# PHYSIOLOGY AND GENETICS OF LEPTIN IN PERIPARTURIENT DAIRY COWS

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# PHYSIOLOGY AND GENETICS OF LEPTIN IN PERIPARTURIENT DAIRY COWS

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# PHYSIOLOGY AND GENETICS OF LEPTIN IN PERIPARTURIENT DAIRY COWS

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# ABSTRACT

In dairy cattle, the increase in milk yield has been accompanied by a more negative energy balance during early lactation and a decrease in fertility. As the hormone leptin is involved in regulation of nutritional status and reproductive function this hormone is an interesting protein to investigate during the periparturient period in dairy cattle when many changes take place both in energy metabolism and reproductive physiology. This study was performed to get insight into the function of leptin during the periparturient period and to perform an association study between polymorphisms in the bovine leptin gene and leptin receptor gene and fertility as well as production traits. Leptin concentrations in the periparturient cow undergo remarkable changes; leptin concentrations were high during late pregnancy and declined to a nadir at parturition. Genetic analysis of the leptin gene indicated that a combination of three polymorphisms located at a 135 bp region of the leptin promoter explained most of the variance in prepartum leptin concentrations. We proposed a putative pregnancy-dependent enhancer to be located at this site on the leptin promoter. The two extreme genotype combinations (high and low prepartum leptin concentrations) could be used to investigate the function of leptin concentrations in pregnant cows. A polymorphism located on intron 2 of the leptin gene explained a significant part of the variation in milk yield. On the promoter region of the leptin gene an SNP was detected that was associated with first postpartum luteal activity. Fertility traits are considered to be important to select for and this SNP could be a candidate marker for fertility in dairy cows. Another SNP on the leptin promoter was associated with energy balance and dry matter intake where a higher dry matter intake occurred together with a higher energy balance. Two genotype combinations of the aforementioned three associated SNPs were defined which had a good milk yield together with a good energy balance and fertility. Calculations of an economical value per trait have to validate if one of these genotype combinations would be a possible candidate to be used in selection.

# CONTENTS

# CHAPTER 1

# General Introduction

## INTRODUCTION

Cattle is mainly kept for human milk and meat consumption in the Western World. For these two products, different breed types of cattle are used: dairy and beef cattle. In dairy cattle selection of sires is mainly based on milk production of their daughters. The last two decades milk yield increased 3-4% per year and approximately half of this improvement was due to genetic selection (Pryce and Veerkamp, 1999). The other half was due to improved environmental factors like nutrition, housing, health and management. The milk yield increase has been accompanied by a decrease in fertility. Several different traits are used to measure fertility, for example a lengthened calving interval and more days to first heat (Veerkamp et al., 2001). An explanation for the antagonistic relation between milk yield and fertility can be that energy intake does not meet the requirements for maintenance and milk production, which results in a period of negative energy balance (Van der Lende, 1998). During this period the cow mobilizes her fat reserves (i.e. adipose tissue) and it appears that all her energy is going to the production of milk, and that other processes like reproduction and immunity, get a lower priority. Because fertility, just as milk production, is also an economically important trait, fertility should be considered as part of a dairy cattle breeding program.

## STRATEGIES TO IMPROVE DAIRY TRAITS

There are several ways of selection for traits like milk yield and fertility. First there is conventional selection. Sires in dairy cattle are mainly selected on estimated breeding values from their daughters' and their relatives' information. One of the disadvantages of this selection method is the long time before daughters of potential sires produce and lactation information becomes available. A further disadvantage is the relative low heritability of fertility traits, mostly between 0.02 and 0.10, which means that a large number of daughters is required for accurate selection for fertility. Second there is selection on using polymorphisms in specific genes or chromosome segments. When traits are controlled differentially by different alleles, then this allele-information can be used in genetic selection. Meuwissen and Goddard (1996) showed that selection can be improved by integrating genetic information with information from phenotypic measurements.

To incorporate DNA information in breeding programs genomic regions have to be identified in which polymorphisms exist that are associated with the traits of interest. Two different approaches are being used to identify these genomic regions (Ovilo et al., 2002).

The first approach is the identification of QTLs (Quantitative Trait Loci) by using DNA markers in genome or chromosome scans (QTL approach). Several genome and chromosome scans have been reported in cattle (Georges et al., 1995; Heyen et al., 1999; Lindersson et al.,

1998; Schrooten et al., 2000). Most of these studies identified markers for traits of interest, but the gene(s) responsible for the effect is often not yet known. In the vicinity of a marker often a large number of genes can be located and only in a few studies using positional cloning techniques the causal gene could be identified (Blott et al., 2003; Grisart et al., 2002).

The second approach is the detection of mutations in candidate genes and an association study with these mutations and economic traits (candidate gene approach). A candidate gene is a gene, which due to its biological function might be responsible for the phenotypic occurrence of a certain characteristic. Compared to the QTL approach, the candidate gene approach is working from the other way, the function of the gene is known and it is investigated whether polymorphisms in this gene affect dairy traits. However, there are often many candidate genes for the trait of interest and it might be more time-consuming to evaluate all of those than it is to do a genome scan (Andersson, 2001). Furthermore, candidate gene tests must also be interpreted with caution because spurious results can occur due to linkage disequilibrium to the actual causative gene or due to gene frequencies being higher in certain subsets of the data (Ovilo et al., 2002). Several studies using the candidate gene approach in cows have been published. For example, the major histocompatibility complex in cattle was found to be associated with susceptibility to clinical mastitis and production traits (Lunden et al., 1990; Sharif et al., 1999; Weigel et al., 1990). Both via the QTL approach (genome scan and fine mapping) and the candidate gene approach (knock-out mice), the myostatin gene was found to have five independent loss-of-function mutations that are associated with double-muscling in cattle (Grobet et al., 1997; Grobet et al., 1998; Kambadur et al., 1997; McPherron et al., 1997; McPherron and Lee, 1997).

## LEPTIN

It can be hypothesized that hormones play an important role in the reduction of fertility following selection for milk yield. One of these hormones might be leptin, a 16 kDa protein that is synthesized by adipose tissue. Leptin is involved in regulation of feed intake, energy balance, fertility, and immune functions (Fruhbeck et al., 1998). Hence current selection might have affected leptin levels and/or indirectly have favored a leptin polymorphism that negatively influences fertility. In rodents and human genetic differences in the leptin gene exist. For example *ob/ob* mice lack functional leptin and are hyperphagic, obese, and infertile (Hamann and Matthaei, 1996). If polymorphisms exist in the leptin gene of dairy cattle, and these are associated with energy balance, fertility, feed intake and/or milk yield, these associations will provide insight into the underlying mechanisms of leptin and the polymorphisms might be used for selection in dairy cows.

## AIM OF THIS THESIS

The aim of this thesis is to obtain a better understanding of the role of leptin in the periparturient (i.e. period around parturition) cow on physiologic and genetic level and to investigate opportunities to use information of the leptin and leptin receptor gene to improve selection for production and reproduction traits in dairy cows.

The first objective was to provide insight into the function of leptin during the periparturient period. Leptin levels were described during the periparturient period in dairy cows and related to differences in fertility and production traits.

The second objective of this study was to detect polymorphisms in the bovine leptin gene and the leptin receptor gene. For this the leptin gene including its promoter region and parts of the leptin receptor gene were sequenced to find polymorphisms, but also to detect putative transcription factor binding sites on the leptin promoter region which might influence leptin expression.

The third objective was to obtain more insight in the role of the leptin and leptin receptor gene in dairy traits and to indicate whether these genes might be useful for selection. For this an association study was performed of all genotyped polymorphisms with fertility and production traits measured in dairy cows during the periparturient period. Also differences in leptin levels between genotypes of all found polymorphisms were analyzed.

## OUTLINE OF THIS THESIS

In Chapter 2 a literature survey regarding leptin and its receptor and their role in metabolic processes and fertility is given. This Chapter also includes recent literature, which was published during the investigations that are described in the other Chapters of this thesis. Chapter 3 describes the association study performed with polymorphisms on the leptin gene. Chapter 4 describes how leptin concentrations behave during the periparturient period in cows and what their relation is with fertility and production traits. Chapter 5 describes the association of leptin polymorphisms with leptin concentrations and Chapter 6 describes one found polymorphism in the leptin receptor gene and its relation with leptin concentrations during late pregnancy. Chapter 7 describes the relation of polymorphisms in the leptin promoter region with fertility, production traits and leptin concentrations. Finally, the findings in this thesis are discussed and put into broader perspective in Chapter 8.

# CHAPTER 2

# Literature Survey

## INTRODUCTION

This chapter will give an overview of the literature about the knowledge that has been achieved after the discovery of leptin (1953) and its receptor (1966) and their genes in 1995 and 1996. First, the position and arrangement of the leptin gene and its receptor on the bovine genome will be discussed. Thereafter the diverse functions of leptin that have been investigated are elucidated. This information is divided into four sections; first the regulatory function of leptin through the hypothalamus will be discussed, then leptin's role in nutritional status and its role in reproductive function. The last section discusses plasma leptin concentrations and its secretory characteristics.

## DISCOVERY OF A GENE RESPONSIBLE FOR FEED INTAKE AND FERTILITY

In the summer of 1949 some very fat young mice were found in the breeding stock of Jackson Laboratory (Bar Harbor, Maine, USA). The fatness was associated with excessive feed intake. These obese, infertile animals increased rapidly in weight until they were about four times the weight of normal animals (Ingalls et al., 1950). Breeding data revealed that this phenomena had to be the result of a recessive mutation and the gene responsible was called the obese gene (*ob*).

In 1953, Kennedy postulated the 'lipostasis theory': the amount of body fat and feeding is regulated by the central nervous system through a blood born product which signals through the hypothalamus by a negative feedback system (Kennedy, 1953). Hervey (1959) finally provided evidence for such a product and the role of the hypothalamus. He surgically linked normal rats, allowing partial exchange of blood between them (parabiosis), and electrically lesioned the hypothalamus of one member of each pair. The lesioned animals became hyperphagic and obese, while their partners developed anorexia and died of starvation. Hervey (1959) reasoned that, with increasing adiposity, a humoral factor accumulates and serves to inhibit food intake through a feedback mechanism within the hypothalamus. The factor would be ineffective in the lesioned rats, but by passing into the circulation of the non-lesioned parabionts their food intake would be suppressed.

## DISCOVERY OF THE LEPTIN GENE

The *ob* gene was discovered in 1994 by positional cloning techniques (Zhang et al., 1994). The 167 amino acid protein product of the *ob* gene was named leptin (derived from the Greek term 'leptos' meaning 'thin'). The leptin gene consists of three exons, of which the

first exon is not transcribed into the leptin protein of 16 kDa. In mice, the first intron is more than 8 kb long and the second intron has a length of 1.6 kb (He et al., 1995; De la Brousse, et al., 1996) (Figure 1). Leptin has 67% sequence identity among species as human, gorilla, chimpanzee, orangutan, rhesus monkey, dog, cow, pig, rat and mouse (Zhang et al., 1997). The human leptin gene is located at HSA 7q31.3, the mouse leptin gene on MMU 6A3.3 (www.ncbi.nlm.nih.gov) and the bovine leptin gene maps to BTA 4q32 (Pfister-Genskow et al., 1996).

To date, several important transcription factor binding domains have been identified in the promoter region of the leptin gene in humans, rodents and ruminants. These include domains for CAAT/enhancer binding proteins (C/EBP) (Hwang et al., 1996; Miller et al., 1996; Taniguchi et al., 2002), adipocyte determination differentiation dependent factor 1/sterol regulatory element binding protein 1 (ADD1/SREBP1) (Kim et al., 1998), peroxisome proliferator activated receptor γ (PPARγ) (Hollenberg et al., 1997), hypoxia-inducible factor 1 (HIF-1) (Grosfeld et al., 2002; Meissner et al., 2003), and Sp1 and LP1 (Mason et al., 1998). C/EBP is a transcription factor important for the transcription of most genes expressed in adipose tissue and for other genes involved in energy metabolism (Darlington et al., 1995). Several groups (Hwang et al., 1996; Mason et al., 1998) mutated the C/EBP site of the leptin promoter in rat and mouse and demonstrated that this site was functional in regulating leptin gene expression. Taniguchi et al. (2002) showed in a transfection study that C/EBP activates the promoter of the bovine leptin gene.

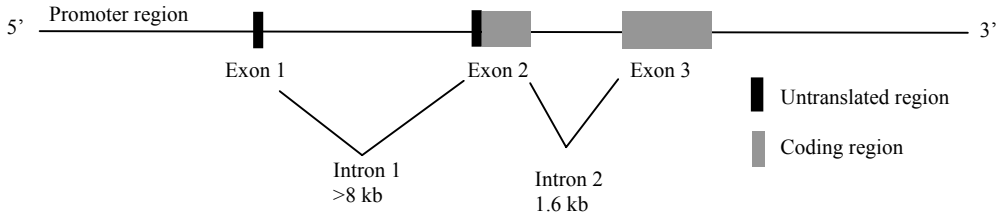


**Figure 1.** *Molecular organization of the leptin gene.*

Nuclear magnetic resonance analysis of a crystalline form of leptin revealed that leptin is a four-helix protein (A-B-C-D) which is similar to the structure of the cytokine-family (Zhang et al., 1997). Leptin contains a single disulfide bond that links two cysteines within the C and D helices and this bond has been proven critical for the structural integrity and stability of leptin (Rock et al., 1996) (Figure 2). Leptin binds to its receptor at the interface of α-helices A and C (Hiroike et al., 2000).

The first 21 amino acids of leptin function as a signal peptide and are cleaved off before the 146 amino acid protein is released into the blood as a circulating protein. In *ob/ob* mice a

cytosine to thymidine change at codon 105 changes the amino acid arginine into a stop codon that causes premature termination of transcription of the gene. This results in the synthesis of a truncated non-functional protein. Mutations in the leptin gene have also been identified in obese humans. Until now, only two obese families have been discovered; a Turkish family, having a missense mutation at codon 105 resulting in an arginine to tryptophan replacement (Strobel et al., 1998; Ozata et al., 1999) and a Pakistani family with a single nucleotide deletion at codon 133, resulting in a frameshift mutation (Montague et al., 1997). Both mutations are rare and therefore probably not responsible for the majority of obese humans.

