eppendorf), and thereafter stored in a –75 °C freezer (Specht Scientific) until use.

2.3 METHOD: GENOTYPIC ASSESSMENT OF THE SELECTED COWS

The genotypic analysis of the selected cows involved: the extraction of DNA from the blood, the amplification of a segment of exon 2 of the *lep* gene, and the restriction of the amplification product to ascertain which alleles were present and thus determine the genotype.

2.3.1 **DNA extraction**

DNA was extracted from frozen blood samples by applying a salting out protocol, which was adapted from Bruford *et al.* (1992). This protocol involved using proteinase K, in the presence of EDTA, and SDS (a detergent), to digest the white blood cells by denaturing proteins and solubilizing the cell membranes. The DNA

was then extracted by salting out and ethanol precipitation (Sambrook and Russell, 2001), which is known to produce high quality and large quantities of DNA from white blood cells of mammals. DNA was extracted in the following way:

- Two lysis buffers were prepared to lyse the white blood cells and expose the DNA.
    - Blood cell lysis (BCL) buffer was prepared (0.32 M sucrose, 10 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$) and stored at a 5 X concentration at room temperature.
    - Before commencement of the extraction process, the 5 X BCL buffer was diluted 1:5 with sterile water and 0.05 volumes 20 % (v/v) Triton X-100 was added.
    - TNE lysis buffer (0.4 M NaCl, 10 mM Tris-Cl pH 8, 2 mM EDTA pH 8) was prepared at a 1 X concentration.
- The blood sample was defrosted in a 37 °C water bath for 10 minutes.
- 400 µl of the blood sample was centrifuged in a 1.5 ml sterile eppendorf tube at 2 000 rpm for 15 minutes at 0 °C.
- The supernatant was carefully removed by decanting, without discarding the buffy coat, which contained the white blood cells.
- The remaining pellet was resuspended in 1 ml of ice-cold 1 X BCL buffer and 14 µl 10 % Triton X-100 and vortexed until the pellet was in suspension.
- The sample was then centrifuged at 2 000 rpm for 20 minutes at 2 °C, the supernatant discarded and the pellet drained briefly.
- The pellet was resuspended in 1 ml ice-cold 1 X BCL buffer and 14 µl 10 % Triton X-100, and vortexed to resuspend the pellet.
- The solution was centrifuged at 2 000 rpm for 15 minutes at 2 °C, after which the supernatant was discarded and the pellet drained for 5 to 10 minutes.
- 500 µl 1 X TNE lysis buffer, 50 µl 1 M Tris-Cl pH 8, 7.5 µl 25 % SDS, 7.5 µl 10 % Triton X-100, and finally 1 µl of 10 mg / ml Proteinase K was added to the pellet.
- The sample was incubated overnight in a 37 °C water bath.
- After incubation half a volume of 5 M NaCl was added to the sample, to salt out the DNA, and then shaken vigorously for 15 seconds.
- The sample was then centrifuged at 5 000 rpm for 15 minutes at room temperature, after which the supernatant, containing DNA, was transferred to a

fresh eppendorf tube.

- The shaking of the sample and the centrifugation step were repeated twice, until the supernatant was clear.
- Two volumes of ice-cold 100 % ethanol was added to the sample, this was mixed by inversion and placed into a –20 °C freezer for 30 minutes to increase the DNA yield through ethanol precipitation. A stringy opaque precipitate became visible at this point.
- The sample was then centrifuged at 13 000 rpm for 15 minutes at room temperature.
- Excess ethanol was poured off and the eppendorf tube blotted on tissue paper.
- One volume of 70 % ethanol was added and the DNA pellet washed by inversion for 5 minutes.
- The sample was then centrifuged at 13 000 rpm for 10 minutes and the excess ethanol decanted, after which the 70 % ethanol wash was repeated.
- Once the final ethanol had been decanted off, the pellet was air dried for 30 minutes on tissue paper.
- The DNA was resuspended overnight in 50 µl 10 mM Tris-Cl pH 8, in a 37 °C water bath.
- The DNA samples, resuspended in Tris-Cl pH 8, were stored in a -20 °C freezer.

2.3.2 **DNA verification and quantification**

Agarose gel electrophoresis and spectrophotometry were used to ensure that the DNA extraction protocol had produced large quantities of high quality DNA, which could be used in the PCR amplification protocol.

Agarose gel electrophoresis

To confirm the successful extraction of the DNA, the samples were run on a 1 % agarose gel, which was able to resolve DNA fragments ranging between 150 bp and 6 kb (Sambrook and Russell, 2001). The gel was prepared with:

- 2 g of agarose,

- 200 ml of 1 X TAE buffer, and
- 10 ml of ethidium bromide (10 mg / ml).

TAE buffer was used instead of TBE buffer, because it provides better resolution of mammalian DNA fragments (Sambrook and Russell, 2001). The extracted DNA was visualized by including 10 ml of a 10 mg / ml stock solution of ethidium bromide in the gel and the TAE buffer at a 0.5 mg / ml concentration (Sambrook and Russell, 2001). Once the gel had set, DNA was loaded into a well using a micropipette. Each well of the gel contained:

- 5 µl of sample genomic DNA,
- 3 µl of sdH_2O, and
- 2 µl of loading buffer (Type III).

Loading buffer ensured that the DNA sample sank evenly to the base of the well by increasing the density of the sample; provided colour to the sample, making loading easier; and allowed for the prediction of the movement of the DNA fragments through the gel, since the loading buffer contains a dye that moves at predictable rates through the gel (Sambrook and Russell, 2001). A molecular weight marker was not included because the purpose of the gel was to indicate the presence of DNA, although two genomic DNA samples which had previously been shown to run successfully on a gel in the laboratory were used as a control to ensure that the gel was running correctly.

A voltage of 120 V was applied to the gel; Sambrook and Russell (2001) suggested a voltage of 1 – 5 V / cm, using the Hoeffer PS500X DC Power Supply Unit (Hoeffer Scientific Instruments) for approximately one hour.

After the gel had been run to completion it was viewed using the Bio-Rad VersaDoc Imaging System (Model 4000), which illuminated the gel with UV light, allowing the ethidium bromide to fluoresce, thus visualizing the DNA bands on the gel.

DNA quantification

The DNA concentration of each sample was determined using a Beckman DU 640 Spectrophotometer, using 10 µl of the DNA sample and 490 µl 1 X TE buffer. The absorbance of each sample was determined at 260 nm and 280 nm. A number of calculations were then carried out to determine the purity and concentration of the DNA, indicated in Table 2-2.

Table 2-2 Calculations used to determine the concentration of the DNA samples.

Purity = A_{260} / A_{280}
DNA concentration = A_{260} x dilution factor x 50 = z µg / µl
Dilution factor = Total volume of spectrophotometry sample / Volume of DNA sample

DNA samples with concentrations greater than 100 ng / µl were then diluted with 10 mM Tris-HCl pH 8, to produce working solutions of 100 ng / µl, by applying the formula $C_1V_1 = C_2V_2$, where C is the concentration and V is the volume (Table 2-3). The concentrations of J24 and J39 were less than 100 ng / µl, and were therefore not diluted. On the other hand, the volumes of samples J14, J15 and J40 were insufficient to produce a 50 µl 100 ng / µl working solution, and were thus not diluted but kept as the initial stock concentrations.

Table 2-3 **Volumes of initial DNA samples and 10 mM Tris-HCl pH 8 used to produce 100 ng / μl working solutions.**

Sample no.	Stock [DNA] (ng / μl)	Volume of stock DNA required (μl)	Working solution [DNA] (ng / μl)	Volume of working solution DNA (μl)	Volume of 10 mM Tris-HCl pH 8 required (μl)
J1	303.0	17	100	50	33
J2	425.5	12	100	50	38
J3	565.5	9	100	50	41
J4	298.0	17	100	50	33
J5	193.0	26	100	50	24
J6	221.8	23	100	50	27
J7	232.3	22	100	50	28
J8	346.5	14	100	50	36
J9	1237.5	4	100	50	46
J10	454.0	11	100	50	39
J11	395.3	13	100	50	37
J12	385.0	13	100	50	37
J13	457.0	11	100	50	39
J16	352.0	14	100	50	36
J17	181.0	28	100	50	22
J18	211.0	24	100	50	26
J19	442.8	11	100	50	39
J20	382.0	13	100	50	37
J21	362.0	14	100	50	36
J22	542.3	9	100	50	41
J23	425.5	12	100	50	32
J25	517.5	10	100	50	40
J26	445.5	11	100	50	39
J27	549.5	9	100	50	41
J28	450.3	11	100	50	39
J29	348.8	14	100	50	36
J30	304.8	16	100	50	34
J31	517.5	10	100	50	40
J32	450.5	11	100	50	39
J33	424.5	12	100	50	38

Sample no.	Stock [DNA] (ng / µl)	Volume of stock DNA required (µl)	Working solution [DNA] (ng / µl)	Volume of working solution DNA (µl)	Volume of 10 mM Tris-HCl pH 8 required (µl)
J34	946.5	5	100	50	45
J35	414.8	12	100	50	38
J36	292.5	17	100	50	33
J37	212.0	24	100	50	26
J38	217.0	23	100	50	27
J41	1678.5	3	100	50	47
J42	677.3	7	100	50	43
J43	736.3	7	100	50	43
J44	590.8	8	100	50	42
J45	203.3	25	100	50	25
J46	1078.5	5	100	50	45
J47	500.0	10	100	50	40
J48	634.3	8	100	50	42
J49	411.3	12	100	50	38
J50	631.5	8	100	50	42

2.3.3 **Amplification of exon 2 of the lep gene**

To amplify exon 2 of the lep gene from the extracted DNA the polymerase chain reaction (PCR) was employed. This allowed only the region of interest to be amplified through the use of the specifically designed primers, namely RFLP-*Kpn* 2I. The RFLP-*Kpn* 2I primers were obtained from Roche Products, and synthesized by Metabion. The forward primer consisted of 22 base pairs, while the reverse primer consisted of 21 base pairs. The specifications of the primers provided included the optical density (OD), the percentage of guanine and cytosine residues (GC %), the molecular weight (MW), the melting temperature (T_m) and the amount of primer provided by the suppliers. These specifications are indicated in Table 2-4.

Table 2-4 **Forward and reverse primer information.**

	Forward primer	Reverse primer
OD	9.2 OD	10.8 OD
GC %	59.1 %	57.1 %
MW	6702	6364
T_m	64.0 °C	61.8 °C
Volume	41.2 nmol	52 nmol

OD = optical density
MW = molecular weight
GC % = percentage of G and C residues
T_m = melting temperature

The RFLP-*Kpn* 2I primers were supplied as a lyophilisiert, and were made up to a 100 μM solution by adding 412 μl of sterile distilled water to the forward primer and 520 μl to the reverse primer. From these solutions, 5 μM working solutions were prepared according to the following formulae:

Primer Calculations

Forward primer:
$C_1V_1 = C_2V_2$
(100 μM)(V_1) = (5 μM)(1000 μl)
V_1 = 50 μl of the 100 μM stock solution + 950 μl dsH_2O to produce the 5 μM working solution.

Reverse primer:
$C_1V_1 = C_2V_2$
(100 μM)(V_1) = (5 μM)(1000 μl)
V_1 = 50 μl of the 100 μM stock solution + 950 μl dsH_2O to produce the 5 μM working solution.

Initial PCR amplification

The PCR reaction conditions proposed by Buchanan *et al.* (2002) had specifically amplified exon 2 of the *lep* gene in Holstein, Ayrshire, Brown Swiss, Canadienne, and Guernsey breeds, as well as in a number of beef breeds, and in six Jersey cows. It was, therefore, decided to test these conditions (Table 2-5) for their applicability to the South African Jersey DNA under the laboratory conditions used in this investigation.

Table 2-5 Initial PCR trial, based on the conditions proposed by Buchanan *et al.* (2002).