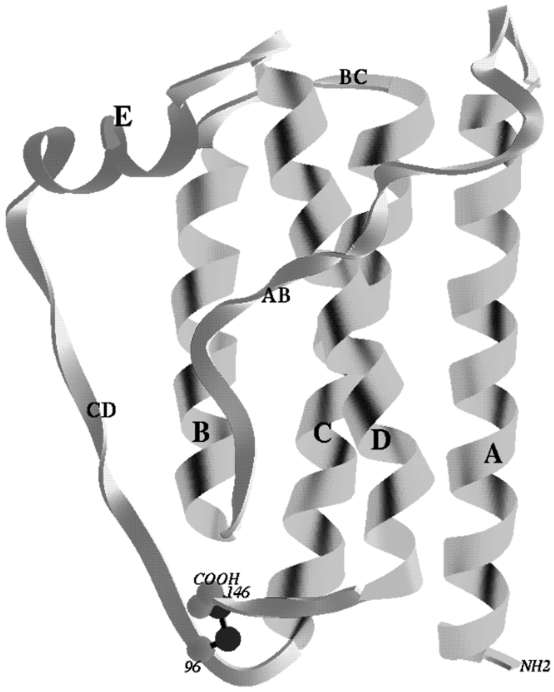


**Figure 2.** *The leptin molecule consists of 146 amino acids which form a four-helix bundle (A-B-C-D). This is consistent with a classification as a cytokine four-helix bundle. Its molecular weight is around 16 kDa and it contains one disulphide bond. Leptin has 67% sequence identity among diverse species. Two cysteines located at amino acids 96 and 146 form a disulphide bridge essential for the stability of the leptin molecule. (reprinted with permission from: Zhang et al., 1997)*

## THE LEPTIN RECEPTOR

In 1966, Hummel et al. discovered a mouse strain with the same characteristics as seen in the *ob/ob* mice, but with more severe symptoms. These mice also developed diabetes and therefore the gene responsible was called *db*. Five years before, a rat strain with the same characteristics was reported, the so-called Zucker rats (Zucker and Zucker, 1961). The parabiotic-mice trial of Coleman (1973) with the *ob/ob* and *db/db* mouse strains tested the effect of a shared blood circulation between fat and lean animals in various combinations. He inferred that the *ob/ob* mouse must lack the circulating satiety factor, while the *db/db* strain fails to respond to it.

In 1995, a year after the discovery of the leptin gene, the leptin receptor gene (Ob-R) was isolated (Tartaglia et al., 1995). The human leptin receptor gene is located at HSA 1p31 and the mouse leptin receptor gene on MMU 4C6. The gene consists of 20 exons (4 kb) divided over a region of approximately 170 kb (human) (www.ncbi.nlm.nih.gov). In bovine the leptin receptor gene maps to BTA 3q33 (Pfister-Genskow et al., 1997).

The leptin receptor is a glycoprotein with a single transmembrane-spanning region. A total of six leptin receptor isoforms in different tissues were reported, which derive from a single gene by alternative splicing. Intracellular domains of variable length characterize these isoforms, that share identical extracellular and transmembrane domains (Tartaglia, 1997) (Table 1, Figure 3).

**Table 1.** *Six isoforms of leptin receptor with the tissues where they have been found predominantly and their function*

| | Tissue | Function |
|---|---|---|
| Ob-Ra | Brain, Adipose Tissue, Placenta | Transport leptin across BBB* and placenta |
| Ob-Rb | Hypothalamus, Placenta | Signal Transduction |
| Ob-Rc | Brain, Adipose Tissue | Transport leptin across BBB* |
| Ob-Rd | Adipose Tissue | Remains to be determined |
| Ob-Re | Heart, Adipose Tissue, Placenta | Binding protein for leptin, transport to fetus |
| Ob-Rf | Low levels in brain | Remains to be determined |

**BBB* = blood brain barrrier.

Ob-Rb is the longest form and is predominantly expressed in the hypothalamus and therefore is thought to play an important role in signal transduction (Vaisse et al., 1996).
Ob-Ra is present at the blood brain barrier (BBB) and choroid plexus. It has been shown that leptin enters the brain by a specific mechanism, independent of insulin (Banks et al., 1996), and it seems that Ob-Ra plays a modulating role in transporting leptin across the BBB (Hileman et al., 2000; Banks et al., 2002). Ob-Rc was found in brain tissues taken from cortex and cerebellum. This indicates that Ob-Rc might also function as a leptin transporter

in the brain, with a different function as or additional to Ob-Ra (Hileman et al., 2002). Ob-Re is a glycosylated circulating form of Ob-R and thought to serve as a binding protein for leptin (Gavrilova et al., 1997). A change in Ob-Re level regulates in part the biological activity of leptin in the circulation (Kado et al., 2003). The functions of Ob-Rd and Ob-Rf remain to be determined (Table 1).

The leptin receptor is a member of the class I cytokine receptor superfamily and acts via the JAK/STAT (Janus Kinases/Signal Transducers and Activators of Transcription) signal transduction pathway as follows: Ob-R is dimerized and leptin binds to this dimerized Ob-R molecule. At the intracellular region JAK proteins phosphorylate two tyrosine residues (Li et al., 1999), which then provide a binding site for a STAT protein. The then activated STAT protein dimerizes and translocates to the nucleus where it binds DNA and activates transcription (Vaisse et al., 1996).

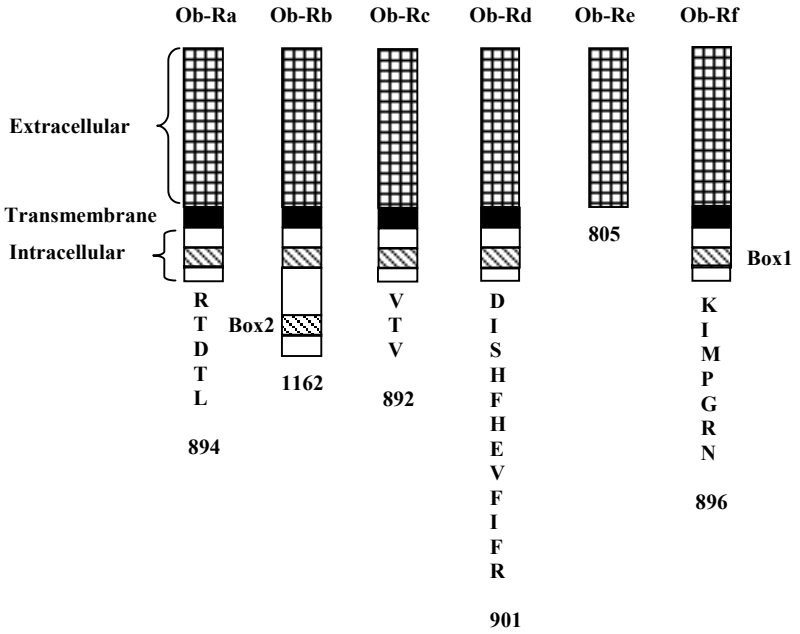


**Figure 3.** *Structures of leptin receptor isoforms, which share a common extracellular leptin-binding domain, but differ at the intracellular domain. Boxes 1 and 2 are intracellular domains that are involved in the JAK/STAT signal transduction, which leads to transcription activation (White et al. 1997). Both boxes are only present in Ob-Rb. The other, short isoforms are between 892 and 901 amino acids long and Ob-Re, the shortest, soluble form of the leptin receptor is 805 amino acids long. (reprinted, with permission, from the Annual Review of Physiology, Volume 62 © 2000 by Annual Reviews, www.annualreviews.org)*

Sequences of the human, mouse, rat and pig leptin receptor gene are known but only partial sequences of sheep and cow leptin receptor have been reported (Dyer et al., 1997; Pfister-Genskow et al., 1997). Sequence identity of human, pig, sheep (partially known) and cow (partially known) vary between the extracellular domain (82%), transmembrane domain (86-90%) and cytoplasmic domain (72-78%). Sequences of rat and mouse show 45% identity with the known domains of human, pig, sheep and cow. However, the leptin binding sequence at the extracellular region shows 90-93% sequence identity between human, pig, sheep, mouse and rat (Ruiz-Cortes et al., 2000).

In obese strains of mice and rats several mutations in the leptin receptor gene were reported. *Db/db* mice are missing the cytoplasmic region of Ob-Rb due to a single point mutation (Chen et al., 1996) that alters the normal splicing pattern (Lee et al., 1996), which also results in a diminished STAT signalling of the leptin receptor (Vaisse et al., 1996). The *fa/fa* Zucker rat has a glutamine to proline mutation in the leptin receptor gene (Phillips et al., 1996) which appears to induce both a reduced affinity for leptin and reduced signal transduction capability (Yamashita et al., 1997; Silva et al., 2002). In humans several polymorphisms were found in the leptin receptor gene, but most of these were not associated with obesity or diabetes (Considine et al., 1996; Echwald et al., 1997; Gotoda et al., 1997). However, Clement et al. (1998) did find a leptin receptor polymorphism on exon 16 which results in a truncated leptin receptor lacking both the transmembrane and the intracellular domains and this polymorphism was associated with obesity and hypophysis dysfunction.

## LEPTIN AND THE HYPOTHALAMUS-HYPOPHYSIS AXIS

Within the central nervous system, the hypothalamus is the main site of leptin action with respect to controlling food intake and energy expenditure. Numerous studies have evaluated the localization of leptin receptor messenger RNA (mRNA) within the hypothalamus of several species including humans (Considine et al., 1996), rodents (Brogan et al., 2000; Garcia et al., 2000; Seeber et al., 2002) and ruminants (Dyer et al., 1997; Ren et al., 2002).

The hypothalamus transduces leptin signals into neural responses, which cause alterations in food intake (Tang-Christensen et al., 1999). Figure 4 presents several pathways of leptin. Neuropeptide Y (NPY) seems to be important for regulation of food intake. Leptin inhibits the signaling of NPY and thus inhibits food intake (Kotz et al., 1998; Jang et al., 2000). Furthermore, mice deficient (*ob/ob*) or unresponsive (*db/db*) to leptin, are characterized by increased levels of NPY mRNA. Direct administration of leptin reversed these changes in *ob/ob* mice (Ahima et al., 2000). Leptin administration also stimulated the production of the gonadotrophins LH and FSH (lutenizing hormone and follicle stimulating hormone) from the hypophysis via GnRH-neurons (gonadotrophin-releasing hormone)

neurons in the hypothalamus (Woller et al., 2001; Watanobe, 2002; Amstalden et al., 2003). Also direct hypophysis effects of leptin on secretion of FSH and LH may exist since full-length Ob-R mRNA was present in anterior hypophysis of sheep (Dyer et al., 1997), pigs (Lin et al., 2000), mice and rats (Jin et al., 2000). Leptin also directly affected basal and GHRH-mediated (growth hormone-releasing hormone) GH (growth hormone) secretion from the hypophysis (McMahon et al., 2001; Zieba et al., 2003a).

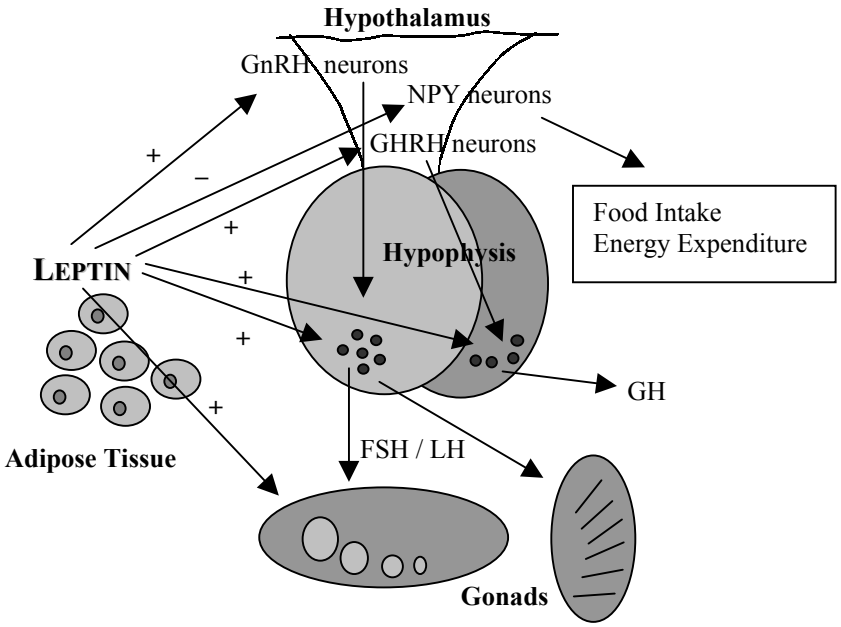


**Figure 4.** *Pathways of leptin. Leptin enters the blood brain barrier and binds to GnRH, NPY and GHRH neurons. GnRH stimulates (+) FSH and LH production in the posterior hypophysis. Leptin inhibits (-) the release of NPY, resulting in decreased food intake and increased energy expenditure. Furthermore, leptin stimulates the basal and GHRH-induced GH release from the anterior hypophysis and also acts directly on the hypophysis and stimulates the release of FSH and LH. Leptin has a local effect on the ovary by stimulating steroidogenesis through leptin receptors on granulosa cells.*

## ROLE OF LEPTIN IN NUTRITIONAL STATUS

### Leptin, Food Intake and Body Weight

Injections of leptin caused a rapid decrease in food intake and body weight in *ob/ob* mice (Campfield et al., 1995), wild-type mice (Halaas et al., 1995), monkeys (Tang-Christensen et al., 1999) and pigs (Barb et al., 1998). When *ob/ob* mice were pair-fed with leptin-treated *ob/ob* animals and thus received the same amount of food, they lost 30% less weight than the leptin treated *ob/ob* mice. This data suggested that besides its effect on food intake via hypothalamic NPY neurons, leptin could also play an important role in regulating fat mobilization (Halaas et al., 1995).

The satiety effects of leptin were also observed in ruminants by administration of recombinant human leptin in ewes for 3 days. This treatment caused a decrease in voluntary dry matter intake to approximately a third of the normal intake (Henry et al., 1999). However, these effects were lost when sheep were underfed and leptin was administered (Henry et al., 2001; Morrison et al., 2001) . This indicates that another signal blocks the effect of leptin on feed intake when the body is in a negative energy balance.