Reagents	Sample volumes (µl)	Initial concentration	Final concentration
PCR buffer	2	10 X (with 15 mM $MgCl_2$)	1 X (with 1.5 mM $MgCl_2$)
MgCl2	1.2	25 mM	1.5 mM
dNTP's	0.4	10 mM each	200 µM
DNA template	1	100 ng / µl	100 ng
Fwd primer	2	5 µM	10 pmol
Rev primer	2	5 µM	10 pmol
Taq DNA pol	5[a]	5 U / µl	1 U / µl
dH_2O	6.4		
Total	**20**		

[a] The *Taq* DNA polymerase was made up as a dilution; sufficient *Taq* was mixed with sterile distilled water for the number of reactions being carried out.

Fwd = forward

Rev = reverse

pol = polymerase

Amplification of exon 2 of the lep gene was achieved through the application of a Roche PCR Core Kit, which contained all the required reagents, excluding the

primers, DNA template, and the sterile distilled water. As suggested by Buchanan *et al.* (2002), the following PCR profile was applied: a two minute denaturation step at 94 °C, followed by 35 cycles of 45 seconds at 94 °C, 45 seconds at 52 °C, and 55 seconds at 72 °C, next was a three minute extension step at 72 °C, after which the reaction held at 4 °C. The *Taq* DNA polymerase was added to the reaction tubes after the initial two minute denaturation step.

Four controls were included in the amplification reactions, to ensure that no contamination occurred, that the reaction was working efficiently, and that the primers were specific to the DNA. The first, a positive control, consisted of plant DNA known to amplify under the conditions being employed. This DNA and specific primers were supplied by Mariaan Ponsie of the School of Botany and Zoology, University of KwaZulu-Natal. The other three controls were all negative controls: one containing all the components of the reaction mixture except DNA template, to test for contamination; one containing all the components of the reaction mixture except *Taq* polymerase; and the third, containing the reaction mixture but substituting cow DNA with plant DNA to ensure that the primers were specific enough to amplify the specified exonic region of the leptin gene.

The reactions were run on the GeneAmp PCR System 2700 (Applied BioSystems).

Optimization of amplification.

The amplification conditions suggested by Buchanan *et al.* (2002) were initially tested. Due to a consistent lack of product, a round of exhaustive optimization trials were conducted on the GeneAmp PCR System 9700 (Applied BioSystems), including alterations to the $MgCl_2$, primer, dNTP and *Taq* polymerase concentrations; the annealing temperature; the annealing time; and the number of cycles (Table 2-6). As no satisfactory amplification product was produced by any of these trials, the ambient temperature, mechanical failure of the PCR machine,

as well as the contamination of the primer working solution were considered. Finally it was found that the primer working solutions were contaminated, and therefore re-constituted and subjected to a second round of amplification.

Amplification was considered to be successful when the product had the expected size of 94 bp (Buchanan *et al.*, 2002), as well as occurring in abundant quantities, thus allowing for subsequent reactions to be conducted.

Table 2-6 **First round of PCR optimization trials to amplify a portion of exon 2 of the *lep* gene.**

Trial	PCR Component	Initial Reaction	Optimization Trials	Reason
1	**$MgCl_2$**	1.5 mM	0.5 mM; 2.5 mM	Increased $MgCl_2$ concentration to improve specificity.
2	**Annealing temperature**	52 °C	54 °C; 56 °C	Increased T_A to improve specificity.
3	**Touch-down PCR**[a]	52 °C	58 °C – 53 °C	To reduce amplification of non-specific products.
4	**Primer concentration**	10 pmol	5 pmol	Decreased primer concentration to decrease spurious priming.
5	**dNTP concentration**	200 µM	100 µM	Reduction of dNTP concentration to improve stringency.
6	***Taq* polymerase concentration**	1 U	0.5 U	Decreased polymerase concentration to improve stringency.
7	**Annealing time**	45 s	30 s	Long annealing times increase spurious priming.
8	**Number of cycles**	35	40	Increase of number of cycles to increase yield.

[a] The T_A starts at 58 °C for the first cycle, and then decreases by 1 °C each cycle, until it reaches 53 °C, where it remains for the remaining 30 cycles.

At the inception of the second round of optimization trials, a novel PCR profile for the RFLP-*Kpn* 2I primer pair was published (Madeja *et al.*, 2004), suggesting a very different PCR profile for these primers than that which had been optimized by Buchanan *et al.* (2002). This profile was therefore also included in the second round of optimization trials, in an attempt to produce acceptable amplification products. The Madeja *et al.* (2004) profile was thus applied to the initial PCR amplification component concentrations (Buchanan *et al.*, 2002), as shown in Table 2-5, and was as follows:

2 min @ 94 ° C; 35 X (45 s @ 94 ° C, 45 s @ 59 ° C, 1 min @ 72 ° C); 5 min @ 72 ° C.

The second round of optimization trials were conducted with the newly prepared primer working solutions, and included the comparison of the altered Buchanan *et al.* (2002) and the Madeja *et al.* (2004) profiles. The altered Buchanan *et al.* (2002) profile differed from Buchanan *et al.*'s original profile in that the altered annealing temperature was 50 ° C, as opposed to 52 ° C in the original profile. After comparison the Madeja *et al.* (2004) profile was optimized to produce the final PCR conditions. The optimization trials carried out can be found in Table 2-7.

Table 2-7 **PCR optimizations tested after the primer working solutions had been remade.**

Trial	PCR Components	Initial Reaction	Optimization Trials	Reason
9	**PCR profile**	Buchanan *et al.* (2002) profile	Altered Buchanan *et al.* (2002) profile	To test this profile with the remade primer working solutions.
10	**PCR profile**	Buchanan *et al.* (2002) profile	Madeja *et al.* (2004) profile	To test this profile because it had a much higher T_A than the Buchanan *et al.* (2002) profile.
11	**Annealing temperature of Madeja *et al.* (2004) profile**	59 ° C	60 ° C; 61 ° C	Increase in T_A to reduce amplification non-specific PCR products.

The final optimized PCR conditions were found to be the same as those of Buchanan *et al.* (2002) with respect to the components of the PCR reaction (Table 3-6); the difference is that the profile is an adjusted version of Madeja *et al.* (2004). The Madeja *et al.* (2004) profile is used but with an increase in the annealing temperature from 59 ° C to 61 ° C.

Confirmation of amplification.

Amplification of the correct 94 bp amplification product was confirmed by running the RFLP-*Kpn*21 amplification product on an agarose gel and thereafter the sequencing of the RFLP-*Kpn*21 amplification product to confirm the correct sequence.

a. Agarose gel electrophoresis

Initially, the RFLP-*Kpn*21 amplification product was run on a 3 % 200 ml agarose gel, composed of:

- 6 g agarose,
- 200 ml of 1X TAE, and
- 10 µl of 10 mg / ml ethidium bromide.

A 50 bp DNA ladder (GibcoBRL, Life Technologies) was included as a size standard, against which the size of the DNA fragments could be estimated. The 50 bp DNA ladder consisted of 16 blunt-ended fragments with sizes between 50 and 800 bp, in multiples of 50 bp, as well as a large fragment of 2 652 bp (GibcoBRL, Life Technologies).

A sample loaded onto the gel was made up of:

- 5 µl PCR product,
- 5 µl sdH_2O, and

- 2 µl loading buffer.

The 50 bp ladder sample was composed of:

- 10 µl sdH_2O,
- 2 µl loading buffer, and
- 0.6 µl of the 50 bp DNA ladder.

The gel was hot started at 120 V for 5 minutes, and then run to completion at 80 V (approximately three hours) using the Hoeffer PS500X DC Power Supply Unit. The gel was then visualized under UV light, using the Bio-Rad VersaDoc Imaging System (Model 4000). Quantity One software (Version 4.5.1) was used to analyse the resulting fingerprint, and thereafter employed to calculate the size of the bands produced on the gel.

b. Sequencing of the amplification product

Due to the remote possibility that the amplification product of 94 base pairs could be the result of the amplification of another area of the genome that produced the same product size, the amplification product was sequenced. The amplification product of sample J20 was sent to the Molecular Biology Unit (MBU) at the University of KwaZulu-Natal, Pietermaritzburg, where it would be sequenced using the dye terminator reaction method of sequencing. However, difficulties were experienced in the MBU sequencing facility, and the sample was thus dispatched to a commercial company, Inqaba Biotechnical Laboratories in Pretoria, for sequencing. The sequencing was repeated twice with different J20 amplification products to confirm the accuracy of the sequence.

The sequences determined were received via e-mail. The BLAST 2 SEQUENCES (Version BLASTN 2.2.10, 2004) tool on the NCBI website (www.ncbi.nlm.nih.gov) was used to determine whether the sequences obtained from sequencing were the same as the *lep* gene sequence as reported by Buchanan *et al.* (2002).

2.3.4 Restriction digestion of the amplification product

A restriction digestion was performed on the amplified DNA of each cow to identify the presence of either the T- or C-allele of the *lep* locus in a particular genotype. The restriction digestion was performed as suggested by Buchanan *et al.* (2002), using the restriction enzyme *Mro* I instead of *Kpn* 2I, which required slight optimization of the protocol. Included in the optimisation steps were a number of controls, including:

- A reaction mixture lacking enzyme thereby indicating that the reaction mixture was not contaminated,
- A reaction mixture where the amplified DNA was replaced by genomic to ensure that the enzyme was cutting,
- A reaction mixture lacking PCR product, to show that no contamination occurred, and
- Reactions containing spermidine and those without, to determine its importance.

Spermidine was initially included in the reactions (Buchanan *et al.*, 2003), but was later excluded since it did not impact the reaction, and was expensive and difficult to obtain. For each sample two different reactions were run; one of which lacked restriction enzyme (uncut) and a second that included the restriction enzyme (cut). The final restriction digestion protocol is shown in Table 2-8.

Table 2-8 **Restriction digestion protocol modified from the digestion carried out by Buchanan *et al.* (2002).**

Reagent	Cut sample	Control 1[a] (100 ng/µl)	Control 2[b]	Control 3[c]	Final concentration
PCR product	15	5[a]	15	-	-
***Mrol* sol^n (µl)**	1	1	-	1	2 U
SuRE / Cut buffer A (µl)	2	2	2	2	-
dsH_2O (µl)	2	12	3	17	-
Total (µl)	20	20	20	20	-

[a] DNA template (genomic DNA) replaces PCR product

[b] No enzyme (uncut)

[c] No DNA template

The samples were incubated at 55 °C for one hour (Buchanan *et al.*, 2003), this was optimized to 37 ° C for two hours to produce optimal results.

2.3.5 **Generation of *lep* locus fingerprints**

Non-denaturing (or native) polyacrylamide gel electrophoresis (PAGE) was used to separate the double stranded DNA fragments produced during PCR and digestion. PAGE is more discerning than agarose gel electrophoresis, and is able to resolve fragments differing in size by 1 bp (Sambrook and Russell, 2001).

A 20 % PAG was used because it is able to discern for sizes ranging from 6 – 100 (Sambrook and Russell, 2001). The volumes of reagents used to cast a 10 ml 20 % polyacrylamide gel are as follows:

- 6.66 ml of 29 % Acrylamide and 1 % bisacrylamide
- 1.27 ml sdH_2O
- 2.00 ml TBE
- 85 µl 10 % ammonium persulfate (APS)
    - APS was made up fresh on a weekly basis
- 10 µl TEMED

The perform PAGE the following steps are undertaken:

- As polyacrylamide is a neurotoxin, face masks were worn in addition to the usual laboratory coats and gloves, during the gel preparation stages.
- The gel was poured and run in 0.5 X TBE buffer, at a low voltage (1 – 8 V/cm) to prevent denaturation of small DNA fragments.
- To condition the gel, and remove any particles caught in the gel, and to equilibrate the gel it was pre-electrophoresed for 30 minutes, at 180 V and 35 mA (Scie-Plas Limited Vertical Electrophoresis Unit V10-CDC) and 150 V and 30 mA (Mighty Small™ II SE250, Hoeffer Scientific Instruments).