Acute or long-term changes in food composition or food restriction caused changes in plasma leptin in ruminants. In pregnant ewes and adult rams, the concentration of plasma leptin increased within 48 hours respectively 5 days after increasing the dietary intake from low to high (Blache et al., 2000; Thomas et al., 2001). Complete food deprivation caused a rapid fall in plasma leptin (Marie et al., 2001) and long-term food restriction decreased plasma leptin concentration in sheep (Delavaud et al., 2000; Ehrhardt et al., 2000; Morrison et al., 2001). Acute changes in plasma leptin were the result of changes in leptin mRNA expression in adipose tissue. Food deprivation reduced the level of adipose tissue leptin mRNA in cows (Amstalden et al., 2000).

### Leptin and Adiposity

There are two definitions of adiposity, the state of being fat (body fatness) or the excessive accumulation of lipids in a site or organ (obesity). In this thesis the first definition is used for the term adiposity. In rodents and humans plasma concentrations of leptin are positively correlated with adiposity (Maffei et al., 1995; Bunger et al., 1999). Similar relationships have been observed between body fatness and leptin in growing and lactating ruminants (Blache et al., 2000; Chilliard et al., 2000; Thomas et al., 2001). A linear relationship was demonstrated in well-fed late lactating dairy cows between plasma leptin and body condition score (BCS) (Ehrhardt et al., 2000). However, other studies in ruminants showed that plasma leptin is more related to the size of adipocytes (fat cells) than to body condition score (BCS) (Delavaud et al., 2002).

**Leptin and Insulin**

Insulin is an important regulator of energy homeostasis. It stimulates glucose, free fatty acid and amino acid uptake by tissues. Therefore it is not surprising that circulating plasma insulin concentrations were positively correlated with leptin mRNA levels in adipose tissue and with circulating plasma leptin concentrations (Maffei et al., 1995). Leroy et al. (1996) showed *in vitro* that adipose tissue cells expressed leptin mRNA and secreted leptin protein after administrating insulin to these cells. Kieffer et al. (1996) reported that leptin receptors were expressed in insulin producing beta-cells within the pancreatic islets, suggesting that leptin might influence insulin secretion through a direct action on these cells. However, results of further studies were conflicting and therefore no clear evidence for this hypothesis exists. Tanizawa et al. (1997) reported stimulation by leptin of insulin secretion whereas others found an inhibition (Kieffer et al., 1997; Poitout et al., 1998) . Some studies did not find any influence of leptin on pancreatic cells (Leclercq-Meyer et al., 1996; Leclercq-Meyer and Malaisse, 1998).

## ROLE OF LEPTIN IN REPRODUCTIVE FUNCTION

**Leptin and Fertility**

Injections of leptin in *ob/ob* mice, which are infertile, increased serum LH concentrations, ovarian weight in females and elevated serum levels of FSH, increased testicular weights, and elevated sperm counts in males (Barash et al., 1996). Furthermore, repeated administration of leptin to female *ob/ob* mice results in ovulation, pregnancy and parturition after insemination (Chehab et al., 1996). Consistent with this study, leptin infusion in the fasted male rhesus monkey, increased mean plasma levels of LH and FSH, LH pulse frequency and LH pulse amplitude (Finn et al., 1998). Leptin administration stimulated GnRH producing neurons in the hypothalamus, but also directly stimulated the hypophysis to produce LH and FSH (Figure 4).

In ruminants, recombinant ovine leptin administration to fasted mature beef cows stimulated LH secretion (Amstalden et al., 2002), and in fasted ovariectomized dairy cows leptin affected LH secretion in a dose-dependent manner; the highest concentration leptin (20 ng/ml) did not affect LH secretion, whereas 0.2 and 2.0 ng/ml leptin stimulated LH secretion 141% and 122% respectively (Zieba et al., 2003b). However, in ovariectomized food-restricted ewes, and in well-fed and undernourished ewe lambs, intracerebroventricular infusions of recombinant ovine leptin did not affect plasma concentrations of LH or FSH, LH pulse frequency or amplitude (Henry et al., 1999; Morrison et al., 2001). Furthermore, Morrison et al. (2002) showed that intravenous administration of leptin did not affect LH secretion in growing prepubertal ewe lambs. Thus the specific effect of leptin on gonadotropin secretion may depend on age (prepubertal/mature), species (cow/sheep),

concentration of administered leptin, and whether or not the animals were operated (ovariectomized/not-ovariectomized).

**Leptin and the Onset of Puberty**

It has been suggested that leptin is a regulator of puberty onset in mice (Ahima et al., 1997; Chehab et al., 1997), rats (Gruaz et al., 1998) and humans (Matkovic et al., 1997). In humans, leptin concentrations increased during puberty and only in males leptin concentrations decreased after puberty due to testosterone inhibition of leptin secretion (Horlick et al., 2000). This also probably explains the gender differences in leptin secretion (Havel et al., 1996; Rosenbaum et al., 1996). Leptin administration advanced the pubertal onset in mice (Ahima et al., 1997; Chehab et al., 1997) and rats (Gruaz et al., 1998).

Leptin concentrations in dairy cattle increased linearly from 16 weeks before until the week of pubertal ovulation in yearling heifers (Garcia et al., 2002) and was correlated with body weight (Diaz-Torga et al., 2001). Block et al. (2003a) did not find this increase and suggested that leptin might be a permissive factor for the onset of pubertal ovulation when metabolic resources are adequate, but that the secretion of leptin alone is not sufficient to initiate puberty.

**Leptin and Pregnancy**

From early to mid pregnancy circulating leptin levels increased and remained elevated until late pregnancy in humans (Mukherjea et al., 1999), rat (Amico et al., 1998; Garcia et al., 2000), mice (Tomimatsu et al., 1997) and sheep (Ehrhardt et al., 2001; Forhead et al., 2002). These elevations were both due to an increase in adiposity as an increase in leptin mRNA expression in adipose tissue (Ehrhardt et al., 2001). The increase in leptin concentration during pregnancy seems to be paradoxical as this is a period of increased nutritional demands, thus not a period in which the actions of leptin are expected to increase. However, as the concentration of circulating Ob-Re is increased during pregnancy, most leptin is present in the bound, not active form. This might induce a state of leptin resistance, or a change in leptin bioavailability (Mounzih et al., 1998; Seeber et al., 2002). In cows no study has been performed yet to measure leptin levels during early and mid pregnancy, but leptin levels are high during late pregnancy (Block et al., 2001).

**Placental Leptin**

Placental production of leptin was measured in rats (Kawai et al., 1997), humans (Masuzaki et al., 1997), baboons (Henson and Castracane, 2000) and bats (Zhao et al., 2003). However, in mice no placental production was measured (Gavrilova et al., 1997; Tomimatsu et al., 1997; Kronfeld-Schor et al., 2001; Zhao et al., 2003), with the exception of one study (Hoggard et al., 1997). Also the ruminant placenta contained negligible levels of leptin mRNA throughout gestation (Bispham et al., 2003). It seems that placental leptin is produced

species-specific, even within the same order of mammals. Furthermore, it seems that expression of leptin in the human placenta is regulated in a different way from that in adipose tissue (Bi et al., 1997) suggesting that a different regulatory site on the promoter of leptin is responsible for placental leptin production.

Placental leptin receptor mRNA was present in humans (Bodner et al., 1999), mice (Hoggard et al., 1997; Yamaguchi et al., 1998), rats (Amico et al., 1998; Garcia et al., 2000) and sheep (Thomas et al., 2001) and their levels increased during pregnancy. Probably the placental leptin receptor transports maternal leptin to the fetus (Smith and Waddell, 2003) and this may provide a promoting signal for placental function and fetal development (Schulz et al., 2000).

**Leptin and Ovarian Function**

Several lines of evidence indicated that leptin has direct action at the level of the ovary. Bovine ovarian granulosa (Spicer and Francisco, 1997) and thecal cells (Spicer and Francisco, 1998) had leptin receptors and leptin modulated steroidogenesis of porcine granulosa cells in a dose-dependent manner (Ruiz-Cortes et al., 2003). Furthermore, a high dose of *in vivo* leptin treatment (30 μg at 3 hourly intervals for 15h) decreased ovulation rate in rats, but did not influence levels of LH and FSH (Duggal et al., 2000). Moreover, in women, the secretion of leptin exhibited a monthly cycle (Hardie et al., 1997) and maintenance of normal ovulatory cycles required a specific serum leptin level (Laughlin et al., 1997). Leptin is expressed at the ovary in rat (Ryan et al., 2003) and human (Loffler et al., 2001) and leptin receptor mRNA has been identified in the ovary of human (Cioffi et al., 1997), rat (Zamorano et al., 1997), and pig (Lin et al., 2000; Ruiz-Cortes et al., 2000).

**Leptin and Lactation**

During pregnancy leptin levels are high and they decline rapidly towards parturition. Eliminating the energetic costs of lactation by preventing milk delivery in rats and cows caused an increase in plasma leptin levels together with an increase in energy balance (Woodside et al., 2000; Block et al., 2001). This indicates that the fall in circulating leptin levels towards and during lactation is due to the energetic costs of milk production. The suckling stimulus itself did not appear to influence the decrease in leptin concentration (Brogan et al., 1999). Pickavance et al. (1998) observed that the food intake-induced leptin increase was eliminated during lactation and they speculated that the hypoleptinemia may be an important factor promoting the hyperphagia of lactation. Overall, these data demonstrated that the onset of the negative energy balance is largely responsible for the declining leptin concentrations towards parturition and that the low leptin levels during lactation probably induce the hyperphagia of lactation.

**Leptin and the Mammary Gland**

In mammary tissue in ruminants, leptin mRNA was present both *in vitro* and *in vivo* (Smith and Sheffield, 2002). Both long and short forms of the leptin receptor were expressed in the ovine mammary gland during pregnancy and lactation. The levels of both forms are high in sheep during early- and mid-pregnancy when active growth of the mammary gland is initiated, and lower during late pregnancy and lactation (Bonnet et al., 2002). Thus, leptin and its receptor could be an important mediator in regulating mammary gland growth and development (Maffei et al., 1995; Laud et al., 1999).

## PLASMA LEPTIN CONCENTRATIONS

Plasma concentration of leptin is affected by the presence of specific binding proteins, variation in nutrition and adiposity, and by changes in physiological stages like pregnancy and lactation. In rodents and humans, leptin circulated in both free and bound form (Houseknecht et al., 1996; Sinha et al., 1996). The soluble isoform of the leptin receptor (Ob-Re) accounted for a major fraction of the leptin-binding activity present in plasma. In rats, 88% of circulating leptin was present in the bound form (Hill et al., 1998), whereas only 24% of bound leptin was reported for humans (Sinha et al., 1996; Diamond et al., 1997). The fraction of bound leptin was greater in lean than in obese individuals (Houseknecht et al., 1996; Diamond et al., 1997) and in humans a relation of gender with free and bound leptin was found (McConway et al., 2000). As leptin was secreted in a pulsatile manner (Sinha et al., 1996), a hypothesis for the function of leptin binding proteins is to flatten the leptin pulsatility in serum, but no studies have been performed yet to investigate this.

Kinetic studies in the rat indicated that free leptin has a size of 16 kDa and a half-life of 3.4 min, whereas bound leptin had a size of 66 kDa with a half-life of 71 min. This indicated that bound leptin was protected from proteolytic degradation (Hill et al., 1998). In humans, the half-life of plasma leptin (bound and free together) was estimated to be 25 min (Klein et al., 1996). Until now, the half-life of bound and free plasma leptin have not yet been determined in any farm animal species.

**Secretory Characteristics**

The pulsatile secretion of leptin had a diurnal rhythm in rats and humans (Sinha et al., 1996; Pickavance et al., 1998). The leptin concentration was highest at night and lowest at noon, which in humans was linked to the time of feeding (Schoeller et al., 1997). Rats however consumed most of their food at night, and thus leptin levels were high during the time of feeding. Recently it was found that leptin levels in the brain, but not in plasma, were linked to the time of feeding in rats (Asakuma et al., 2003), which might direct to differences in leptin transport across the blood brain barrier. Additional factors like obesity, gender, diet

and endocrine factors possibly affected this rhythm (Langendonk et al., 1998; Licinio et al., 1998; Saad et al., 1998; Ahren, 2000). During the hyperphagia of lactation in the rat, this rhythm was completely eliminated (Pickavance et al., 1998). In ruminants, plasma leptin appeared to be secreted in a pulsatile manner, but diurnal changes in leptin concentration in relation to the light:dark cycle were not present (Blache et al., 2000; Tokuda et al., 2000; Block et al., 2001). However, plasma leptin was decreased when exposing sheep to a short day-length for 3 weeks (Bocquier et al., 1998). In long term studies, the effect of photo-period on leptin was suggested to be secondary to changes in live weight and adiposity rather than a direct effect of photo-period (Marie et al., 2001).

## SUMMARY

Since the discovery of leptin in 1995 a lot of research has been done to reveal its function. Studies in humans and rodents have elucidated many functions of leptin. Also the genes of leptin and leptin receptor were object of investigation and several associations with found polymorphisms have been described in these species. However, at the start of this study the knowledge of leptin in ruminants was poor. Therefore the objective of this thesis was to get more insight in the physiology and genetics of leptin in periparturient dairy cows.

# CHAPTER 3

# Associations between Leptin Gene Polymorphisms and Production, Live Weight, Energy Balance, Feed Intake and Fertility in Holstein Heifers

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## ABSTRACT

Leptin is a 16 kDa protein synthesized by adipose tissue and is involved in regulation of feed intake, energy balance, fertility and immune functions. Since evidence of a genetic correlation between start of luteal activity and energy balance, milk yield and live weight is present, we investigated the association of genetic differences in the bovine leptin gene with these traits. Between 1990 and 1997, a total of 613 Holstein-Friesian heifers of two genetic groups with known pedigree were followed from parturition until 105 days of lactation. During the first 15 weeks of lactation live weight, feed intake, and milk yield were measured for 565 cows. The start of luteal activity was set at the first day with a milk-progesterone concentration higher than 3 ng/ml. In addition to the interval between calving and start of luteal activity, analyses were performed for average milk, fat, protein and lactose yield, percentage fat, protein, and lactose in milk, dry matter intake, feed intake, energy balance, and live weight over the first 15 wk of lactation. All 613 cows were genotyped for two restricted fragment length polymorphisms and for the BM1500 microsatellite, all located at the leptin gene locus. Significances of the genotype effects were estimated using the approximated F-statistic provided by ASREML. Fixed effects were year-season, genetic group, and a quadratic polynomial for age at calving. Animal was fitted as a random effect including the additive relationship between animals to account for background genes. Firstly, each genotype effect was fitted in turn, and secondly the other restriction fragment length polymorphisms were fitted as a cofactor to take into account effects of linkage disequilibrium. Thirdly, sire x genotype interaction was investigated. Heifers with the RFLP1 *AB*-genotype produce 1.32 kg/d more milk and consume 0.73 kg/d more food compared with the RFLP1 *AA*-genotype. No effects were found for start of luteal activity. When linkage disequilibrium with the other markers was taken into account and dry matter intake was included as fixed effect in the model a 0.96 kg/d higher milk yield was still found. Assuming that no pleiotropic effects on traits such as immunity and milk production in later lactations exist, future breeding programs favoring the RFLP1 *B*-allele can yield a higher milk production without negatively affecting energy balance and fertility. The prospects are good because in this study the frequency of the RFLP1 *AB*- and *BB*-genotypes were only 18.5% and 0.2% respectively.

## INTRODUCTION

Leptin is a 16 kDa protein that is synthesized by adipose tissue and is involved in regulation of feed intake, energy balance, fertility, and immune functions (Fruhbeck et al., 1998). Leptin binds to a receptor mainly localized on neuropeptide Y neurons, which results in a reduction of feed intake and an increase of energy expenditure. Neuropeptide Y is also involved in the control of reproductive function (Magni et al., 2000).

Genetic differences in the leptin gene were first observed in mice; *ob/ob* mice lack functional leptin and are hyperphagic, obese, and infertile (Hamann and Matthaei, 1996). When leptin is administered, fertility is restored and body fat mass is reduced (Halaas et al., 1995). The sterility of *ob/ob* female mice is caused by an insufficiency of hormones at the hypothalamic-hypophysis level rather than physical hindrance of excess adipose tissue (Chehab et al., 1996).

Polymorphisms in the human leptin gene were associated with low circulating leptin levels (Hager et al., 1998), birthweight (Orbak et al., 2001) and obesity (Ohshiro et al., 2000). Four polymorphisms in the porcine leptin gene were associated with fatness (Jiang and Gibson, 1999). Although polymorphisms in the bovine leptin gene have been described (Pomp et al., 1997; Fitzsimmons et al., 1998; Haegeman et al., 2000) and an association with fat deposition in beef cattle was reported (Fitzsimmons et al., 1998) no association studies have been reported yet in dairy cows. Lindersson et al. (1998) reported QTLs for milk production traits close to the leptin gene (82.8 cM). They found QTLs for milk, fat, and protein yield at 65 and 85 cM, and for fat and protein percentage at 75 and 95 cM respectively. However, when these authors tested for a direct effect of the obese locus with BM1500 and BM1501 microsatellite, no associations were found with milk production traits.

Selection for milk production has a negative influence on the fertility of dairy cows (Pryce et al., 2000). Dairy cows have a slight to severe negative energy balance during early lactation, which influences the duration of the postpartum anestrus period (Jolly et al., 1995). Since evidence suggests that a genetic correlation between start of luteal activity and energy balance, milk yield and live weight exist (Veerkamp et al., 2000), in which only phenotypic records were analyzed, it could be hypothesized that polymorphisms at the leptin gene locus might play a role. If associations between leptin polymorphisms and milk yield, live weight, feed intake, or fertility exist, these associations will provide insight into the underlying mechanisms of leptin, and results may be used in future breeding programs.

The aim of this study is to relate polymorphisms at the bovine leptin gene locus with variation in energy balance, milk production, feed intake, live weight, and fertility traits.

## MATERIALS AND METHODS

### Animals and Traits

Between 1990 and 1997, a total of 613 Holstein-Friesian heifers with known pedigree were followed during the first 15 wk of lactation. A total of 450 cows participated in the breeding program of CR-Delta (Arnhem, The Netherlands) and 163 cows originated from the ID-Lelystad farm ('t Gen). All cows were fed ad libitum. During the first 15 wk of lactation live weight, feed intake, and milk yield were measured for 565 of these cows. Milk samples were taken at a fixed day of the week for measurement of fat, protein, and lactose yields. Feed intake was recorded daily using automated feed intake units. Energy balance (MJ/day) was calculated as the difference between energy intake and calculated energy requirements for milk, fat, and protein yields and maintenance costs as a function of live weight. Progesterone profiles were used to determine the interval between calving and commencement of luteal activity (CLA) *[in following Chapters: FPLA]*. For more details on the dataset and traits, see Veerkamp et al. (2000).

Traits analyzed were average yields of milk (MY), fat (FY), protein (PY), and lactose (LY), percentages fat (F%), protein (P%) and lactose (L%) in milk, dry matter intake (DMI), feed intake (FI), energy balance (EB), and mean live weight over the first 15 wk of lactation (LW). Additionally, live weight in wk 1 (LW1), wk 15 (LW15) and minimum live weight (LWM) during these 15 wk were analyzed. Live weight change was analyzed as the difference between LW15 and LW1 (ΔLW). The differences between LWM and LW1 (ΔLW1M) and between LWM and LW15 (ΔLW15M) were also included in the analyses.

### RFLPs and Microsatellite

All 613 cows were genotyped for two restriction fragment length polymorphisms (RFLPs) (Pomp et al., 1997; Haegeman et al., 2000) and one microsatellite (Fitzsimmons et al., 1998) in the leptin gene locus at bovine chromosome 4. Polymerase chain reactions (PCRs) for all three polymorphisms were performed in 20-μl reactions with a standard PCR mix, containing 1.5 mM $MgCl_2$, 200 μM dNTP, 0.3 μM primer, 10 mM Tris HCl and 50 mM KCl. Allele *A* in the RFLP-studies was the allele not digested by the restriction enzyme, allele *B* was the restriction enzyme digested PCR product.

The first polymorphism (RFLP1) is located in the intron between two exons of leptin. The PCR reaction, as described by Pomp et al. (1997) turned out to be problematic because of PCR artifact bands. Therefore, new primers were designed (forward primer: 5'-*TGGAGTGGCTTGTTATTTTCTTCT*-3'; reverse primer: 5'-*GTCCCCGCTTCTGGCT ACCTAACT*-3') closer to the polymorphic site, which resulted in a PCR-product of 400 bp. For the PCR-reaction 0.5U of Taq polymerase and approximately 50 ng DNA were used. Thermal cycling conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94, 55, and 72°C (1 min each) and ending with a final extension for 15 min at

72°C. Digestion of the PCR product with 5U of *Sau*3AI (3.1.21.4; Invitrogen) overnight at 37°C revealed products of 100 and 300 bp.

The second polymorphism (RFLP2) is situated in exon 3 of the leptin gene (Haegeman et al., 2000) and causes an amino acid change from Alanine to Valine. These amino acids both belong to the group of aliphatic amino acids, but Valine is more hydrophobic. Primers were used as described by Haegeman et al. (2000). The PCR reaction was performed with 0.5U of Taq polymerase and approximately 100 ng genomic DNA. Thermal cycling conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s, followed by a final extension for 15 min at 72°C. Digestion of the PCR product of 331 bp with 5U of *Hph*I (3.1.21.4; Invitrogen) overnight at 37°C revealed products of 311 and 20 bp. The PCR reaction resulted in an artifact band of 600 bp which did not interfere because it was not digested by the enzyme.

Primers for the BM1500 microsatellite, which is located 3.6 kb downstream of the stop codon, were used as described by Fitzsimmons et al. (1998). The PCR reaction was performed with 0.2U of Taq polymerase and approximately 100 ng genomic DNA. PCR reaction was performed as follows: denaturation at 94°C for 2 min, 35 cycles of 94, 55 and 72°C (30 s each) and a final extension for 15 min at 72°C. After the PCR reaction was completed, 2 µl of formamide, 0.5 µl of loading buffer and 0.5 µl of M13 ladder were added to 1 µl PCR product. The samples were denaturated for 2 min at 95°C and then put on ice. Samples were loaded in a 6% polyacrylamide gel and run for 1 h and 30 min in an ABI 373A sequencer. The gel was analyzed with the programs Genescan Analysis 2.0.2 and Genotyper 1.1.1. Three alleles of *136* (allele *A*), *144* (allele *B*), and *146* (allele *C*) bp were detected.

**Analysis**

Contrasts between leptin genotypes for the measured production and reproduction traits were estimated using ASREML (Gilmour et al., 2001). Apart from the genotype effects, fixed effects were year-season where there are four seasons in a year (year-seasons with a low number of data were pooled with adjacent data, n=20), genetic group (Delta or 't Gen; n=2), and a quadratic polynomial for age at calving. Animal was fitted as a random effect including the additive genetic relationship between animals to account for background genes. The pedigree file contained 1240 animals. The 613 heifers were daughters of 98 sires, the largest sire group contained 36 heifers. The study had 45 sires with three or fewer daughters and to ensure that leptin genotype effects were not confounded with selection in the sires, sire was included as a fixed effect as well. Therefore most information for the genotype effects come from a within sire halfsib comparison. The $\chi^2$-test was used to determine linkage equilibrium between the three polymorphisms.

Significance of the genotype effects were estimated using the approximated F-statistic provided by ASREML (Gilmour et al., 2001). First, each genotype effect (RFLP1 with n=2, RFLP2 with n=3, BM1500 with n=6) was fitted in turn (i.e. single genotype model), and

secondly the other RFLPs were fitted as cofactor to take into account effects of linkage disequilibrium (multiple genotype model). Thirdly, we tested for sire x genotype interactions to account for different genotype effects within families.

**Table 1.** *Genotypic frequencies of the three markers. Allele A in the RFLP studies was the allele not digested by the restriction enzyme, allele B was the restriction enzyme digested PCR product. The BM1500 microsatellite shows 3 alleles of 136 (A), 144 (B), and 146 (C) bp*

| Genotypes | AA | AB | BB | AC | BC | CC |
|---|---|---|---|---|---|---|
| RFLP1 | 0.813 | 0.185 | 0.002 | X | X | X |
| RFLP2 | 0.581 | 0.329 | 0.090 | X | X | X |
| BM1500 | 0.230 | 0.381 | 0.126 | 0.126 | 0.086 | 0.051 |

## RESULTS

Genotype frequencies of the three polymorphisms are listed in Table 1. Genotypes are distributed according to the Hardy and Weinberg equilibrium. Allele *A* in the RFLP studies was the allele not digested by the restriction enzyme, allele *B* was the restriction enzyme digested PCR product. The BM1500 microsatellite shows three alleles with lengths of *136* (*A*), *144* (*B*), and *146* (*C*) bp. There was only one animal with the RFLP1 *BB*-genotype, which was excluded from the analysis. Four animals for RFLP2 and one animal for the microsatellite could not be typed. Linkage disequilibrium was found for the pairwise comparison of the three polymorphisms ($p<0.001$; Table 2).

An overview of all analyses (single and multiple genotype models) for all three polymorphisms are listed in Table 3 and the most important effects ($p<0.05$) and tendencies ($0.05<p<0.1$) for RFLP1 are listed in Table 4. The *AA*-genotype was used as zero reference to show the differences between the genotypes. In the multiple genotype analysis, i.e. taking linkage disequilibrium into account, RFLP1 showed significant differences between genotypes *AA* and *AB* for MY, PY, FI, and LY. With multiple analysis instead of single analysis the difference between the MY increased from 1.23 kg/d to 1.32 kg/d. Significance levels for DMI, mean live weight, and live weight at wk 15 were between 0.05 and 0.10. In the multiple genotype analysis DMI tended to be 0.39 kg/d higher in RFLP1-*AB* cows ($p=0.087$). The difference for live weight at wk 15 between the two genotypes of RFLP1 was 10.8 kg ($p=0.077$) and for mean live weight over the first 15 wk of lactation the difference was 9.1 kg ($p=0.097$); in both cases, genotype RFLP1-*AB* tended to show the higher live weight in comparison with genotype RFLP1-*AA*. For feed intake, there was no significant difference in the single genotype model, whereas in the multiple genotype model the *AB*

genotype had a higher feed intake than the *AA*-genotype (Δ=0.73 kg/d; *p*=0.043). In the single genotype model, the genotype effects for RFLP2 tended to be significant for ΔLW15M (*p*=0.087), where the *AA*-genotype showed 5 kg less weight gain after its minimum weight than the other two genotypes, and L% (*p*=0.085), where the *AA*-genotype showed the lowest percentage of lactose compared with the other two genotypes. For BM1500 a tendency was seen with L% (*p*=0.089).

**Table 2.** *Pairwise linkage disequilibrium of RFLP1, RFLP2 and BM1500. Differences between observed and expected percentages are shown (Δ = % observed - % expected) (p<0.001)*

| | | RFLP2 | | | BM1500 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | AA | AB | BB | AA | AB | BB | AC | BC | CC |
| RFLP1 | AA | -3.7 | 2.3 | 1.4 | 3.3 | -2.3 | -1.6 | 1.1 | -0.4 | 0.0 |
| | AB | 3.6 | -2.2 | -1.4 | -3.3 | 2.4 | 1.6 | -1.2 | 0.4 | 0.0 |
| RFLP2 | AA | | | | 7.2 | -2.2 | -6.4 | 2.7 | -0.3 | -0.8 |
| | AB | | | | -5.5 | 4.8 | 1.2 | -1.7 | 0.6 | 0.6 |
| | BB | | | | -1.6 | -2.6 | 5.2 | -1.0 | -0.2 | 0.1 |

To investigate the role of increased DMI to the increased yield, DMI was added to the analysis of MY as fixed effect (covariable) which resulted in a significant positive difference favoring the genotype *AB* (single genotype analysis: Δ=0.94 kg/d, *p*=0.064; multiple genotype analysis: Δ=0.96 kg/d, *p*=0.089).