- 5 µl of each restriction digestion product was loaded with 2 µl of loading buffer, and a 50 bp DNA ladder (GibcoBRL, Life Technologies) was run on each gel (0.6 µl ladder with 5 µl sdH_2O and 2 µl loading buffer).
- The restriction digestion products were run in pairs (uncut and cut) on the gel.
- The gel was run at 180 V and 35 mA (Scie-Plas Limited Vertical Electrophoresis Unit, V10-CDC) or 150 V and 30 mA (Mighty Small™ II, SE250, Hoefer Scientific Instruments) for approximately two hours.
- The gel was stained in a 1 X TAE and ethidium bromide (10 mg / ml) solution for half an hour.
- After which it were visualised using the Bio-Rad VersaDoc Imaging System (Model 4 000).

2.3.6 **Identification of the genotypes**

The fingerprints generated with PAGE allowed the restriction digestion products to be separated, resulting in the identification of two alleles, the C- and the T-allele. The T-allele is not digested during restriction, and so remains as a 94 bp fragment. The C-allele is digested during the restriction, thus producing 75 bp and 19 bp fragments. It should be noted, however, that the 19 bp product of the C allele is not seen because of its small size. The fingerprints represented three possible genotypes, namely homozygous CC and TT, and heterozygous CT.

The uncut control of each sample was used as a reference to confirm the presence of either the 94 bp fragment or the 75 bp fragment in the cut sample, as a means to determining the genotype of each sample.

Once the digested samples have been separated on 20 % polyacrylamide gels, the genotype of each individual is recorded as TT, CT, or CC.

2.3.7 **Determination of genetic population statistics**

On completion of the identification of the genotypes of the RFLP-*Kpn* 2I locus of

the selected cows, a number of population statistics were calculated. Calculations to determine the genotypic frequencies and allele frequencies, as well as a chi-squared test to assess Hardy-Weinberg equilibrium were conducted. This test was performed using the Chi-Square Goodness of Fit function of GenStat Version 8.1 (Genstat8, 2005). In addition the inbreeding coefficient (F_{IS}) was calculated to measure the deviation of the heterozygous genotype from the frequency expected under Hardy-Weinberg equilibrium.

2.4 METHOD: PHENOTYPIC ASSESSMENT OF THE SELECTED SARSDEN JERSEY HERD

The phenotypic performance of the selected Sarsden Jersey herd was assessed to establish how this herd performed relative to the average expected performances of registered South African Jersey cows. The selected herd sample was analyzed for six different economically important dairy traits, namely:

- Milk yield (kg) – adjusted to 305 days,
- Butterfat percentage,
- Protein percentage,
- Lactose percentage,
- Inter-calving period in days (ICP), and
- Services per conception (SPC).

The mean and standard error for each of these traits were calculated using SPSS 11.5 for Windows (SPSS for Windows, 2002), and the mean performance was compared to the mean performance of the entire Sarsden Jersey herd, over the investigation period, using the ANOVA function of SPSS 11.5 for Windows (SPSS for Windows, 2002).

2.5 METHOD: ASSESSMENT OF THE RELATIONSHIP BETWEEN PHENOTYPIC PERFORMANCE AND GENOTYPE OF THE SELECTED SARSDEN JERSEY HERD

An investigation into the possible association between the genotypes of a particular SNP in exon 2 of the *lep* gene and the phenotypic performance of six economically important dairy traits was undertaken by applying a statistical model.

This investigation required the inclusion of milk production and reproductive performance data, which would effectively reflect possible relationships. Milk production and reproductive performance measurements were taken at completion of the first lactation, except in the case of ICP, which was taken as the period between the first and second lactation. The first lactation of each individual was considered as the most suitable measurement for three reasons:

- First lactation records were available for the largest number of individuals, namely 48 out of the 50 cows genotyped,
- According to Theron *et al.* (2000) the best approach for comparing cows is to use their completed first lactation records, provided the cows are from the same herd, in the same year and from the same status. This approach was confirmed through personal communication with Graham Hallowell, the Programme Manager of the NDAIS (Hallowell, 2005), and
- Repeatability of milk production traits is relatively high; 0.50 for milk yield, and 0.60 for butterfat percentage. The repeatability of reproductive performance traits is not as high, but this is controlled by the use of the lifetime SPC records (r = 0.15), and the ICP (r = 0.15) between the first and second calvings (Bourdon, 1997).

2.5.1 Assessment of a possible association between genotype and milk production traits

The identification of an association between economically important milk production traits and the genotypes of the *lep* gene is vital in the development of possible molecular markers for use in marker assisted selection (MAS) in animal production. Four production traits were investigated for an association to the exon 2 SNP genotype, namely:

- Milk yield,
- Butterfat percentage,
- Protein percentage, and
- Lactose percentage.

Although the primary aim of this investigation was to establish the whether a particular genotype of an individual had any effect on the above mentioned production traits, a number of other factors, namely, age at first calving, calving year, and calving season, were included in the analysis, as covariates, in order to account for their influence on the production traits, and to reduce the experimental error of the models (Ward, 2005).

A General Linear Model (GLM) was applied to determine the effect of the genotype on milk production traits. The GLM Univariate procedure of the SPSS 11.5 for Windows software package (SPSS for Windows, 2002) was utilized as it accounted for secondary factors that may have influenced the phenotypic performance, such as the environment, as well as accounting for the unbalanced nature of the datasets. The GLM procedure carried out a regression analysis, as well as an analysis of variance, for one dependent variable by one or more factors, and included the effect of covariates and covariate interactions with factors. The production trait was included as the response variate; the genotype was the categorical predictor, or factor, and the three covariates (age at first calving,

calving season, and calving year) were treated as continuous predictors, or covariates. The genotype factor consisted of three treatment levels: CC, CT, and TT.

The first step in the analysis involved testing whether the interaction between the factor and the three covariates was significant, or whether only the main effects of each were considerable. This was determined by execution of the GLM Univariate procedure with a customized model containing an interaction term between genotype and the covariates age at first calving; calving season, and calving year. These three covariates were investigated because they were the effects that could have most influenced the production and reproductive performance of the cows. The null hypothesis (H_o) tested whether the coefficient of the covariate was homogeneous across all levels, and thus accounted for a negligible amount of variation compared to the error term. If the interaction was non significant it was excluded from the final model, however, if it were found to be significant, it was included in the model (SPSS for Windows, 2002).

When the interaction term was non significant the GLM Univariate procedure was repeated, without the interaction term in the model. This produced an analysis of covariance, which assessed the effect of genotype on the various traits, while controlling for any effect from age at first calving, calving year and calving season. The null hypothesis (H_o) to be tested is that all genotypes produce equal means ($\mu_1 = \mu_2 = \ldots = \mu_j$). The level of significance was set at 5 %.

The general linear model used to assess the influence of genotype on the milk production traits was:

$$Y_{ijkm} = \mu + g_i + a_j + s_k + c_m$$

Where:

y_{ijkm} = observation of the ijkmth cow either for milk yield, butterfat %, protein

%, or lactose %, adjusted for the j^{th}, k^{th}, and m^{th} covariates.

μ = overall mean.

g_i = fixed effect of the i^{th} genotype (i = CC, CT, TT).

a_j = value of the j^{th} covariate (age at first calving) (j = 1, 2, ...,).

s_k = value of the k^{th} covariate (calving season) (k = 1, 2, 3, 4).

c_m = value of the m^{th} covariate (calving year) (m = 1997, 1998, ..., 2004).

When significant effects were observed for the covariates, they were further analyzed individually using one-way ANOVA, and least square analysis (LSD), as well as linear regression analysis. In this case the covariates were treated as factors, while the traits remained as the dependent variable. The null hypothesis in these cases was once again that the means of each group were equal ($H_O = \mu_1 = \mu_2 = \ldots = \mu_j$).

2.5.2 **Assessment of a possible association between genotype and reproductive performance traits**

In this investigation two fertility traits are analyzed for an association to the exon 2 SNP genotype, these were the inter-calving period (ICP) and the services per conception (SPC). ICP is the number of days between two consecutive calvings, while SPC is the number of services or artificial inseminations (AI's) per conception.

The measurement of ICP for the sample was taken as the number of days between the first and second calvings. SPC is a lifetime ratio, dividing the total number of lifetime services by the total number of conceptions, and so does not relate to a particular lactation.

The primary aim of this investigation was to establish the whether the genotype of an individual has any affect on the reproductive performance or fertility traits. As for the previous investigation on production traits, a number of covariates were

included in the analysis to account for their influence on the fertility traits, and to reduce the experimental error of the models. These factors were age at first calving, calving year, and calving season. Age at first calving was not included in the model for ICP.

The same analysis was used to analyze the association between genotype and reproductive performance traits, as was used to investigate the production traits.

The general linear model to assess the impact of genotype on the two reproductive performance traits was:

$$Y_{ijkm} = \mu + g_i + a_j + s_k + c_m$$

Where:

y_{ijkm} = observation of the ijkmth cow either for SPC or ICP, adjusted for the j^{th}, k^{th}, and m^{th} covariates.

μ = overall mean.

g_i = fixed effect of the i^{th} genotype (i = CC, CT, TT).

a_j = value of the j^{th} covariate (age at first calving) (j = 1, 2, ...,).

s_k = value of the k^{th} covariate (calving season) (k = 1, 2, 3, 4).

c_m = value of the m^{th} covariate (calving year) (m = 1997, 1998, ..., 2004).

An important relationship in the dairy farming industry is that between milk yield and fertility. This is an interesting relationship to look at because there is a negative correlation between milk yield and fertility. A regression analysis and correlation was performed to determine the strength and size of this relationship. These analyses were performed with SPSS 11.5 for Windows (SPSS for Windows, 2002).

CHAPTER 3 RESULTS

3.1 INTRODUCTION

The *lep* gene genotypes were characterized in a selected group of Jersey cows through amplification and fingerprinting of a section of exon 2 of the *lep* gene. Awareness of the different genotypes of the *lep* gene could provide knowledge about their influence on economically important dairy traits. In this investigation the relationships between RFLP-*Kpn* 2I genotypes and milk production and reproductive performance were investigated. An SNP in exon 2 carries two known alleles, the C- and the T-allele. These alleles were identified by amplifying a specific primer pair (RFLP-*Kpn* 2I) designed by Buchanan *et al.* (2002), which identifies either of these alleles by introducing a purposeful mismatch. This mismatch creates a recognition sequence within the amplification products; this allows the two alleles to be differentiated in a subsequent restriction digestion, as the T-allele does not contain the recognition sequence for the restriction enzyme, while the C-allele does.

The results of this investigation are presented as follows:

- Initial phenotypic assessment of the Sarsden herd
- Genotypic assessment of the selected cows
    - DNA extraction
    - DNA quantification
    - Amplification of the *lep* gene
    - Generation of individual fingerprints
    - Determination of individual genotypes
    - Genetic description of the selected cows
- Phenotypic assessment of the selected cows

- Assessment of a possible association between the phenotypic performance and the genotypes of the selected cows

The output from the various statistical programmes are taken up in Appendix B.

3.2 INITIAL PHENOTYPIC ASSESSMENT OF THE SELECTED SARSDEN JERSEY HERD

The selection of a herd for inclusion in this investigation required prior knowledge of the inherent genetic variation of the herd. This was important since it was desirable that all possible genotypes would have the potential for being represented in the sample population. Therefore, an assessment of phenotypic variation, as an indicator of possible genotypic variation, was conducted (Table 3-1). The data revealed that in the large Sarsden Jersey herd some phenotypic variation did exist, suggesting, through assumption of a uniform environment, possible genotypic variation, this was confirmed through consultation with the farmer.