To investigate possible effects within sire families, significance of the sire x genotype interaction was estimated. Significant sire x genotype interactions were found for LW and LWM (RFLP1, BM1500), LW1, and LW15 (BM1500), P% and F% (RFLP2). However, when investigating the sire families for RFLP1 and RFLP2, most of these effects could be ascribed to a few animals that received the deviating allele most likely from their dam. The many genotypes possible for BM1500 resulted in a too few daughters per genotype per sire to be sure of the interactions found for BM1500. Only the interaction effect on F% for RFLP2 was likely to come from true linkage, as the effect was observed within 17 sire families, and the distribution of daughters across the genotypes suggested that these sires were likely to be heterozygous.

**Table 3.** *Level of significance for the effect of each of the three polymorphisms in the bovine leptin gene region on traits. In the multiple analysis the other two polymorphisms are fitted as co-factors, i.e. linkage disequilibrium is taken into account. Significant effects (p<0.05) are printed in bold and tendencies (0.05< p<0.1) are underlined*

| | Single Genotype Analysis | | | Multiple Genotype Analysis | | |
|---|---|---|---|---|---|---|
| | RFLP1 | RFLP2 | BM1500 | RFLP1 | RFLP2 | BM1500 |
| LW1 (kg) | 0.262 | 0.712 | 0.386 | 0.144 | 0.566 | 0.301 |
| LW15 (kg) | 0.183 | 0.638 | 0.970 | 0.077 | 0.326 | 0.835 |
| ΔLW (kg) | 0.699 | 0.113 | 0.535 | 0.572 | 0.157 | 0.662 |
| LW (kg) | 0.230 | 0.914 | 0.715 | 0.097 | 0.507 | 0.507 |
| LWM (kg) | 0.222 | 0.970 | 0.723 | 0.102 | 0.657 | 0.579 |
| ΔLW1M (kg) | 1.000 | 0.560 | 0.856 | 0.841 | 0.507 | 0.814 |
| ΔLW15M (kg) | 0.655 | 0.087 | 0.416 | 0.549 | 0.228 | 0.776 |
| FI (kg/d) | 0.095 | 0.343 | 0.883 | **0.043** | 0.336 | 0.799 |
| DMI (kg/d) | 0.140 | 0.440 | 0.919 | 0.087 | 0.445 | 0.876 |
| EB (MJ/d) | 0.639 | 0.275 | 0.940 | 0.806 | 0.262 | 0.930 |
| MY (kg/d) | **0.024** | 0.852 | 0.940 | **0.027** | 0.970 | 0.963 |
| FY (g) | 0.185 | 0.869 | 0.950 | 0.206 | 0.951 | 0.945 |
| PY (g) | **0.012** | 0.631 | 0.769 | **0.014** | 0.961 | 0.791 |
| LY (g) | **0.020** | 1.000 | 0.895 | **0.021** | 0.844 | 0.988 |
| F% (g/kg) | 0.258 | 0.566 | 0.746 | 0.308 | 0.787 | 0.731 |
| P% (g/kg) | 0.740 | 0.432 | 0.342 | 0.841 | 0.638 | 0.460 |
| L% (g/kg) | 0.862 | 0.085 | 0.061 | 0.920 | 0.142 | 0.089 |
| CLA (d) | 0.777 | 0.198 | 0.821 | 0.823 | 0.281 | 0.869 |

*Abbreviation key:* CLA: first corpus luteum activity, DMI: dry matter intake, EB: energy balance, FI: feed intake, FY: fat yield, F%: percentage fat in milk, LW: mean live weight wk1 to wk15, LWM: minimum live weight, LW1: live weight in wk 1, LW15: live weight in wk15, LY: lactose yield, L%: percentage lactose in milk, ΔLW: LW15-LW1, ΔLW1M: LWM-LW1, ΔLW15M: LW15-LWM, MY: milk yield, PY: protein yield, P%: percentage protein in milk.

## DISCUSSION

In this study, we associated polymorphisms in the bovine leptin gene locus with genetic variations in energy balance, milk production, live weight, and fertility traits. Evidence is present that a genetic correlation between CLA and EB, MY, and LW exists in the animals used in this study (Veerkamp et al., 2000) and we found a genetic association between one of the mutations at the leptin gene locus and milk yield.

As a consequence of the increased milk yield more protein and lactose is produced by the RFLP1-*AB* cows; while P% and L% did not differ between the genotypes. Energy balance and CLA seemed not to be negatively influenced. Leptin affects many different features and, due to the small dataset, although relatively large for the traits measured here, the power and design of the experiment is probably too limited to exclude effects with significance levels between 0.05 and 0.1.

For MY, the genotypic standard deviation is 2.2 kg/d and for DMI 1.2 kg/d (Veerkamp et al., 2000). The increase in milk yield in the RFLP1-*AB* cows is more than 0.5 genetic standard deviation, whereas a 0.25 genetic standard deviation was found for DMI. When MY is corrected for DMI and linkage disequilibrium, a 0.96 kg/d higher milk yield was found. So the increase of DMI (0.39 kg/d) accounted for 0.36 kg/d milk. The energy needed for the 0.96 kg of milk must come from other sources such as a more negative energy balance, less weight or a better feed efficiency. It is surprising, however, that the data suggested that despite the higher milk production of the RFLP1-*AB* genotype, these heifers tended to be heavier and did not have a more negative energy balance. A hypothesis might be that these heifers have lower leptin levels, which results in more feed intake and less (basal) energy expenditure, or that the efficiency of feed expenditure (net efficiency) of the RFLP1-*AB* heifers is higher. This hypothesis, however, conflicts with studies investigating genetic differences in net efficiencies (Veerkamp and Emmans, 1995), because these studies found no evidence that genetic differences in net efficiencies exist.

**Table 4.** *Summary of differences between the RFLP1 genotypes with the AA-genotype as zero-reference for the most important effects (p<0.05, bold) and tendencies (0.05<p< 0.1, underlined). In the multiple analysis the other two polymorphisms are fitted as co-factors, i.e. linkage disequilibrium is taken into account*

| | Single Genotype Analysis | | Multiple Genotype Analysis | |
|---|---|---|---|---|
| | AB | P | AB | P |
| LW15 (kg) | 7.4 | 0.183 | 10.8 | 0.077 |
| LW (kg) | 6.0 | 0.230 | 9.1 | 0.097 |
| DMI (kg/d) | 0.31 | 0.140 | 0.39 | 0.087 |
| FI (kg/d) | 0.55 | 0.095 | 0.73 | **0.043** |
| MY (kg/d) | 1.23 | **0.024** | 1.32 | **0.027** |
| PY (g) | 37.4 | **0.012** | 40.3 | **0.014** |
| LY (g) | 58.9 | **0.020** | 64.7 | **0.021** |

Because the interval from parturition to first ovulation correlates significantly with the interval from parturition to the leptin nadir in high-producing dairy cows (Kadokawa et al., 2000), we expected to find an association of one or more of the molecular genetic markers with CLA. Woodside et al. (1998) showed that leptin influences the length of the anestrus period in rats suffering from severe negative energy balance due to food deprivation. As high producing cows also suffer from a negative energy balance due to lactation, leptin may influence the postpartum anestrus period in early-lactating cows. The association of CLA with RFLP2 revealed a *p*-value of 0.198. Although not significantly different from zero, the effect for RFLP2-*BB* and -*AA* was respectively -5.68 and -2.93 d postpartum, compared with RFLP2-*AB*. Therefore, at this stage we can not exclude that RFLP2 is associated with CLA.

Leptin has an impact on many processes, and the route that leptin works on yield might work by many physiological/morphological pathways. Therefore it is important to investigate what other QTLs are found on chromosome 4. Lindersson et al. (1998) used the BM1500 and BM1501 microsatellites to test whether QTL-effects on milk yield were associated with the obese locus. Similar to our study, no associations were found. Their study did not exclude the existence of a QTL at RFLP1 as both microsatellites are located 2.5 and 3.6 kb downstream of the leptin coding sequence and do not flank the RFLP1 polymorphism. This would have limited the power to detect the RFLP1 effect on yield. Fitzsimmons et al. (1998) reported an association between microsatellite BM1500 and fat deposition in beef cattle. For the RM188 marker, located 58.1 cM upstream of the leptin locus, an association was found for herdlife but not for milk production traits and somatic cell score (SCS) (Heyen et al., 1999). This is in contrast with the results published by Zhang et al. (1998), who found a QTL for SCS in between the markers RM188 and TGLA116 (located 33.9 cM upstream of the leptin locus). Spelman et al. (1999) published an association of marker TGLA116 with overall opinion of the cow by the dairy farmer, and Ashwell and Van Tassell (1999) detected several loci affecting milk, health, and type traits in a genome scan but only found an association for teat length on chromosome 4 (marker BL21, 63.9 cM). Schrooten et al. (2000) reported an association of gestation length between markers TGLA159 and TGLA420 (17 cM) but found no associations for other functional traits.

It is also possible that a gene closely linked to the leptin gene, rather than the leptin gene itself, is the gene actually responsible for some of the effects currently detected by the markers. The markers might then be differently linked to the QTL within sire families. Therefore, sire x genotype interactions were estimated. In this study, however, the genotypes were not divided equally within a sire family, thus results were not reliable. Only RFLP2 seems to be a marker for F% because 17 heterozygous sires were responsible to the interaction found. QTLs for F% were found on positions 75 cM and 95 cM on chromosome 4 (Lindersson et al., 1998). Obviously, other effects might still exist around the leptin gene, but these are not linked to the RFLP or microsatellite investigated here.

In conclusion, RFLP1 is associated with milk production but feed intake and live weight might also be affected. RFLP2 might be a marker for fat percentage in milk. Future breeding programs favoring the RFLP1 *B*-allele can yield a higher milk production without negatively affecting the energy balance and fertility. The prospects are good because in this study the frequency of the *AB*- and *BB*-genotypes are 18.5% and 0.2% respectively. Before starting a selection procedure, more has to be known about pleiotropic effects of the leptin locus. For example, the effects on traits like immunity and milk production in later lactations have to be studied.

# CHAPTER 4

# Leptin Concentrations in Relation to Energy Balance, Milk Yield, Intake, Live Weight, and Estrus in Dairy Cows

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**ABSTRACT**

The objective of this study was to describe fluctuations in leptin concentrations during late pregnancy and lactation and to investigate how those fluctuations are related to energy balance, milk yield, milk components, dry matter intake, live weight, first postpartum luteal activity, and first observed estrus during lactation. Live weight, dry matter intake, energy balance, and milk yield were measured weekly on 304 primiparous Holstein cows for the first 80 days of lactation. The first postpartum luteal activity was determined by measuring milk progesterone, and independently, first observed estrus. For measuring leptin concentrations from 30 days before until 80 days after calving, blood samples were taken at two-week intervals at a fixed time of the day after milking but before feeding. Leptin concentrations were high during pregnancy and declined to a nadir at parturition. It seems that leptin concentrations reflect the state of energy balance during lactation; plasma leptin concentrations were lower in cows with a mean negative energy balance during lactation. Those cows usually produced more milk, consumed less feed, and had a lower live weight compared with cows having a mean positive energy balance. The recovery of leptin concentrations from the leptin nadir at parturition seemed to depend on the extent and duration of the negative energy balance, thus probably on the amount of fat that was re-accumulated. Although there was lack of a relationship between leptin and first postpartum luteal activity, higher leptin concentrations associated with shorter intervals to first observed estrus might indicate a relationship between leptin and expression of estrus.

## INTRODUCTION

Leptin, mainly produced in adipose tissue, inhibits feed intake (Morrison et al., 2001) and down-regulates adipose tissue deposition (Halaas et al., 1995). Block et al. (2001) showed that leptin is negatively correlated with the amount of non-esterified fatty acids, which reflects the amount of fat mobilization. Furthermore, there is evidence that leptin positively influences fertility. For example, leptin was reported to restore fertility in leptin deficient *ob/ob* mice (Chehab et al., 1996) and to accelerate the onset of puberty in normal female rodents (Ahima et al., 1997). Also, fluctuations in plasma leptin concentrations appear to be related to LH concentrations in sheep (Nagatani et al., 2000).

As leptin affects both fat deposition and LH concentrations, it could play an important role in the processes occurring during the lactation period in dairy cows. During early lactation, cows are in a state of negative energy balance (EB) and fat stores are first used for lactation, maintenance, and growth with reproductive processes receiving the lowest priority (Mwaanga and Janowski, 2000). The negative EB suppresses the LH pulse frequency, resulting in a delayed first ovulation (Jolly et al., 1995). Canfield and Butler (1990) showed that the interval from parturition to the nadir in EB is positively correlated with the interval from parturition to first ovulation. Veerkamp et al. (2000) found a genetic correlation between start of luteal activity and energy balance of -0.60.

Although leptin concentrations during lactation have been described in dairy cows (Kadokawa et al., 2000; Block et al., 2001), to our knowledge, no study linking leptin with production and reproduction traits has been reported. Therefore the objective of this study was to describe fluctuations in leptin concentrations during late pregnancy and lactation and relate these to differences in energy balance, milk yield, milk components, dry matter intake, live weight**,** first postpartum luteal activity, and first observed estrus during lactation.

## MATERIALS AND METHODS

### Animals and Traits

Between 1990 and 1997, 304 Holstein-Friesian primiparous cows were followed from 4 wk before until 15 wk after calving. Of those 304 cows, 230 cows participated in the breeding program of CR-Delta (Arnhem, The Netherlands) and 74 cows originated from the ID-Lelystad farm (’t Gen). During the first 15 wk of lactation dry matter intake (DMI), milk yield (MY) and live weight (LW) were measured. Milk samples were taken at a fixed day of the week for measurement of fat, protein, and lactose yields. Cows were housed inside with a controlled lighting in addition to daylight (total 17h/d light). Temperature was not regulated. Cows were fed ad libitum with a complete ration of dried grass (mixed pasture containing mainly rye grass), corn silage and concentrates. Food intakes were recorded daily using

automated food intake units. Energy balance (MJ/day) was calculated as the difference between energy intake and calculated energy requirements for milk, fat, and protein yields and maintenance costs as a function of live weight. Milk progesterone was measured twice a week for the first 100 days of lactation to determine the interval between calving and first postpartum luteal activity (FPLA), and independently, first estrus (FE) was recorded by farm staff until 250 days after parturition. For more details on this material, see Veerkamp et al. (2000).