Table 3-1 Mean, standard deviation and range of production statistics of the Sarsden Jersey herd for 1997 to 2005.

Trait	No. of cows (n)	Range	Mean	Std. Deviation (2σ)[a]
Milk Yield (305 days)	6054	11314	5801.44	1336.562
Fat%	6054	4.7	4.513	0.4077
Protein%	6054	1.96	3.5642	0.22504
Lactose%	6054	3.91	4.7766	0.22338
ICP (days)	3794	1069	414.92	78.826

[a] The probability that x, the value for an individual, lies within the interval of two standard deviations either side of the mean is 0.95 (Stewart, 1978).

3.3 GENOTYPIC ASSESSMENT OF THE SELECTED SARSDEN JERSEY HERD

The determination of the individual's genotypes was achieved by the collection of DNA from the blood of 50 randomly selected Jersey cows on the Sarsden dairy farm, this DNA was then subjected to amplification and allele identification.

3.3.1 **DNA extraction**

High quality, high molecular weight DNA was extracted from the 50 selected Jersey cows using a salting-out technique adapted from Bruford *et al.* (1992). Figure 3-1 shows an example of the confirmation agarose gel displaying bands of high molecular weight, unsheared DNA.

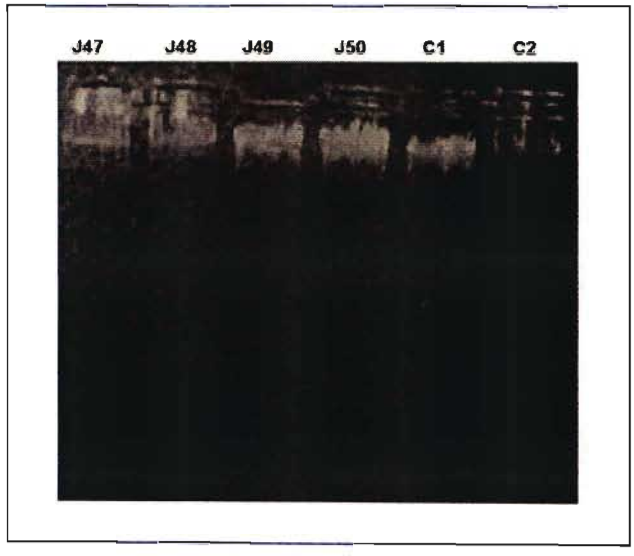


Figure 3-1 **High molecular weight extracted DNA of four individuals, including the gel controls, run on a 1 % agarose gel at 120 V.**

3.3.2 DNA quantification

DNA quality and concentration was determined through spectrophotometric analysis. The concentration of the extracted DNA ranged from 0.0873 μg / μl (JJ24), which is a rather low concentration, to 1.6785 μg / μl (J41), which would provide a large amount of DNA for further reactions. In the case of the DNA purity, most of the samples were close to the ideal purity ratio of 1.8 (Sambrook and Russell, 2001). The results of this analysis are displayed in Table 3-2, where the absorption readings at 260 nm and 280 nm, the calculated purity and the calculated concentrations are given.

Table 3-2 Quality and quantity of DNA samples produced by the extraction protocol.

Sample no.	Absorption (A_{260})	Absorption (A_{280})	Purity (A_{260} / A_{280})	[DNA] (μg / μl)
J1	0.1212	0.0688	1.7616	0.3030
J2	0.1702	0.1269	1.3412	0.4255
J3	0.2262	0.1658	1.3643	0.5655
J4	0.1192	0.0653	1.8254	0.2980
J5	0.0772	0.0446	1.7309	0.1930
J6	0.0887	0.0559	1.5868	0.2218
J7	0.0929	0.0512	1.8145	0.2323
J8	0.1386	0.0855	1.6211	0.3465
J9	0.4950	0.2289	2.1625	1.2375
J10	0.1816	0.1048	1.7328	0.4540
J11	0.1581	0.0945	1.6730	0.3953
J12	0.1534	0.0891	1.7217	0.3850
J13	0.1828	0.0927	1.9720	0.4570

Sample no.	Absorption (A_{260})	Absorption (A_{280})	Purity (A_{260} / A_{280})	[DNA] (μg / μl)
J14	0.0590	0.0332	1.7771	0.1475
J15	0.0412	0.0202	2.0396	0.1030
J16	0.1408	0.0701	2.0086	0.3520
J17	0.0724	0.0368	1.9674	0.1810
J18	0.0844	0.0477	1.7694	0.2110
J19	0.1771	0.0443	3.9977	0.4428
J20	0.1528	0.0861	1.7747	0.3820
J21	0.1448	0.0776	1.8660	0.3620
J22	0.2169	0.1110	1.9541	0.5423
J23	0.1702	0.1138	1.4956	0.4255
J24	0.0349	0.0178	1.9607	0.0873
J25	0.2070	0.1061	1.9510	0.5175
J26	0.1782	0.0913	1.9518	0.4455
J27	0.2198	0.1152	1.9080	0.5495
J28	0.1801	0.0998	1.8046	0.4503
J29	0.1395	0.0754	1.8501	0.3488
J30	0.1219	0.0867	1.4060	0.3048
J31	0.2507	0.1699	1.4756	0.5175
J32	0.1802	0.1180	1.5271	0.4505
J33	0.1698	0.0873	1.9450	0.4245
J34	0.3786	0.3327	1.1380	0.9465
J35	0.1659	0.0899	1.8454	0.4148
J36	0.1170	0.0615	1.9024	0.2925

Sample no.	Absorption (A_{260})	Absorption (A_{280})	Purity (A_{260} / A_{280})	[DNA] (µg / µl)
J37	0.0848	0.0570	1.4877	0.2120
J38	0.0868	0.0463	1.8747	0.217
J39	0.0367	0.0200	1.8350	0.0918
J40	0.0498	0.0267	1.8652	0.1245
J41	0.6714	0.3503	1.9166	1.6785
J42	0.2709	0.1370	1.9774	0.6773
J43	0.2945	0.1506	1.9555	0.7363
J44	0.2363	0.1192	1.9824	0.5908
J45	0.0813	0.0418	1.9450	0.2033
J46	0.4314	0.2178	1.9807	1.0785
J47	0.2000	0.1004	1.9920	0.5000
J48	0.2537	0.1276	1.9882	0.6343
J49	0.1645	0.0851	1.9330	0.4113
J50	0.2526	0.1278	1.9765	0.6315
Average	**0.17846**	**0.09948**	**1.846672**	**0.444012**

3.3.3 **Amplification of lep gene**

Exon 2 of the *lep* gene was amplified to determine the genotypic constitutions of the selected cows. Initially, during a number of optimization trials, the results remained unsatisfactory, producing little or no amplification product, and often smears of DNA on the confirmation gel. After extensive optimization trials, the results suggested a possible contamination of the original primer working solutions. After re-constituting these solutions, amplification was achieved, this required minimal optimization. The reaction conditions were a modification of

Buchanan *et al.* (2002), while the PCR profile was slightly modified from Madeja *et al.* (2004). The optimized reaction conditions and PCR profile are provided in Table 3-3.

Table 3-3 Optimized PCR reaction and PCR profile.

Optimized PCR components		
PCR component	**Buchanan *et al.* (2002) conditions**	**Optimized reaction conditions**
$MgCl_2$ concentration	1.5 mM	1.5 mM
dNTP concentration	200 µM	200 µM
DNA template concentration	100 ng	100 ng
Primer concentration	10 pmol	10 pmol
***Taq* DNA polymerase concentration**	1 U / µl	1 U / µl
Optimized PCR profile (modified from Madeja *et al.* (2004)		
2 min @ 94 ° C; 35 X (45 s @ 94 ° C, 45 s @ 61 ° C, 1 min @ 72 ° C); 5 min @ 72 ° C		

The amplification product was of a high concentration, and was acceptable for the subsequent digestion step. The clear and sharp bands of the putative 94 bp amplification product of exon 2 of the *lep* gene are clearly visible in the 3 % agarose confirmation gel in Figure 3-2.

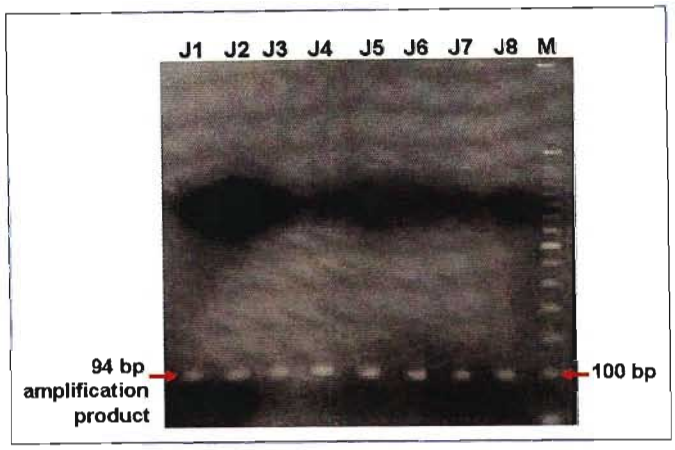


Figure 3-2 PCR amplification products of samples J1 to J8.

3.3.4 Sequencing of the PCR amplification products

The amplification product of individual J20 was sequenced to confirm that the product was the expected 94 bp product as reported by Buchanan *et al.* (2002). Three attempts to sequence the sample were undertaken, the first by the PCR the Molecular Biology Unit (MBU), at the Pietermaritzburg Campus of the University of KwaZulu-Natal, and the second and third by a commercial company, Inqaba Biotechnical Laboratories in Pretoria. The first two attempts were unsuccessful; however the third produced a sequence that proved that the amplification product was the expected product, after comparison with the sequence published by Buchanan *et al.* (2002).

The forward and reverse sequences of the amplification product were supplied and are shown in Figure 3-3. The NCBI BLAST website programme BLAST 2 SEQUENCES was used to analyse the sequence of the amplification product. The reverse *lep* sequence showed a 94 % similarity to the *lep* sequence reported by Buchanan *et al.* (2002), and the forward *lep* sequence showed a 92 % similarity, which was of a sufficiently high similarity to be accepted as the correct amplification product.

```
Lepseq:   atgcgctgtggacccctgtatcgattcctgtggctttggccctatctgtcttacgtggaggctgtgc
          ||||||||||||||| |||||||||||||||||||||||| |||||||||||| ||||||||||
Revseq:   atgcgctgtggaccc-tgtatcgattcctgtggctttggcctgatctgtcttacg-ggaggctgtgc
```

Figure 3-3 **Sequence analysis of exon 2 of the lep gene. The comparison of the J20 reverse sequence with the lep gene reported by Buchanan *et al.* (2002).**

3.3.5 Generation of fingerprints for individuals

The two different alleles, the T- and the C-alleles, at the SNP in exon 2 of the *lep* gene were revealed through the amplification of the Buchanan *et al.* (2002) primer pair. The T-allele contained a thymine nucleotide at the site of the SNP, while the C-allele contained a cytosine nucleotide at the SNP. The two different alleles were identified by the introduction of an intentional mismatch in the reverse primer region. The C-allele amplification product allowed subsequent cleavage of the 94 bp product with digestion by the *Mro* I restriction enzyme, producing a 75 bp and a 19 bp restricted product. The T-allele, on the other hand, was not digested with *Mro* I restriction enzyme, keeping the 94 bp amplification product intact. The PAG's were able to visualize the 94 bp and 75 bp products, while the 19 bp product ran off the gel due to its small size. From this it follows that three possible genotypes could occur, namely the homozygous CC and TT genotypes, and the heterozygous CT genotype. Figure 3-4 provides a graphical representation of the three possible genotypes at the SNP, as well as a PAG of the three genotypes. Individual J41, in Figure 3-4, has a CC genotype; both alleles have been cut by the enzyme to produce a 75 bp DNA fragment. J42 is a typical TT individual; no digestion of the PCR product took place, and 94 bp bands are observed on the gel. Individual J43 is heterozygous at the locus, one of the alleles was restricted and the other was not.