**Leptin RIA**

From 30 days before up to 80 days after calving, blood samples were taken at 2-wk intervals at a fixed time of the day after milking but before feeding. Leptin concentrations were determined by RIA essentially as described by Delavaud et al. (2000) but with three slight modifications: (1) antibody 8172 was used instead of antibody 7137 because this antibody showed higher titers, (2) a final dilution of 1:45,000 was used instead of 1:15,000 and (3) bound and free ligands were separated using anti-rabbit-SACCEL (IDS Ltd. England) instead of anti-rabbit ram plasma. The coefficients of variation were 11% within assay and 8.5% between assays.

**Analyses**

*Lactation curve for leptin.* Initially, leptin concentrations were described by fitting a smoothing spline function in ASREML (Gilmour et al., 2001) for days in milk (DIM) using all 2066 measurements on 304 primiparous cows. Fixed effects were sample date, except when the effect of months of measurement was estimated, genetic group (Delta or 't Gen; n=2) and a quadratic polynomial for age at calving. The spline function gave mean leptin concentrations for any DIM, while adjusting individual records for fixed effects as well as random effects for sire and animal to account for genetic and permanent environmental differences between the observations. To get an overview of the relationships between traits, groups of cows differing ± one SD of the mean were taken for each trait, including mean leptin concentrations per cow before (LEPpre) and after (LEPpost) parturition. For these groups the mean ±SE was calculated for the other traits. The Student's t-test was performed to indicate significant differences between the groups ($p<0.05$).

*Effect of traits on leptin curve.* The effect of each trait (average over the first 80 days of lactation) on leptin concentrations during late pregnancy and lactation was modeled by fitting an interaction between a spline describing leptin concentrations as a function of DIM, and a spline describing leptin concentrations as a function of trait values. The complete model estimates a function that describes leptin curves at all different levels for the traits. Rather than presenting function parameters, the ASREML predicted function was used to predict leptin (±SED) at different DIM for plus and minus one SD for each trait (i.e. DMI, MY, LW, EB, FPLA, FE and milk components). To indicate significant differences the

Student's t-test was performed for several DIM, and a value of $p<0.05$ was determined to be significant. To clarify relationships with leptin further, additional analyses were performed where the correlated traits DMI, MY, and LW were adjusted for each other and the residuals were included in the statistical model. Similar as before, leptin curves at different DIM were predicted for plus and minus one standard deviation of the residuals for each trait.

*Reproduction traits*. The same model was used to estimate a spline function and predict leptin concentrations at different DIM for FPLA and FE. In addition, a new model was used to investigate changes of leptin around the day of FPLA and FE. Therefore we expressed leptin concentrations for DIM relative to FPLA and, separately, leptin concentrations relative to FE. Leptin concentrations were determined by fitting a spline describing leptin concentrations as a function of FPLA or FE.

**Table 1.** *Averages and standard deviation for all traits analyzed (n=304). The values of plus and minus one standard deviation were used to predict leptin concentrations at different DIM*

| Item[1] | Mean | SD | Mean - SD | Mean + SD |
|---|---|---|---|---|
| DMI (kg/d) | 18.3 | 1.6 | 17 | 20 |
| LW (kg) | 529.9 | 41.6 | 488 | 572 |
| MY (kg/d) | 31.2 | 4.3 | 27 | 36 |
| EB (MJ/d) | -2.58 | 11.38 | -14 | 9 |
| FPLA (d) | 30.2 | 17.5 | 13 | 48 |
| FE (d) | 70.7 | 40.9 | 30 | 112 |
| FY (g) | 1251 | 172.7 | 1075 | 1425 |
| PY (g) | 1066 | 121.7 | 950 | 1200 |
| LY (g) | 1479 | 207 | 1275 | 1675 |
| F% (g/kg) | 40.3 | 4.2 | 36 | 45 |
| P% (g/kg) | 34.3 | 2.1 | 32 | 37 |
| L% (g/kg) | 47.4 | 1.2 | 46 | 49 |

[1]Fat (F), protein (P), and lactose (L) are presented both as total yields (FY, PY, LY) and in proportion to milk yield (F%, P%, L%).

## RESULTS

### Fixed Effects

Table 1 presents averages and standard deviations for all measured traits. Mean performance ±SE for groups of cows differing ± one SD of the mean for each separate trait and for leptin concentrations pre- and postpartum are presented in Table 2. Cows with highest milk production had a higher intake, were lower in EB, and had a longer anestrous period (FPLA and FE) than lower producing cows. Heavier cows had higher prepartum leptin concentrations and cows in a more positive EB had higher postpartum leptin concentrations. Also, cows in a more positive EB had 11.3 fewer days to FPLA. Furthermore, low leptin concentrations during pregnancy were associated with low leptin concentrations during lactation and vice versa.

**Table 2.** *Characteristics (mean ± SE) of groups of cows differing ± one SD for each separate trait and for leptin concentrations. LEPpre = mean concentration of leptin during the prepartum period, LEPpost = mean concentration of leptin during the postpartum period, n = number of cows in each group*

| | n | DMI (kg/d) | LW (kg) | MY (kg/d) | EB (MJ/d) | FPLA (d) | FE (d) | LEPpre (ng/ml) | LEPpost (ng/ml) |
|---|---|---|---|---|---|---|---|---|---|
| DMI < 17 | 63 | - | 500 ± 5[a] | 28.6 ± 0.5[a] | -7.5 ± 1.3[a] | 27.9 ± 2.2 | 66.5 ± 4.6 | 4.4 ± 0.2 | 4.0 ± 0.1 |
| DMI > 20 | 41 | - | 570 ± 7[b] | 33.8 ± 0.7[b] | 4.3 ± 1.7[b] | 32.5 ± 3.0 | 76.5 ± 7.0 | 4.7 ± 0.2 | 4.4 ± 0.2 |
| LW < 488 | 40 | 16.8 ± 0.2[a] | - | 29.6 ± 0.6 | -2.0 ± 1.8 | 35.4 ± 3.7 | 84.0 ± 6.2 | 3.9 ± 0.2[a] | 4.0 ± 0.2 |
| LW > 572 | 50 | 19.6 ± 0.2[b] | - | 31.0 ± 0.6 | 1.3 ± 1.7 | 26.8 ± 2.1 | 61.5 ± 5.8 | 5.0 ± 0.2[b] | 4.2 ± 0.1 |
| MY < 27 | 49 | 17.5 ± 0.2[a] | 525 ± 7 | - | 8.9 ± 1.2[a] | 22.5 ± 2.0[a] | 65.4 ± 5.0[a] | 4.7 ± 0.2 | 4.3 ± 0.1 |
| MY > 36 | 39 | 19.4 ± 0.2[b] | 540 ± 5 | - | -10.6 ± 1.5[b] | 36.4 ± 3.2[b] | 89.7 ± 7.4[b] | 4.4 ± 0.2 | 3.9 ± 0.2 |
| EB < -14 | 55 | 17.4 ± 0.2[a] | 528 ± 5 | 34.5 ± 0.5[a] | - | 34.8 ± 2.7[a] | 72.6 ± 5.2 | 4.7 ± 0.2 | 3.6 ± 0.1[a] |
| EB > 9 | 45 | 19.4 ± 0.2[b] | 549 ± 8 | 27.1 ± 0.6[b] | - | 23.5 ± 2.2[b] | 59.0 ± 5.9 | 4.7 ± 0.2 | 4.3 ± 0.1[b] |
| FPLA < 13 | 17 | 18.1 ± 0.4 | 540 ± 11 | 27.9 ± 1.1[a] | 3.8 ± 2.8[a] | - | 59.5 ± 10.6[a] | 4.8 ± 0.3 | 4.4 ± 0.2 |
| FPLA > 48 | 46 | 18.6 ± 0.3 | 525 ± 6 | 32.8 ± 0.5[b] | -5.3 ± 1.6[b] | - | 90.0 ± 4.9[b] | 4.2 ± 0.1 | 3.9 ± 0.1 |
| FE < 30 | 45 | 18.4 ± 0.2 | 546 ± 7 | 30.0 ± 0.6[a] | 0.3 ± 1.5 | 21.2 ± 1.2[a] | - | 4.6 ± 0.2 | 4.0 ± 0.1 |
| FE > 112 | 46 | 18.5 ± 0.3 | 522 ± 6 | 32.7 ± 0.6[b] | -5.0 ± 1.4 | 35.9 ± 3.5[b] | - | 4.5 ± 0.2 | 4.0 ± 0.1 |
| LEPpre < 3.1 | 37 | 18.1 ± 0.3 | 515 ± 6[a] | 32.6 ± 0.6 | -4.2 ± 2.1 | 28.8 ± 2.5 | 79.1 ± 6.5 | - | 3.3 ± 0.1[a] |
| LEPpre > 5.8 | 41 | 18.6 ± 0.2 | 544 ± 5[b] | 31.3 ± 0.7 | -3.6 ± 2.0 | 25.8 ± 1.8 | 61.5 ± 5.1 | - | 4.7 ± 0.1[b] |
| LEPpost < 3.0 | 35 | 18.3 ± 0.2 | 525 ± 7 | 33.0 ± 0.6[a] | -8.1 ± 1.7[a] | 29.9 ± 2.8 | 66.5 ± 7.0 | 3.4 ± 0.2[a] | - |
| LEPpost > 4.9 | 54 | 18.7 ± 0.2 | 537 ± 6 | 30.3 ± 0.7[b] | 1.7 ± 1.6[b] | 29.1 ± 2.3 | 66.8 ± 5.2 | 5.5 ± 0.2[b] | - |

[a,b] When two rows are significantly different they are identified with different superscripts.

Initially, month of calving was included in the model and leptin concentrations differed significantly between months (Figure 1). From February until May, leptin concentrations were lower than in the other months. Numbers of blood samples for leptin determination varied from as few as 79 in August to 334 in January. Because of these monthly differences, month of measurement should be taken as fixed effect in the further calculations. However, for a more precise adjustment, sample date instead of month of measurement was taken as fixed effect in the models. Genetic group and age at calving had no influence on leptin concentrations.

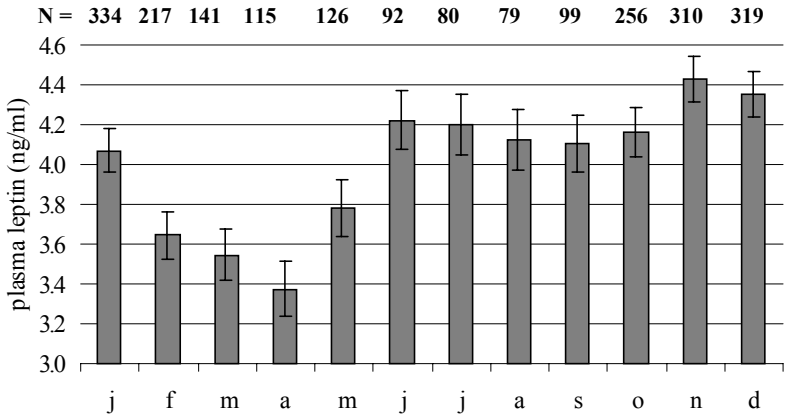


**Figure 1.** *Plasma leptin concentrations (± SE) per month of measurement after adjustment for DIM. N = number of samples in each month.*

**Leptin Concentrations**

Leptin concentrations were high during late pregnancy and declined rapidly to a nadir at parturition (Figure 2). Shortly after parturition leptin concentrations seemed to rise, but they soon declined again to the nadir level at calving. This appeared to be constant until at least 70 days after parturition.

**Leptin Concentrations and Traits**

The two curves representing leptin concentrations at different DIM for plus and minus one SD for each trait are given in Figures 3 to 5. Cows that consumed 17 kg/d DM during the first 15 wk of lactation had significantly lower ($p$<0.05) concentrations of leptin during lactation than cows consuming 20 kg/d DM (Figure 3a); cows that consumed more food had higher leptin concentrations. Adjustments for potential effects of MY and LW (Table 2) only slightly affected the leptin curves for high and low DMI (Figure 3d).

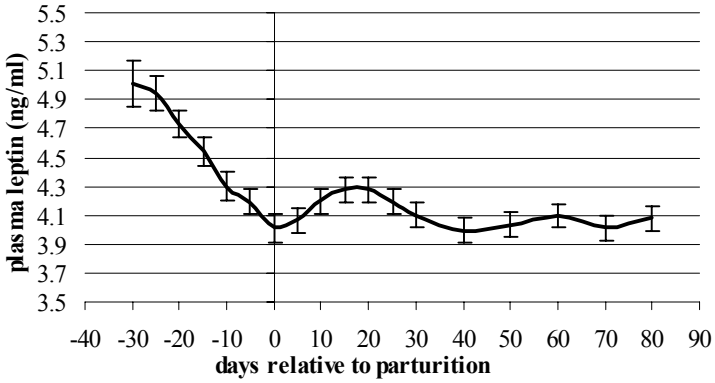


**Figure 2.** *The lactation curve for leptin (± SE). The concentration of leptin was measured by a RIA (Delavaud et al., 2000). The spline function used gives mean leptin concentrations and standard errors for any DIM, taking into account fixed and random effects included in the model.*

Low weight cows (488 kg) showed significantly lower leptin concentrations than high weight cows (572 kg) (Figure 3b). Although LW was measured postpartum, the leptin difference was larger pre-partum than during lactation. After adjusting for DMI and MY, there were only significant differences during pregnancy and very early lactation (Figure 3e).

High and low producing cows showed differences in leptin concentrations from 25 days of lactation onwards (Figure 3c). After adjusting for DMI and LW, significant differences were present after day 5 of lactation (Figure 3f). From that day onwards high producing cows (36 kg/d) showed significantly lower plasma leptin concentrations than low producing cows (27 kg/d) ($p<0.05$).

Cows with a mean EB of -14 MJ/d during the first 15 weeks of lactation did not differ in prepartum leptin concentrations from cows with a mean EB of +9 MJ/d (Table 2, Figure 4). However during lactation, cows with an overall positive mean EB of 9 MJ/d showed significantly higher leptin concentrations than cows with a negative EB of -14 MJ/d. Furthermore, cows in a negative EB showed even lower leptin concentrations during lactation than the nadir level reached at parturition. Recovery of leptin to higher concentrations after the nadir at parturition was observed for cows in a positive EB. It seemed that these cows recovered sooner from the nadir level observed at parturition than cows in a negative EB.