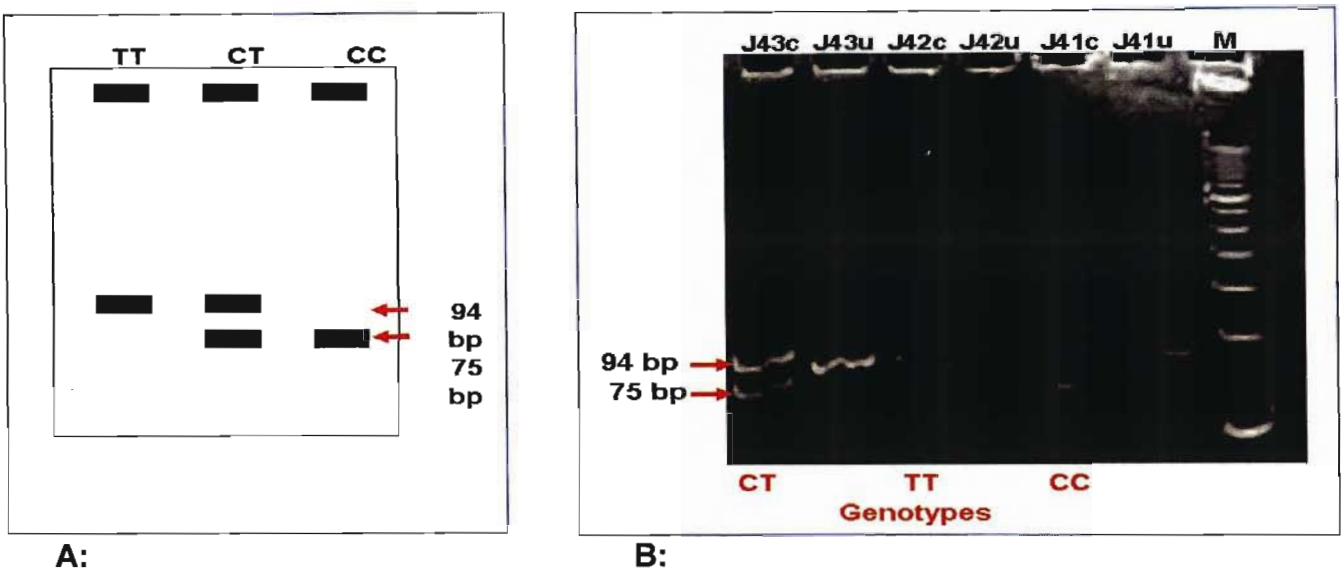


Figure 3-4 **Gel electrophoresis shows the three genotypes at the SNP in exon 2 of the *lep* gene. A: A graphical representation of the three possible genotypes. B: PAG of three individuals' genotypes, showing the cut and uncut samples.**

The genotypes of each individual were compiled and are listed in Table 3-4, where TT and CC represent homozygous individuals and CT represents heterozygous individuals.

Table 3-4 **Genotypes of each cow.**

Sample no.	Cow ID	Genotype	Sample no.	Cow ID	Genotype
J1	98163	TT	J26	R48	TT
J2	99100	CT	J27	0036	CT
J3	9951	TT	J28	00192	CT
J4	97119	CT	J29	99182	TT
J5	99176	CT	J30	0019	CT
J6	0174	CT	J31	01242	CT
J7	0010	CT	J32	0135	TT
J8	019	CT	J33	S178	CT
J9	01186	CT	J34	01280	CT
J10	00180	CT	J35	0115	TT
J11	99223	TT	J36	01111	TT

Sample no.	Cow ID	Genotype	Sample no.	Cow ID	Genotype
J12	0145	CT	J37	97141	TT
J13	0040	CT	J38	9983	CT
J14	98169	CT	J39	99203	CT
J15	9856	CT	J40	01154	CT
J16	98116	CT	J41	99164	CC
J17	98154	TT	J42	9758	TT
J18	97149	CT	J43	97170	CT
J19	00185	CT	J44	00172	CT
J20	0085	CT	J45	99160	TT
J21	0082	TT	J46	99154	TT
J22	S28	TT	J47	00203	TT
J23	S138	CT	J48	00120	TT
J24	00199	CC	J49	9712	CT
J25	013	CC	J50	00143	CT

3.3.6 **Genetic characterization of the selected sample population**

The genetic characterization of the selected sample population revealed that all three genotpyes were represented in the population (Table 3-5). The homozygous condition CC was the rarest genotype, followed by the other homozygote, TT. The heterozygote CT was the most prevalent genotype. From these data the allele frequencies were determined, revealing that the C-allele was present in the lowest proportion of 36 % and the T-allele accounting for 64 % of the alleles (Table 3-5). Thus, it can be concluded that the T-allele is nearly twice as prevalent as the C-allele.

Table 3-5 **Genotypic and allelic frequencies of the sample population.**

Genotype	Observed number of each genotype	Genotypic frequency	Alleles	Allele frequency
CC	3	0.06	C	0.36
CT	30	0.60		
TT	17	0.34	T	0.64
Total	50	1.00		1

A chi-square test was performed to assess whether the sample population was in Hardy-Weinberg equilibrium, which is indicative of any external forces influencing the sample population, such as selection, and non-random mating. The calculated χ^2 value (Table 3-6) at one degree of freedom revealed a p value greater than 0.025, but less than 0.05, verifying that this sample was not in Hardy-Weinberg equilibrium, at a significance level of 5 %.

Table 3-6 **Chi-squared test to determine whether the sample population was in Hardy-Weinberg equilibrium.**

Classes	Observed no. (O)	Expected no. (E)	$(O - E)^2 / E$
CC	3	6.48	1.8689
CT	30	23.04	2.1025
TT	17	20.48	0.5913
	50	50	$\chi^2 = 4.5627$

The F-statistic, F_{IS}, is a measure to determine the reduction in heterozygosity due to inbreeding, and was calculated, using the formula $F_{IS} = (H_{exp} - H_{obs}) / H_{exp}$, where H_{exp} is the heterozygosity expected under Hardy-Weinberg equilibrium, as being – 0.29. This negative value indicates an overrepresentation of heterozygotes, when compared to a random mating population, which suggests the active introduction of unrelated semen into the herd by the farmer, for utilization in his artificial insemination (AI) programme. The value of the fixation index provided support to the result of the chi-square test.

3.4 PHENOTYPIC CHARACTERIZATION OF THE SELECTED COWS

The phenotypic characterization of the selected cows from the Sarsden Jersey herd allowed the performance of the sample population to be compared with that of the entire Sarsden Jersey herd, over the investigation period. This assessment indicated whether or not the sample population was a good representation of the entire herd, thus allowing conclusions to be reached as to the accuracy of the final results.

Prior to the assessment, the dataset containing the phenotypic data on the performance of the sample population was evaluated for its suitability for the following analysis, and all incomplete records were removed. Two lactation records were excluded from the dataset.

The economically important dairy traits characterised included two broad groups of traits, namely milk production traits and reproductive performance traits.

3.4.1 **Phenotypic description of milk production traits**

An analysis of variance (ANOVA) was used to compare the performance of four milk production traits between the sample population and the entire Sarsden herd, over the investigation period (1997 – 2005).

To summarize the performance of the cows on the farm, Table 3-7 includes the number of observations, the arithmetic mean, the standard error of the mean, and the range of all the traits averaged over all the lactations, as well as the means of the entire Sarsden herd, over the investigation period. When the means of the

sample population and the Sarsden herd were compared, using one-way ANOVA, the differences between the means, for all four milk production traits, were found to be non significant ($p > 0.251$). This suggested that the sample population was a good representation of the herd as a whole.

3.4.2 **Phenotypic description of reproductive performance traits**

The two reproductive performance or fertility traits investigated were inter-calving period (ICP) and services per conception (SPC). Table 3-7 summarizes the ICP and SPC records of the sample population and the ICP records of the entire Sarsden herd, over the period of the investigation. SPC records were not available for the entire Sarsden herd. The average ICP for this sample was 439.64, which was considerably longer than the ideal of 365 dats; one calf per annum. In the case of SPC, the ideal would be to obtain pregnancy with each service. The average SPC for this sample was 1.84; much greater than the ideal of one. The means of the sample population and the entire herd were compared to determine if a significant difference existed between the two groups, a one-way ANOVA showed the differences between the means to be non significant ($p = 0.063$).

Table 3-7 **Mean milk production performances and reproductive performances of the sample population and entire Sarsden herd.**

	Sample population				Sarsden herd			
Trait	**No. of cows (n)**	**Range**	**Mean**	**Std. Error**	**No. of cows (n)**	**Range**	**Mean**	**Std. Error**
Milk yield[a] (kg)	48	2791	5518.79	83.82	919	7 705	5 426.16	25.15
Butterfat %	48	1.31	4.6440	0.0483	919	4	4.59	0.01
Protein %	48	0.73	3.4942	0.0222	919	1.7	3.472	0.006
Lactose %	48	1.48	4.7752	0.0306	919	2.25	4.8023	0.0055
ICP	42	348	439.64	12.82	1 039	630	417.39	2.26
SPC	48	2.33	1.8396	0.0950	-	-	-	-

[a] Milk yield is the 305 day adjusted measurement.

3.5 ASSESSSMENT OF POSSIBLE ASSOCIATIONS BETWEEN PHENOTYPIC PERFORMANCE AND GENOTYPE OF THE SELECTED COWS

Knowledge of the RFLP-*Kpn* 2I genotypes of the selected cows, and their phenotypic performance allows for an investigation into possible associations between the genotypes and the phenotypes. If such associations existed, it could prove to be useful in the development of markers that could be used in marker assisted selection (MAS) in breeding programmes. Such associations have been identified by other researchers studying the same SNP and dairy traits, thus linking these genotypes to economically important dairy traits (Buchanan *et al.*, 2003). This study investigated such possible associations between the RFLP-*Kpn* 2I genotypes of the selected cows, and the phenotypic performance of these cows, in terms of economically important dairy traits, including milk production and reproductive performance traits.

This investigation into possible associations between RFLP-*Kpn* 2I genotype and phenotype was carried out by using a general linear model (GLM), which determined whether genotype had a significant effect on the various milk production and reproductive performance traits, while taking into account any influence due to age at first calving, calving year, and calving season. There were two steps in this investigation; the first to determine if the interaction between RFLP-*Kpn* 2I genotype and age at first calving, calving year, and calving season was significant, and the second to determine whether the genotype was associated to the economically important traits.

3.5.1 Assessment of the interaction between genotype and age at first calving, calving year, and calving season

The first step in determining if the effect of the genotype was significant was

to determine if the effect of the genotypes was constant over different calving years and seasons, and at different ages at first calving. This required establishing whether the interactions between genotype and the three covariates (age at first calving, calving year, and calving season) were significant, using an ANOVA.

When the interaction between RFLP-*Kpn* 2I genotype and the three covariates was considered, the results were non significant for all production traits, except lactose percentage; and significant for SPC, but non significant for ICP. A non significant interaction suggested that the effect of RFLP-*Kpn* 2I genotype was the same, regardless of the calving year, season, or age at first calving; the interaction component was thus excluded from the final GLM model. With respect to lactose percentage and SPC, the effect of the RFLP-*Kpn* 2I genotype differed as the value or period of the covariates changed, thus the interaction component was included in the assessment of the relationship between RFLP-*Kpn* 2I genotype and phenotype for these two traits.

3.5.2 **Relationship between genotype and milk production traits**

The relationship between the RFLP-*Kpn* 2I genotypes, CC, CT, and TT, and milk production was investigated in terms of four milk production traits, namely:

- Milk yield adjusted for 305 days (kg),
- Butterfat percentage,
- Protein percentage, and
- Lactose percentage.