For percentages of milk components, significant differences were found (results not shown). Cows with a higher leptin level during pregnancy had a higher percentage fat in milk during lactation. Cows with a higher leptin level during pregnancy and early lactation had a higher percentage lactose and cows with a higher leptin level during the whole period

had a higher percentage protein in milk. For yields of milk components (fat, lactose and protein yields in kg), no significant differences were found, probably because percentages of milk components often are lower at higher milk yields.

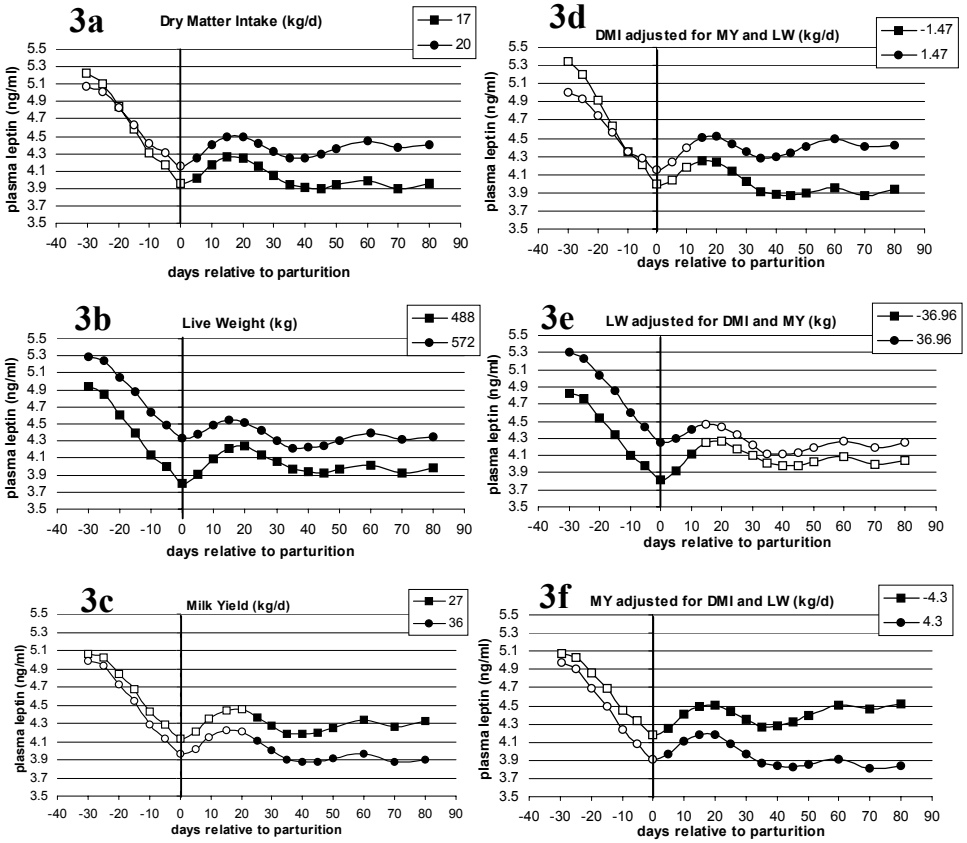


**Figure 3.** *Leptin concentrations were modeled using two spline functions (and their interaction) for leptin, one function for DIM and one function for either DMI (a), LW (b) or MY (c). Leptin was predicted at different DIM for plus and minus one SD as two curves for each trait. In Figures d, e and f DMI, LW and MY were adjusted for each other and the residuals were taken to predict leptin at different DIM. Filled squares and circles indicate significant differences ($p<0.05$), open squares and circles indicate no significant differences ($p>0.05$).*

**Leptin Concentrations and Reproductive Measures**

There were no differences in leptin concentration between cows with an early or a delayed FPLA but from day 15 of lactation onwards leptin concentrations were higher for cows that showed their FE earlier (day 30) than cows that had a delayed FE (day 112) (Figure 5). Changes of leptin before and after FPLA and FE were also investigated. Leptin concentrations rose linearly before and after FPLA and FE (Figure 6). For FPLA, standard errors were high, but for FE leptin concentrations on day -250, -50, and 50 differed significantly.

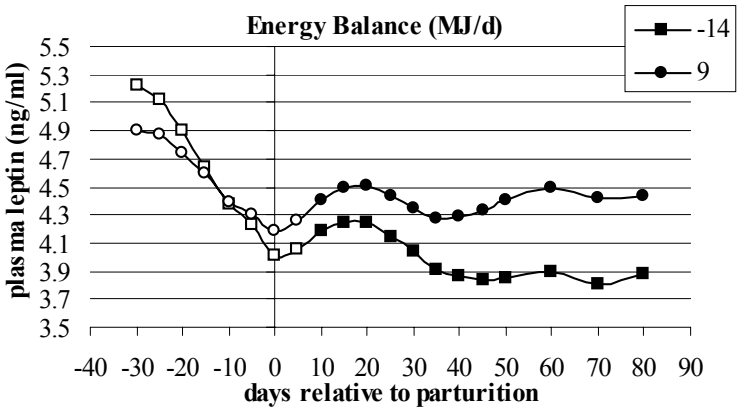


**Figure 4.** *Relationship of energy balance with leptin concentrations. Leptin concentrations were modeled by fitting a spline for leptin on these traits with DIM included in the model. Leptin was predicted at different DIM for plus and minus one SD for mean EB during the first 80 days of lactation (-14 and 9 MJ/d). Filled squares and circles indicate significant differences ($p<0.05$), open squares and circles indicate no significant differences ($p>0.05$).*

## DISCUSSION

The aim of this study was to describe fluctuations in leptin concentrations during late pregnancy and lactation and to relate these fluctuations to dry matter intake, milk yield, live weight, energy balance, milk components, start of luteal activity, and first observed estrus.

**Fixed Effects**

We observed a decreased leptin concentration in the period from February until May (spring). In our experiment cows were housed inside with controlled lighting (17h/d light) additional to daylight. These results are in contrast with Mann et al. (2000) who showed that leptin concentrations in monkeys reach a nadir in late summer (Aug. to Sept.) and a peak in late winter (Jan. to March). However, those monkeys were housed outdoors with an attached

temperature-controlled indoor area. Reist et al. (2001) reported that leptin concentrations are higher in cows calving in spring than in fall and Bocquier et al. (1998) reported that leptin concentrations are decreased when pair-fed sheep are exposed to a shorter photoperiod (8h/d vs. 16 h/d light). These contrasts in results can not be due to an unequal distribution of calving dates between the months, because in the analysis we adjusted for sample date. Also an unequal distribution of the number of samples between the months did not have an effect, because the number of samples was lowest in August (n=79) and highest in January (n=334). The differences between these studies could be due to differences between the studies in environment, feeding patterns or photoperiod. Genetic group and age at calving did not seem to influence leptin concentrations.

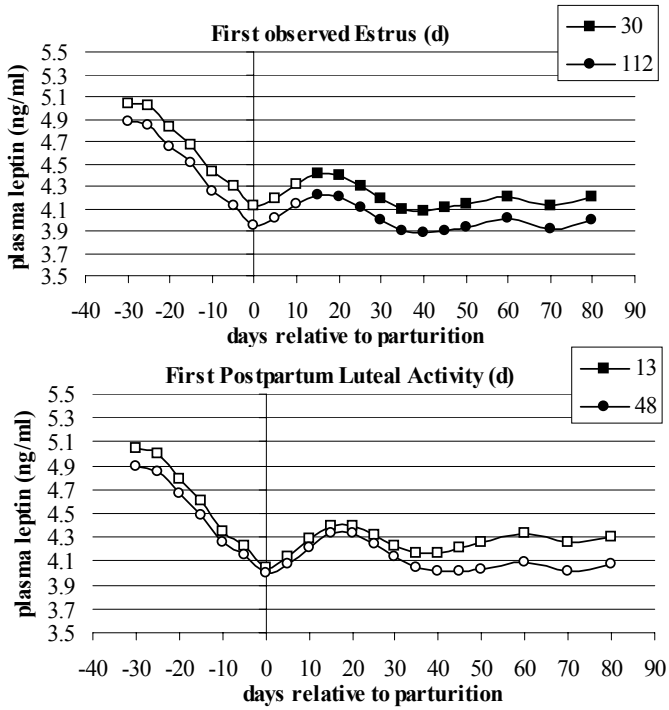


**Figure 5.** *Relationship of reproductive traits with concentrations of leptin. Concentrations of leptin were modeled by fitting a spline for leptin on these traits with DIM included in the model. Leptin was predicted at different DIM for plus and minus one SD as two curves for FE and FPLA. Filled squares and circles indicate significant differences ($p<0.05$), open squares and circles indicate no significant differences ($p>0.05$).*

**Leptin Concentrations**

This study clearly shows that plasma leptin concentrations are high during late pregnancy and decline to a nadir at parturition. This is in agreement with earlier reports in cows and sheep (Kadokawa et al., 2000; Block et al., 2001; Ehrhardt et al., 2001; Holtenius et al., 2001; Kokkonen et al., 2002; McFadin et al., 2002).

Leptin concentrations may be high during pregnancy as a result of the high energy intake necessary for the coming lactation period. This could be due to a specific decrease of the long-active form of the leptin receptor at hypothalamic level (Garcia et al., 2000), which

means that there are less signals to reduce food intake. On the other hand, an increase in the concentrations of the soluble binding protein of the leptin receptor during pregnancy (Gavrilova et al., 1997) could also be responsible for the increased food intake. Assuming that the leptin-binding protein inhibits leptin signaling, the high concentrations observed would cause leptin resistance.

It has been speculated that the placenta produces leptin and that the decline around parturition is caused by the expulsion of the placenta (Hoggard et al., 2001), but in our study the decline already started by 30 days before parturition. Block et al. (2001) and Reitman et al. (2001) reported that in mice, ovine, and bovine species, leptin might not be synthesized by placental tissue. In non-ruminant species, the decline in leptin concentration near parturition may be important for inducing the hyperphagia that is necessary for milk production (Brogan et al., 1999). However, the expected increase in leptin concentrations in response to this hyperphagia is abolished or actively suppressed during lactation (Pickavance et al., 1998).

**Leptin Concentrations and Traits**

Our results show that feed intake influences the production of leptin during the lactation period; a higher DMI is associated with higher leptin concentrations. These results are in agreement with data in non-pregnant, non-lactating sheep and cattle that received a higher feeding level of energy and showed higher leptin concentrations (Delavaud et al., 2000; Chilliard et al., 2001; Delavaud et al., 2002). Buyse et al. (2001) reported that a higher DMI increases insulin concentrations and this positively influences the production of leptin in adipose tissue.

Figure 3b shows that LW affects leptin concentrations during pregnancy and lactation, but adjusting for effects of feed intake and milk yield reduced the effect of LW on leptin concentrations during lactation (Figure 3e). Table 2 shows that this reduced effect during lactation mostly can be ascribed to differences in DMI, because cows with LW>572 kg (n=50) have a higher DMI than cows with LW<488 kg (n=40) whereas MY did not differ significantly between the high- and low-weight cows.

Milk yield influences leptin concentrations during lactation as higher MY was related to lower leptin production, particularly after adjustment for LW and DMI. Again, Table 2 shows significant differences in DMI between high- and low- producing cows; thus DMI is the more important adjustment factor. Mann and Blache (2002) did not find a relationship between MY and leptin concentrations but they used the RIA developed by Blache et al. (2000) which gave lower values (0.4 to 1.2 ng/ml) than the RIA (2 to 14 ng/ml) developed by Delavaud et al. (2000) and Ehrhardt et al. (2000).

A mean positive or negative energy balance during lactation influences circulating leptin concentrations. During lactation, cows in a positive EB have significantly higher leptin concentrations than cows in a negative EB (Figure 4 and Table 2). Table 2 shows that cows

with a mean positive EB (9 MJ/d) or more have a higher feed intake, a higher weight (NS) and a lower milk yield than cows with a mean negative EB (-14 MJ/d). If we look at Figure 3, we see that these groups of cows all have higher leptin concentrations. Because energy balance is calculated from components like DMI, MY and LW, the relationship of leptin with DMI, MY and LW can be ascribed to the relationship of leptin with EB. This is confirmed by studies of Block et al. (2001) and Reist et al. (2001) who also postulated a link between EB and leptin. The suggestion of Reist et al. (2001) that leptin variance could be linked more to individual cows than to either body condition score, energy balance or live weight may suggest that there is a genetic component affecting leptin concentrations. This would not be surprising as genetic components play a major role in physiological pathways. To estimate the genetic and individual cow component during the lactation period, a more sophisticated sire and animal component needs to be included in the model.

Apart from the differences in leptin concentrations we also observed differences in the recovery of leptin concentrations from the nadir level at parturition. This recovery seemed to depend on the extent of negative EB. In this study we see that cows in a positive EB, which have less fat mobilization, show a recovery of leptin concentrations after parturition and that cows in a negative EB even reach leptin concentrations below the nadir level at parturition. The recovery of leptin concentrations after parturition seems to depend on the quantity of fat deposition.

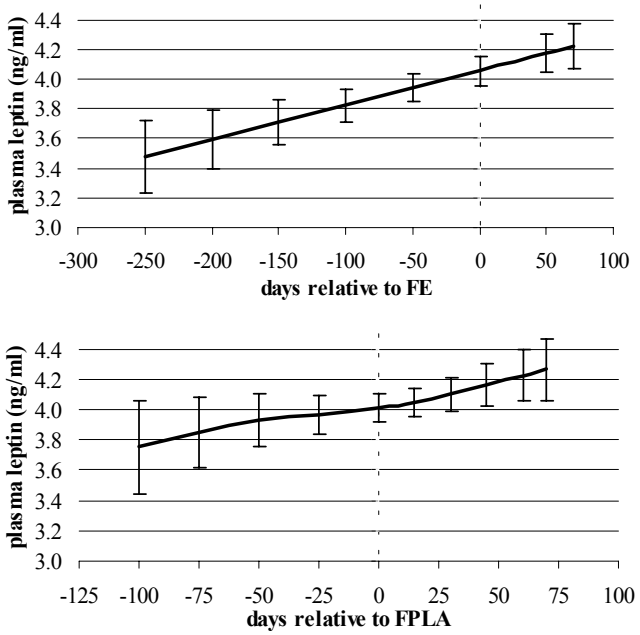


**Figure 6.** *Leptin concentrations before and after first observed estrus (FE) and start of luteal activity (FPLA). The lactation curve of leptin was included in the model to adjust for the effect of DIM on leptin concentrations.*
*Leptin concentrations were measured from day -30 until day 80. FPLA was measured from day 0 until day 100. FE was measured from day 0 until day 250. When a cow has an FE at day 200, it is possible to have leptin concentrations at 230 days before FE.*

**Leptin Concentrations and Reproduction**

In our study no relationship between leptin and FPLA was found, but significant differences in postpartum leptin concentrations were observed for FE. The first observation is in agreement with Holtenius et al. (2002) who reported that postpartum leptin concentrations were not related to time to return to cyclicity. An explanation for the absence of a relationship between FPLA and leptin concentrations might be that leptin concentrations are not low enough to negatively affect ovulatory cycles.