The phenotypic data used in this investigation included only the completed

first lactation data for 48 of the 50 selected cows. A general linear model (GLM) was used to determine if genotype had a significant effect on the various milk production traits, while taking into account any influence due to age at first calving, calving year, and calving season. This involved applying the full general linear model (either including the interaction term, for lactose percentage, or excluding it) to the data.

The mean and standard error of each milk production trait, classified by RFLP-*Kpn* 2I genotype, and the significance of the genotype, are given in Table 3-8. Visually, the homozygous CC genotype had a larger mean for three of the four milk production traits, specifically milk yield, protein percentage and lactose percentage, and the second highest mean performance for butterfat percentage (Figure 3-5). The homozygous TT genotype had the lowest mean for all three milk component percentages (butterfat, protein and lactose), but produced, on average, the second most kilograms of milk.

Although marginal differences did exist between the three genotypes for the milk production traits, the GLM analysis showed that these differences were non significant for all the milk production traits, except lactose percentage. In other words, genotype did not influence the performance of the sample population in terms of milk yield, butterfat and protein percentage; no association was found to exist.

Table 3-8 **Means of 305 day milk yield (kg), butterfat percentage, protein percentage, and lactose percentage, in terms of the different genotypic groups, and the significance of the genotypic effect.**

Trait	Genotype / Statistic	CC	CT	TT	Significance
Milk Yield (kg)	Mean ± SE	5713.33 ± 186.807	5440.64 ± 119.696	5613.18 ± 126.815	ns
BF %	Mean ± SE	4.66 ± 0.05033	4.6689 ± 0.06728	4.60 ± 0.08072	ns
Protein %	Mean ± SE	3.5033 ± 0.11260	3.4975 ± 0.02516	3.4871 ± 0.04510	ns
Lactose %	Mean ± SE	4.8733a ± 0.06936	4.8054a ± 0.02240	4.7082b ± 0.07600	s

Row means with common alphabetical letters are not significantly different ($P > 0.05$).

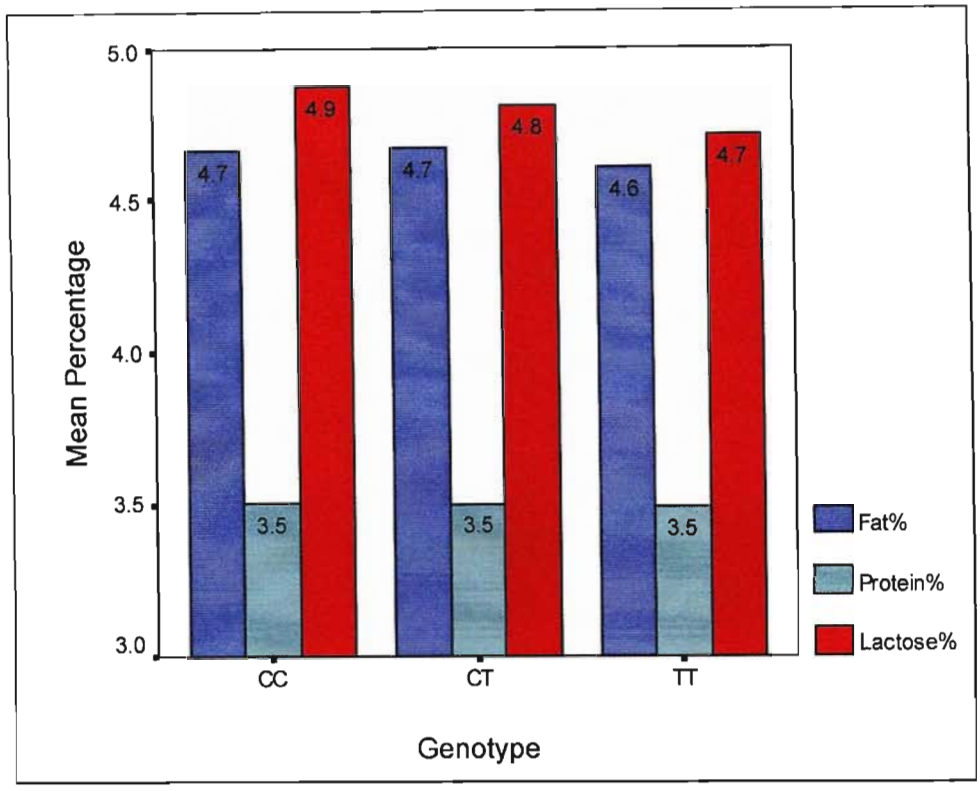


Figure 3-5 **Mean percentages of butterfat, protein, and lactose per genotype.**

3.5.3 Relationship between genotype and reproductive performance traits

Two reproductive performance traits were investigated for a possible association with RFLP-*Kpn* 2I genotype, namely ICP and SPC. The phenotypic data utilized in this investigation included the lifetime mean SPC for the selected cows, and the ICP between the first and second lactations of the selected cows. A general linear model (GLM) was used to resolve whether genotype had a significant effect on the two reproductive performance traits, while accounting for any influence attributable to age at first calving, calving year, and calving season. This concerned applying the full general linear model (either including the interaction term, for SPC, or excluding it) to the data.

The mean and standard error of each reproductive performance trait, classified by genotype, and the significance of the genotype, are given in Table 3-9. The

heterozygous CT genotype, on average, outperforms both homozygous genotypes with respect to both reproductive performance traits. The homozygous CC genotype has the highest mean ICP and SPC, while the TT genotype is intermediate between the CC and CT genotypes.

Although visual differences in the genotypic means did exist for the two traits, the GLM showed that these differences were non significant for both traits, suggesting no association between genotype and reproductive performance in terms of these traits.

Table 3-9 **Means and standard errors of the ICP and SPC for the sample of cows included in the investigation, and the significance of the association with genotype.**

Genotype	ICP		SPC	
	No. of cows (n)	Mean ± SE	No. of cows (n)	Mean ± SE
CC	3	561.00 ± 66.403	3	2.2767 ± 0.54606
CT	28	428.87 ± 15.758	28	1.7625 ± 0.10104
TT	17	432.38 ± 18.600	17	1.8894 ± 0.19230
Significance	Ns		ns	

Another interesting relationship in dairy farming is that between milk yield and fertility. The influence of milk yield on reproductive performance was investigated due to the well known unfavourable correlation between fertility and milk yield. The effect of milk yield was investigated by comparing the number of services required to achieve conception for cows with milk yield. This was achieved by performing a regression analysis, and determining the correlation between milk yield and SPC. From Figure 3-6 it is clear that as milk yield increases, so does the SPC ratio. The correlation between milk yield and SPC for this sample is 0.164. This confirms the fact that the positive correlation between SPC and milk yield, means an unfavourable correlation between yield and fertility.

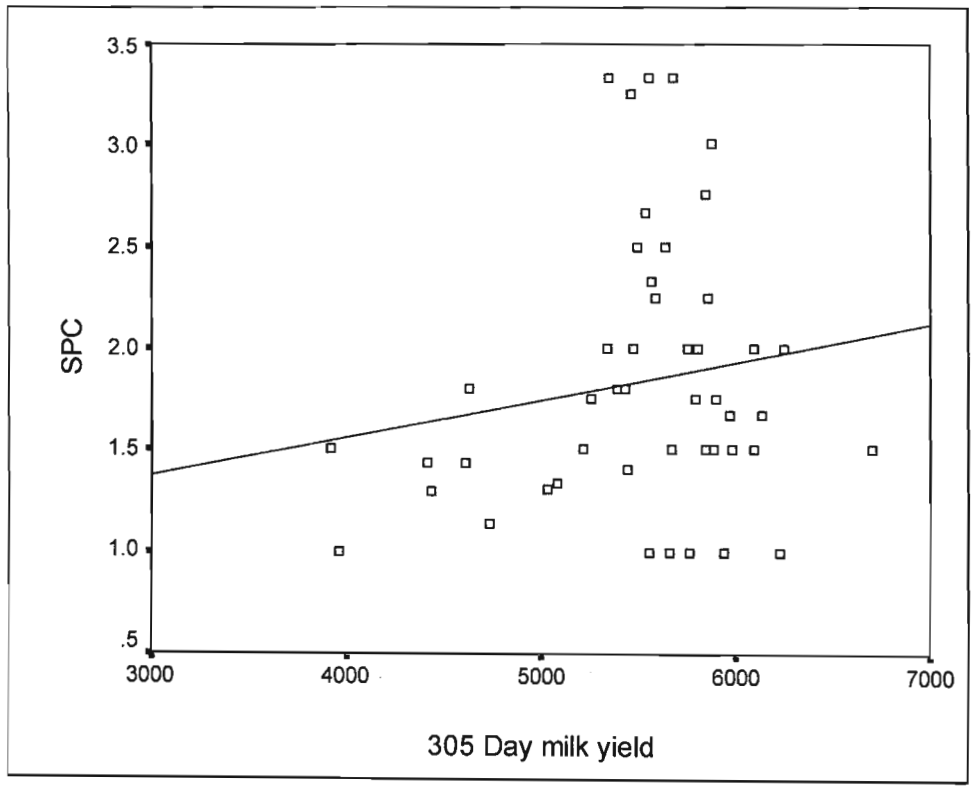


Figure 3-6 **Relationship between milk yield (kg) and fertility (SPC).**

CHAPTER 4 DISCUSSION AND CONCLUSIONS

Dairy production has become one the major agricultural industries in the world and in South Africa, providing a range of different milk products (Taylor and Field, 1998). Depending on the needs of the market, the industrial practices are constantly revised and changed. Farmers and breeders are continually striving to improve the performance of dairy cows through genetic modification and improved management practices.

Over the past half century great progress has been made in improving cow performance. This improvement is mostly attributable to improved breeding practices and management conditions. Over the past 20 years a milk yield increase of between three and four percent has been observed, half of which is due to improved genetic selection, while the other half can be attributed to improved environmental factors, such as nutrition, health and housing (Pryce and Veerkamp, 1999).

Together with the economically important trait of milk production, the trait of fertility is of vital importance to the success of breeding. One of the complicating factors in dairy breeding is the unfavourable correlation that exists between the two traits, milk yield and fertility (Lucy, 2001); as milk yield is increased, so fertility is decreased. The relationship between milk yield and fertility, in terms of SPC was investigated in the Sarsden population. SPC was found to increase as milk yield increased, an antagonistic relationship that has also been reported by Oltenacu (1991) and Nebel and McGilliard (1993).

It has, however, been shown that high milk yielding cows are able to be fertile as well. These superior cows produce up to 18 000 kg of milk per lactation. Farmers and breeders therefore, aim to develop entire herds where the majority of the cows produce 18 000 kg of milk per lactation and display high levels of fertility, through careful planning and selection (Cassell, 2001).

Genetic improvement is achieved through selection of appropriate individuals to be the parents of the next generation. Dairy breeding programmes involve selecting sires to mate to the available cows, and thus produce offspring that will serve as replacements in the dairy herd. Several selection strategies are used in the selection of suitable parent stock.

The more traditional side of the selection practices involves the use of high ranking AI bulls. These sires are selected based upon the performance of their daughters through progeny testing. The average performances of the daughters are used to assess the extent of the genetic contribution of the sire to the phenotype of the progeny. The extent of the sire's contribution is reflected as the estimated breeding value (EBV) of an individual sire. Although traditional progeny testing programmes work reliably, they do have a number of disadvantages. Firstly, there is a time delay before daughters of potential sires become milk producers, when their performance can be assessed. Milk production records, from which EBVs can be determined, are thus only available once daughters start to lactate. Secondly, the average heritability (h^2) estimates for milk production traits are moderate, and are low for fertility traits, thus making phenotypic performance a poor indicator of breeding value (Bourdon, 1997).

The introduction of molecular technologies that are able to assess the genotype directly is fast becoming an important component in breeding in the cattle industry. The identification of markers associated with the economically important traits, has the potential to facilitate early and more accurate identification of superior alleles/genotypes. These sophisticated modern methods utilize identifiable DNA polymorphisms in specific regions of DNA that are associated with the phenotypic traits of interest. It is envisaged that marker assisted selection (MAS) will become a standard component of breeding strategies in the cattle industry, which will thus allow for the evaluation and selection of young bulls before they reach maturity, earlier than is possible through progeny testing.

A number of DNA markers have been identified in dairy cows that have possible associations with economically important dairy traits. Markers with possible linkages to milk yield, butterfat and protein percentage have been identified for the bovine *lep* gene. This gene was first cloned in 1994 by Friedman *et al.*, and encodes the hormone leptin, which has been shown to play a role in fat metabolism, feed intake, reproductive functioning, and immune system control (Houseknecht *et al.*, 1998). Further research has suggested that polymorphisms in the *lep* gene may influence the phenotypic performance of cows in terms of the different milk production traits, resulting in an increased interest in the *lep* gene over the past few years.

Several polymorphisms have been identified in the *lep* gene to date, including SNPs and microsatellites with possible associations with economically important dairy traits. One of these was a single nucleotide polymorphism (SNP) in exon 2 by Buchanan *et al.* (2002). This SNP, named RFLP-*Kpn* 2I, has been thoroughly researched for its putative involvement with body conformational change by the altered leptin hormone. This SNP contains an arginine to cysteine amino acid change (Buchanan *et al.*, 2002). In 2003, Buchanan *et al.* investigated this SNP for an association with a number of economically important dairy traits, thereby contributing to the ever increasing body of *lep* gene knowledge. This work initiated many investigations into this SNP by other research groups searching for information on its links to important phenotype traits.

The investigation, based upon the work by Buchanan *et al.* (2003), was carried out to obtain a better understanding of the relationship between *lep* RFLP-*Kpn* 2I alleles and economically important dairy traits in the South African Sarsden commercial Jersey dairy herd. Fifty cows were genotyped for the RFLP-*Kpn* 2I polymorphism and assessed for possible associations with economically important dairy traits.

The two alleles at the RFLP-*Kpn* 2I locus, identified by other investigations

(Buchanan *et al.*, 2002; Buchanan *et al.*, 2003; Lagonigro *et al.*, 2003; Liefers *et al.*, 2003; Madeja *et al.*, 2004; Barendse *et al.*, 2005) were also found to be present in the Sarsden herd. The frequency of these two alleles varies across different breeds (Table 4-1). The T-allele has been found to be rarer than the C-allele in the majority of the breeds/herds investigated (nine out of fourteen), ranging from 0.06, in Guernsey cattle, to 0.62 in an Ayrshire herd (Buchanan *et al.*, 2002; Buchanan *et al.*, 2003; Madeja *et al.*, 2004; Barendse *et al.*, 2005). In the Sarsden herd the T-allele proved to have the greater frequency, which was similar to the frequency determined by Buchanan *et al.* (2003) for a sample of 17 Ayrshire animals.

Table 4-1 **Allele frequencies of a variety of cattle breeds for the RFLP-*Kpn* 2I alleles, C and T.**

Breed	No. of animals	T-allele frequency	C-allele frequency	Reference
South African Jersey	50	0.64	0.36	-
Jersey	20	0.53	0.47	Buchanan *et al.* (2003)
Holstein	416	0.46	0.54	Buchanan *et al.* (2003)
Ayrshire	17	0.62	0.38	Buchanan *et al.* (2003)
Brown Swiss	21	0.45	0.55	Buchanan *et al.* (2003)
Canadienne	9	0.11	0.89	Buchanan *et al.* (2003)
Guernsey	16	0.06	0.94	Buchanan *et al.* (2003)
Angus	60	0.58	0.42	Buchanan *et al.* (2002)
Charolais	55	0.34	0.66	Buchanan *et al.* (2002)
Hereford	22	0.55	0.45	Buchanan *et al.* (2002)
Simmental	17	0.32	0.68	Buchanan *et al.* (2002)
Polish Black and White	117	0.46	0.54	Madeja *et al.* (2004)
Angus	821	0.48	0.52	Barendse *et al.* (2005)
Shorthorn	742	0.18	0.82	Barendse *et al.* (2005)

The genotypic frequencies of the Sarsden herd were not in Hardy-Weinberg equilibrium. This expected result can be explained by the relatively small sample and the history of the herd; a limited number of cows are used to produce progeny

for herd replacement.

The RFLP-*Kpn* 2I alleles of the cows in the Sarsden herd did not impact on 305 day milk yield in a significant way ($p > 0.05$), this finding was in accordance with a similar study by Madeja *et al.* (2004), who found no association between breeding value for milk yield in Black-and-White Polish bulls and genotype. Buchanan *et al.* (2003) report contradictory results, having identified a significant association between genotype and milk yield in Holstein cows. An explanation for these results may be that this SNP in the *lep* gene does not influence milk yield.

Genotype also did not significantly affect the butterfat percentage of the milk ($p > 0.05$); this finding was in accordance with both Buchanan *et al.* (2003) and Madeja *et al.* (2004). This suggests that this SNP may not affect butterfat percentage, although this does not exclude the possibility that other polymorphisms in the *lep* gene may affect butterfat percentage.

Protein percentage was not significantly influenced by the RFLP-*Kpn* 2I genotype ($p > 0.05$). This confirms the results produced by Madeja *et al.* (2004) in Polish Black-and-White bulls for the breeding value of protein percentage; but was in contrast to the report of Buchanan *et al.* (2003) who suggested that a significant association does exist between protein percentage and the RFLP-*Kpn* 2I genotype. It is possible that the SNP in question is not the critical *lep* gene mutation involved in protein content; further analysis of larger numbers of individuals may be required.

It was, however, interesting to note that an association between the RFLP-*Kpn* 2I alleles and lactose percentage was found ($p < 0.05$), reported on here for the first time. Individuals with the genotype CC produced milk with the highest lactose content, followed by homozygous TT individuals, with heterozygous CT individuals producing the lowest amount of lactose. One would expect the heterozygote genotype to be intermediate, since it contains one C allele, thus suggesting that

the CT genotype should produce a higher lactose percentage than the TT genotype individuals. This association could be the subject of further investigations as lactose intolerance is a relatively common occurrence in today's society. If it were possible to select animals with lower lactose content in their milk, then it may be possible to produce specialized milk for lactose intolerant individuals.

The association between the RFLP-*Kpn* 2I SNP and fertility, measured using ICP and SPC, was undertaken in this investigation for the first time. The leptin hormone has been shown to play an important role in the regulation of the reproductive axis (Liefers *et al.*, 2002) and for this reason all possible links between *lep* gene polymorphisms and fertility traits need to be investigated. It was, however, found that there was no significant association between the RFLP-*Kpn* 2I genotypes and the two fertility traits, ICP and SPC, in the Sarsden herd ($p > 0.05$).

The lack of association between most of the traits and the genotypes can possibly be attributed to the relatively small sample studied; differences in the samples investigated and breed composition differences. Also, milk and reproductive traits are quantitative traits and, as such, are affected by many factors, which include, besides genetic factors, many environmental factors which require intensive investigation. Thus, even if an association had been identified between the locus of interest and the traits under consideration, it would only have accounted for a portion of the total phenotypic variation, as the traits investigated are quantitative. Known environmental factors include the age of the cow, the lactation number and stage of lactation, as well as the health of the cow, with special consideration for the health of the mammary gland. Other environmental factors include calving year and season. Some of these factors were included as covariates in the statistical model in this investigation, but the other factors may require more specific attention in further investigations.

The limitation of most of the molecular investigations into the genotypic association of the *lep* locus with economically important traits is that of the relatively small sample size. Certainly, the possible associations between the different genotypes and phenotypic performance will only be clarified with more intensive investigations involving large numbers of individuals. Such investigations should also include extensive assessments of different breeds, as it cannot be accepted that all breeds will show similar associations. It will also be necessary to determine and account for as many possible environmental factors as possible, by incorporating them into the statistical evaluation model.

Although little association could be identified between this particular RFLP-*Kpn* 2I SNP in the *lep* gene, this hormone's importance in milk production and fertility has been recognised and is currently the subject of numerous investigations world wide. Other polymorphisms may be identified in the future providing a more accurate tool to employ in marker assisted selection in dairy cattle breeding. Such polymorphisms would permit farmers and breeders to make earlier selection decisions on which heifers to use as replacements, and which bulls to mate with, instead of having to wait for progeny testing. This will provide for more accurate selection practices, and will save time and, most important of all, will reduce the costs of dairy farming and breeding.

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APPENDICES

Appendix A Recipes

DNA Isolation Reagents

5 X BCL Buffer (without Triton X-100)

- 57.4 g sucrose
- 2.5 ml 2 M Tris-Cl pH 7.5 (MW = 121.1 g / 1 000 ml)
    - 0.30275 g in 2.5 ml sdH_2O
- 2.5 ml $MgCl_2$ (MW = 203.31 g / 1 000 ml)
    - 0.508275 g in 2.5 ml sdH_2O

Dissolve the Tris-Cl in 2.5 ml sdH_2O, and then add to the sucrose, which was dissolved in 80 ml sdH_2O. Adjust the pH to 7.5 with HCl, and then ad the $MgCl_2$. Make up to 100 ml. Autoclave and store at room temperature (RT).

Before use, dilute 1:5 with sdH_2O, and then add 0.05 vol 20 % Triton X-100. Store in a sterile McCartney bottle, on ice.

Lysis Buffer (per isolation reaction)

- 1 X TNE (0.4 M NaCl; 10 mM Tris-HCl pH 8; 2 mM EDTA pH 8)
    - 0.4 M NaCl (MW = 58.443 g / 1 000 ml) – 0.23372 g NaCl
    - 10 mM Tris-HCl pH 8 (MW = 157.56 g / 1 000 ml) – 0.1576 g Tris-HCl
    - Add NaCl and Tris-HCl to approximately 80 ml of sdH_2O, and adjust pH to 8 with NaOH, before adding the EDTA.
    - 2 mM EDTA (MW = 292.25 g / 1 000 ml) – 0.05845 g
    - Dissolve using stirrer. Make up to 100 ml. Autoclave and store at RT.
- 1 M Tris-HCl pH 8 (MW = 157.56 g / 1 000 ml)
    - 15.756 g Tris-Hcl in approximately 80 ml sdH_2O
    - Adjust pH to 8 using 3 M NaOH, and make up the volume to 100 ml.
    - Autoclave and store at RT.
- 25 % SDS
    - 6.25 g in 25 ml sdH_2O
    - Do not autoclave, store at RT.
- 10 % Triton X-100
    - 1 ml Triton X-100 in 9 ml sdH_2O

- Proteinase K (10 mg /ml)
  - 0.01 g Proteinase K in 1 ml of sdH_2O

5 M NaCl (MW = 58.443 g / 1 000 ml)

- 29.2215 g NaCl in 100 ml sdH_2O
- Autoclave and store at RT.

20 % Triton X-100

- 4 ml Triton X-100 in 16 ml sdH_2O

10 mM Tris-HCl pH 8

- 200 µl 1 M Tris-HCl pH 8 in 1 800 ml sdH_2O

Agarose gel electrophoresis reagents

10 X TAE Buffer

- 400 mM Tris
- 200 mM Na-acetate
- 10 mM EDTA

Loading Buffer – Type III

- 0.25 % bromophenol blue
- 0.25 % xylene cyanol FF
- 30 % glycerol in H_2O

Spectrophotometry reagents

10 X TE

- 100 mM Tris-Cl pH 7.5
- 100 mM EDTA
- Adjust pH of Tris with concentrated HCl, and then add the EDTA.
- Autoclave and store at RT.

PAGE reagents

Acrylamide: bisacrylamide (29:1) (% w / v)

- 29 g acrylamide
- 1 g bisacrylamide
- 100 ml sdH_2O

- Store in a foil wrapped bottle at 4 ° C.

Ammonium persulfate (10 % w / v)

- 0.0228 g APS in 1 ml sdH_2O
- Must be made up fresh weekly.

5 X TBE electrophoresis buffer (1 M Tris; 1 M Boric acid; 05 M EDTA; pH 8.3)

- 1 M Tris
  - 12.11 g
- 1 M Boric acid
  - 6.183 g
- EDTA solution
  - 4.4 ml
    - 2.9225 g in 10 ml
    - Adjust pH to 8 with NaOH before adding to Tris and Boric acid solution.
- Adjust pH to 8.3 with HCl.
- Autoclave and store at RT.

Appendix B Statistical programme output

Effect of genotype on milk production and reproductive performance traits

GLM Univariate analysis for milk production and genotype

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Tests of Between-Subjects Effects
Dependent Variable: Milk Yield_305

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	8404559.614(a)	8	1050569.952	5.502	.000	.530
Intercept	7290397.444	1	7290397.444	38.183	.000	.495
GENOTYPE	71822.274	2	35911.137	.188	.829	.010
CALVING	124883.761	1	124883.761	.654	.424	.016
V8	7463003.123	1	7463003.123	39.087	.000	.501
AGE_AT_1	280578.324	1	280578.324	1.470	.233	.036

GENOTYPE * CALVING * V8 * AGE_AT_1	179621.355	3	59873.785	.314	.815	.024
Error	7446382.302	39	190932.880			
Total	1477789892.000	48				
Corrected Total	15850941.917	47				

a R Squared = .530 (Adjusted R Squared = .434)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Descriptive Statistics

Dependent Variable: Milk Yield_305

GENOTYPE	Mean	Std. Deviation	N
CC	5713.33	323.559	3
CT	5440.64	633.370	28
TT	5613.18	522.871	17
Total	5518.79	580.736	48

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Milk Yield_305

F	df1	df2	Sig.
.207	2	45	.814

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+CALVING+V8+AGE_AT_1+GENOTYPE

Tests of Between-Subjects Effects

Dependent Variable: Milk Yield_305

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	8224938.259(a)	5	1644987.652	9.060	.000	.519
Intercept	7368944.441	1	7368944.441	40.584	.000	.491
CALVING	126418.411	1	126418.411	.696	.409	.016
V8	7530701.390	1	7530701.390	41.475	.000	.497
AGE_AT_1	189628.182	1	189628.182	1.044	.313	.024
GENOTYPE	667670.374	2	333835.187	1.839	.172	.081
Error	7626003.657	42	181571.516			
Total	1477789892.000	48				
Corrected Total	15850941.917	47				

a R Squared = .519 (Adjusted R Squared = .462)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28

TT	17

Tests of Between-Subjects Effects
Dependent Variable: Fat%

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.946(a)	8	.118	1.068	.405	.180
Intercept	.010	1	.010	.094	.761	.002
GENOTYPE	.066	2	.033	.297	.745	.015
AGE_AT_1	.146	1	.146	1.320	.258	.033
CALVING	.005	1	.005	.042	.838	.001
V8	.018	1	.018	.163	.689	.004
GENOTYPE * AGE_AT_1 * CALVING * V8	.081	3	.027	.244	.865	.018
Error	4.315	39	.111			
Total	1040.445	48				
Corrected Total	5.261	47				

a R Squared = .180 (Adjusted R Squared = .011)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Descriptive Statistics
Dependent Variable: Fat%

GENOTYPE	Mean	Std. Deviation	N
CC	4.6600	.08718	3
CT	4.6689	.35601	28
TT	4.6000	.33283	17
Total	4.6440	.33456	48

Levene's Test of Equality of Error Variances(a)
Dependent Variable: Fat%

F	df1	df2	Sig.
.694	2	45	.505

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+AGE_AT_1+CALVING+V8+GENOTYPE

Tests of Between-Subjects Effects
Dependent Variable: Fat%

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.865(a)	5	.173	1.652	.167	.164
Intercept	.030	1	.030	.290	.593	.007
AGE_AT_1	.403	1	.403	3.846	.057	.084
CALVING	.402	1	.402	3.840	.057	.084
V8	.041	1	.041	.395	.533	.009

GENOTYPE	.004	2	.002	.017	.983	.001
Error	4.396	42	.105			
Total	1040.445	48				
Corrected Total	5.261	47				

a R Squared = .164 (Adjusted R Squared = .065)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Tests of Between-Subjects Effects

Dependent Variable: Protein%

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.369(a)	8	.046	2.435	.031	.333
Intercept	.101	1	.101	5.300	.027	.120
GENOTYPE	.053	2	.026	1.389	.261	.067
V8	.115	1	.115	6.076	.018	.135
AGE_AT_1	.036	1	.036	1.872	.179	.046
CALVING	.003	1	.003	.174	.679	.004
GENOTYPE * V8 * AGE_AT_1 * CALVING	.066	3	.022	1.162	.337	.082
Error	.740	39	.019			
Total	587.151	48				
Corrected Total	1.109	47				

a R Squared = .333 (Adjusted R Squared = .196)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Descriptive Statistics

Dependent Variable: Protein%

GENOTYPE	Mean	Std. Deviation	N
CC	3.5033	.19502	3
CT	3.4975	.13313	28
TT	3.4871	.18594	17
Total	3.4942	.15362	48

Levene's Test of Equality of Error Variances(a)

Dependent Variable: Protein%

F	df1	df2	Sig.

.966	2	45	.388

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+V8+AGE_AT_1+CALVING+GENOTYPE

Tests of Between-Subjects Effects
Dependent Variable: Protein%

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.303(a)	5	.061	3.162	.016	.274
Intercept	.094	1	.094	4.883	.033	.104
V8	.108	1	.108	5.605	.023	.118
AGE_AT_1	.135	1	.135	7.013	.011	.143
CALVING	.035	1	.035	1.825	.184	.042
GENOTYPE	.010	2	.005	.267	.767	.013
Error	.806	42	.019			
Total	587.151	48				
Corrected Total	1.109	47				

a R Squared = .274 (Adjusted R Squared = .187)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Tests of Between-Subjects Effects
Dependent Variable: Lactose%

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.860(a)	8	.108	3.357	.005	.408
Intercept	.419	1	.419	13.077	.001	.251
GENOTYPE	.210	2	.105	3.281	.048	.144
AGE_AT_1	.236	1	.236	7.370	.010	.159
CALVING	.153	1	.153	4.770	.035	.109
V8	.377	1	.377	11.770	.001	.232
GENOTYPE * AGE_AT_1 * CALVING * V8	.306	3	.102	3.181	.034	.197
Error	1.250	39	.032			
Total	1096.636	48				
Corrected Total	2.110	47				

a R Squared = .408 (Adjusted R Squared = .286)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Descriptive Statistics
Dependent Variable: Lactose%

GENOTYPE	Mean	Std. Deviation	N
CC	4.8733	.12014	3
CT	4.8054	.11856	28
TT	4.7082	.31335	17
Total	4.7752	.21188	48

Levene's Test of Equality of Error Variances(a)
Dependent Variable: Lactose%

F	df1	df2	Sig.
1.667	2	45	.200

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+GENOTYPE+AGE_AT_1+CALVING+V8+GENOTYPE * AGE_AT_1 * CALVING * V8

Tests of Between-Subjects Effects
Dependent Variable: Lactose%

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.860(a)	8	.108	3.357	.005	.408
Intercept	.419	1	.419	13.077	.001	.251
GENOTYPE	.210	2	.105	3.281	.048	.144
AGE_AT_1	.236	1	.236	7.370	.010	.159
CALVING	.153	1	.153	4.770	.035	.109
V8	.377	1	.377	11.770	.001	.232
GENOTYPE * AGE_AT_1 * CALVING * V8	.306	3	.102	3.181	.034	.197
Error	1.250	39	.032			
Total	1096.636	48				
Corrected Total	2.110	47				

a R Squared = .408 (Adjusted R Squared = .286)

GLM Univariate analysis for reproductive performance and genotype

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Tests of Between-Subjects Effects
Dependent Variable: SPC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	7.027(a)	8	.878	2.572	.023	.345
Intercept	.449	1	.449	1.314	.259	.033
GENOTYPE	1.635	2	.818	2.394	.105	.109
CALVING	2.388	1	2.388	6.993	.012	.152

V8	.427	1	.427	1.251	.270	.031
AGE_AT_1	1.640	1	1.640	4.803	.034	.110
GENOTYPE * CALVING * V8 * AGE_AT_1	4.969	3	1.656	4.849	.006	.272
Error	13.320	39	.342			
Total	182.782	48				
Corrected Total	20.347	47				

a R Squared = .345 (Adjusted R Squared = .211)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	28
	TT	17

Descriptive Statistics

Dependent Variable: SPC

GENOTYPE	Mean	Std. Deviation	N
CC	2.2767	.94585	3
CT	1.7625	.53465	28
TT	1.8894	.79286	17
Total	1.8396	.65796	48

Levene's Test of Equality of Error Variances(a)

Dependent Variable: SPC

F	df1	df2	Sig.
1.588	2	45	.216

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+GENOTYPE+CALVING+V8+AGE_AT_1+GENOTYPE * CALVING * V8 * AGE_AT_1

Tests of Between-Subjects Effects

Dependent Variable: SPC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	7.027(a)	8	.878	2.572	.023	.345
Intercept	.449	1	.449	1.314	.259	.033
GENOTYPE	1.635	2	.818	2.394	.105	.109
CALVING	2.388	1	2.388	6.993	.012	.152
V8	.427	1	.427	1.251	.270	.031
AGE_AT_1	1.640	1	1.640	4.803	.034	.110
GENOTYPE * CALVING * V8 * AGE_AT_1	4.969	3	1.656	4.849	.006	.272
Error	13.320	39	.342			
Total	182.782	48				
Corrected Total	20.347	47				

a R Squared = .345 (Adjusted R Squared = .211)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	23
	TT	16

Tests of Between-Subjects Effects
Dependent Variable: ICP (days)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	114688.462(a)	7	16384.066	3.313	.009	.405
Intercept	7080.260	1	7080.260	1.432	.240	.040
GENOTYPE	46139.889	2	23069.944	4.665	.016	.215
CALVING	153.114	1	153.114	.031	.861	.001
V8	7267.925	1	7267.925	1.470	.234	.041
GENOTYPE * CALVING * V8	33066.683	3	11022.228	2.229	.103	.164
Error	168145.180	34	4945.446			
Total	8400839.000	42				
Corrected Total	282833.643	41				

a R Squared = .405 (Adjusted R Squared = .283)

Univariate Analysis of Variance

Between-Subjects Factors

		N
GENOTYPE	CC	3
	CT	23
	TT	16

Descriptive Statistics
Dependent Variable: ICP (days)

GENOTYPE	Mean	Std. Deviation	N
CC	561.00	115.013	3
CT	428.87	75.574	23
TT	432.38	74.400	16
Total	439.64	83.056	42

Levene's Test of Equality of Error Variances(a)
Dependent Variable: ICP (days)

F	df1	df2	Sig.
.463	2	39	.633

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: Intercept+CALVING+V8+GENOTYPE

Tests of Between-Subjects Effects
Dependent Variable: ICP (days)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	81621.780(a)	4	20405.445	3.752	.012	.289
Intercept	28157.004	1	28157.004	5.178	.029	.123
CALVING	878.577	1	878.577	.162	.690	.004

V8	28968.589	1	28968.589	5.327	.027	.126
GENOTYPE	28415.608	2	14207.804	2.613	.087	.124
Error	201211.863	37	5438.158			
Total	8400839.000	42				
Corrected Total	282833.643	41				

a R Squared = .289 (Adjusted R Squared = .212)