The lack of a relationship between FPLA and leptin concentrations in combination with a negative relationship between FE and leptin might indicate a relationship between and problems with estrous expression (e.g. silent estrus and subestrus). In the present study we found a difference of almost 40 days (30.2 and 70.7 d, respectively) between mean FPLA and FE values (Table 1) and a correlation between FPLA and FE of 0.26 ($p<0.01$). Assuming reliable estrous detection, these results might indicate that animals with higher leptin concentrations have a better estrous expression. Schopper et al. (1993) suggested a relationship between high milk yield (i.e. low leptin concentration in this study) and poor estrous expression.

It seems that a minimum level of leptin is required to induce the first postpartum LH surge as previously suggested by Huszenicza et al. (1999). In our study leptin concentrations rose linear before and after FPLA and FE and it seemed that a permissive level of 4 ng/ml had to be reached to induce both FPLA and FE. Bocquier et al. (1998) also suggested this in case of a sheep model.

We observed no clear nadir in leptin concentrations before FPLA or FE. This is in contrast with the results of Kadokawa et al. (2000) who showed a relationship between days to leptin nadir and days to first ovulation.

In summary, leptin concentrations during lactation reflect changes in EB and those fluctuations were not directly related to onset of luteal function. However, it seems that leptin concentrations may be related to expression of estrus. The recovery of leptin concentrations after the nadir at parturition seemed to be associated with the level of negative energy balance and thus the amount of fat that is re-accumulated during lactation.

## ACKNOWLEDGEMENTS

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# CHAPTER 5

# Association of Leptin Gene Polymorphisms with Serum Leptin Concentration in Dairy Cows

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**ABSTRACT**

Leptin is a hormone produced by adipocytes, and its expression is regulated by body fatness and energy balance. This study describes the association of four leptin gene polymorphisms in dairy cows (R4C, A59V, RFLP1, and BM1500) with circulating leptin concentrations during the periparturient period. A59V is located at a between-species conserved region of leptin, and R4C might have effect on the tertiary structure of the leptin protein because of the presence of an extra cysteine. RFLP1 is an intronic SNP and BM1500 is a microsatellite located 3.6 kb downstream of the leptin locus. The four polymorphisms were genotyped in 323 Holstein Friesian heifers with known pedigree. Leptin concentrations were determined biweekly from 30 days before until 80 days after parturition. The effect of genotype on leptin concentrations was modeled by fitting a spline in ASREML describing leptin concentrations as a function of days relative to parturition for each genotype/allele. Surprisingly, associations were found during pregnancy, but not during lactation. This indicates that the polymorphism could be more effective during pregnancy. If further studies demonstrate that more leptin-binding protein (Ob-Re) is present in this stage, it is hypothesized that a structural difference in the leptin protein could cause a sub-optimal binding stringency to Ob-Re. Free leptin could be cleared faster than bound leptin, and this could result in lower leptin concentrations during pregnancy for the polymorphism. The effects found might be ascribed to R4C. However, more study on the Ob-Re receptor, like binding stringency's between R4C and wild-type leptin and glycosylation during pregnancy, would provide more insight in the results found.

## INTRODUCTION

Leptin is a hormone secreted by adipocytes and contributes to the control of energy balance. It affects the regulation of food intake and energy expenditure. Circulating leptin concentrations reflect in part the amount of adipose tissue in the body in rodents and humans (Friedman and Halaas, 1998) and ruminants (Chilliard et al., 2001).

In cattle, the leptin gene is located on Chr 4 (Stone et al., 1996a) and consists of three exons. The last two exons contain the coding sequence and are separated from the promoter and first exon by a large intron of more than 8 kb (He et al., 1995; De la Brousse et al., 1996; Gong et al., 1996). Two microsatellites (BM1500 and BM1501) have been identified at the leptin locus (Stone et al., 1996b; Konfortov et al., 1999). Six exonic polymorphisms have been described, two of which are missense mutations (Konfortov et al., 1999).

Associations between polymorphisms at the leptin gene locus and economically important traits have been described in cattle. In a previous study (Liefers et al., 2002), we found that a polymorphism located in the second intron (Pomp et al., 1997) is associated with milk yield in dairy cattle. Fitzsimmons et al. (1998) found an association of the BM1500 microsatellite with fat characteristics in beef cattle, but no evidence was found for an association of BM1500 with dairy traits (Lindersson et al., 1998; Liefers et al., 2002).

Although fluctuations in leptin concentrations during late pregnancy and lactation in dairy cattle have been described (Kadokawa et al., 2000; Block et al., 2001; Liefers et al., 2003a), associations between leptin polymorphisms and circulating leptin concentrations have not yet been reported. In this present study, we investigated the presence of mutations in the coding sequence of the leptin gene in Dutch Holstein Friesian heifers, and the relationship between polymorphisms at the leptin gene locus and circulating leptin concentrations during late pregnancy and lactation.

## MATERIALS AND METHODS

### Animals

Between 1990 and 1997, a total of 323 Holstein-Friesian heifers with known pedigree were sampled from 30 days before until 80 days after parturition. A total of 243 cows participated in the breeding program of CR-Delta (Arnhem, The Netherlands), and 80 cows originated from the ID-Lelystad farm (’t Gen) (Liefers et al., 2003a). The 323 heifers were daughters of 54 sires. Mean number of daughters per sire was 8; the largest sire group contained 34 heifers, and there were 7 sires with 10 or more daughters and 28 sires with 3 or fewer daughters. Cows were fed ad libitum with a complete ration of dried grass (mixed pasture containing mainly rye grass), corn silage and concentrates.

**Sequencing**

PCR amplifications on exon 2 and exon 3 of the bovine leptin gene were carried out for 60 animals in 20-µl reactions containing 50 ng of genomic DNA, a standard PCR mix containing 1.5 nM $MgCl_2$, 200 µM dNTP, 0.3 µM of each primer, 10 mM Tris HCl, 50 mM KCl, and 0.5 U Taq DNA polymerase. Exon 2 and 3 are located at basepairs 1108-1251 and 3006-3365 (GenBank Accession no. U50365). Primers are listed in Table 1. For the amplification of exon 2 primer pair Leppr2A and Leppr2B was used, primer pair Leppr3A and Leppr3B was used for exon 3. Annealing temperatures were 60˚C for amplifying exon 2 and 57˚C for amplifying exon 3.

**Table 1.** *Primers that were used to amplify exon 2 (Leppr2A, Leppr2B) and exon 3 (Leppr3A, Leppr3B). For the sequence reactions, primers Lepprex2 and Lepprex3 were used. In the SnaPshot reaction, the primer LepprSS was used*

| *Name* | *Sequence* | *Position U50365* |
|---|---|---|
| Leppr2A | 5'-GTGGGGGATACAGGGGGAGTTTT-3' | 715-737 |
| Leppr2B | 5'-ACGGGATGGCCACGGTTCTAC-3' | 1295-1275 |
| Leppr3A | 5'-GAGGCAAAGGGCAGGGTGGTT-3' | 2780-2800 |
| Leppr3B | 5'-CCGGTGGGCGTGGAATC-3' | 3565-3549 |
| Lepprex2 | 5'-GTGCCTTTCATTACTGTCA-3' | 903-921 |
| Lepprex3 | 5'-CAGGGAGGATGGTGTGGA-3' | 2879-2896 |
| LepprSS | 5'-AAAAAAAAAAAGTGTCATCCTGGACCTTGC-3' | 1161-1179 |

Exons 2 and 3 are located at basepairs 1108-1251 and 3006-3365, respectively (GenBank Accession no. *U50365).*

PCR products were purified by using a Fine Sephadex column, 17 µl PCR product, and 10 µl distilled water. The plate was centrifuged at 15˚C, for 5 min at 910g. The sequence reaction contained 1 µl twice-diluted, purified PCR product, 4 µl BigDye Terminatormix, 4 µl distilled water, and 10 pmol primer. Primers used for the sequence reaction were Lepprex2 for exon 2 and Lepprex3 for exon 3. The sequence reaction required 30 cycles at 96˚C for 20s and 60˚C for 2 min. A second purification took place by using a Superfine Sephadex column and centrifugation for 5 min at 15˚C at 910g. Purified sequence products were sent to Greenomics (Wageningen, The Netherlands) for sequencing. Analyses were performed on the Lasergene software program Seqman (DNAstar inc, Madison, Wis., USA). For exon 2 a product of 581 basepairs was sequenced (EMBL Accession No. *AJ512638*), and for exon 3 this was a 786-basepair product (EMBL Accession No. *AJ512639*).

**Detection of PCR-RFLP and Microsatellite Polymorphisms**

All 323 cows were genotyped for four polymorphisms at the leptin locus: (1) R4C, a *C/T* SNP on exon 2, which leads to an arginine (R) to cysteine (C) substitution at amino acid

4 in the leptin molecule (EMBL Accession no. *AJ512638* Variation: 466); (2) A59V, a *C/T* SNP on exon 3, which leads to an alanine (A) to valine (V) change at amino acid 59 in the leptin molecule (EMBL Accession no. *AJ512639* Variation: 321); (3) RFLP1, a *C/T* SNP on the second intron (EMBL Accession no. *AJ236854* Variation: 1185); and (4) the BM1500 microsatellite located 3.6 kb downstream the leptin gene (GenBank Accession no. *G18586*).

R4C was genotyped by single nucleotide extension using the SnaPshot Multiplex Kit (Applied Biosystems, Foster City, Calif., USA) with the primer LepprSS which locates just before the mutation. Primers Lepprex2 and Leppr2B were used to amplify exon 2, which resulted in the amplification (30 cycles at 94ºC, 50ºC, and 72˚C, 1 min each) of a 393-bp fragment. The PCR products were purified according to the EXOSAP-IT protocol (USB Corporation, Cleveland, Ohio, USA). Labeled *C* and *T* and the primer LepprSS were added in the SnaPshot reaction of 25 cycles of 10 s at 96˚C, 5 s at 50˚C, and 30 s at 60˚C. The product was purified once more with SAP (USB Corporation) and separated on a 5% polyacrylamide sequence gel. Results were analyzed with the program Genescan 3.1.2. (ABI Prism 377-96 Collection Software, Launcher-Genetics).

Genotyping of A59V, RFLP1, and BM1500 was done as described by Liefers et al., (2002). A59V has three genotypes: *CC*, *CT* and *TT*. The BM1500 microsatellite shows three alleles with lengths of *136*, *144*, and *146* bp. There was only one animal with the RFLP1-*TT* genotype, which was excluded from the analysis, so RFLP1 has only 2 genotypes: *CC* and *CT*.

**Leptin RIA**

Two to eight blood samples per animal were taken biweekly from 30 days prepartum until 80 days postpartum at a fixed time of the day, after milking but before feeding. This resulted in a total of 2166 measurements on 323 cows. The average measurements per cow were 6.7, of which one was before parturition and the others after parturition. Leptin concentrations were determined by RIA essentially as described by Delavaud et al. (2000) but with three slight modifications: (1) antibody 8172 was used instead of antibody 7137 because this antibody showed higher titers; (2) a final dilution of 1:45,000 was used instead of 1:15,000; and (3) bound and free ligands were separated by using anti-rabbit-SACCEL (IDS Ltd. England) instead of anti-rabbit ram plasma. The coefficients of variation were 11% within assay and 8.5% between assays.

**Statistical Analysis**

Initially, leptin concentrations were described by fitting a smoothing spline function in ASREML (Gilmour et al. 2001) for days in milk (DIM), by using all measurements. Fixed effects were sample date, except when the effect of months of measurement was estimated, genetic group (Delta or ‘t Gen; n=2), and a quadratic polynomial for age at calving. The spline function gave mean (±SE) leptin concentrations for any DIM, while adjusting

individual records for fixed effects as well as random effects for sire and animal to account for genetic and permanent environmental differences between the observations.

The $\chi^2$-test was used to test linkage disequilibrium (LD) between the polymorphisms. The effect of genotypes on leptin concentrations was modeled by fitting a spline (ASREML, Gilmour et al., (2001)) describing leptin concentrations as a function of DIM for each genotype/allele. Fixed effects were sample date, genetic group, age at calving (linear and quadratic), DIM, and the interaction of genotype with DIM. Animal was fitted as a random effect including the additive genetic relationship between animals to account for background genes. Rather than presenting function parameters, the ASREML predict function was used to predict leptin levels (±SED) at different DIM for each genotype. In the case of the BM1500 microsatellite, two analyses were performed. Firstly, the six possible microsatellite genotypes were used in the analysis, and secondly, the presence of a specific allele (one or two alleles versus no allele present) in the genotype was used in the analysis. To indicate significant differences between the genotypes or alleles, the Student's t-test was performed for a range of DIM.

## RESULTS

For 60 cows the second and third exon of the leptin gene were sequenced together with the intronic boundaries. In introns 1 and 2, four SNPs were found. These were located at 46 bp (53%*C*/47%*T*), 264 bp (67%*C*/33%*T*), 287 bp (60%*G*/40%*C*), and 564 bp (56%*G*/44%*C*) (*AJ512638*). These intronic polymorphisms were not used in the analysis. On the exonic sequences, two missense mutations were found (basepair 466 on *AJ512638* and basepair 321 on *AJ512639*). The first exonic SNP will lead to an Arg to Cys change of the fourth amino acid (R4C) and is located at the non-conserved region of the A-helix of the leptin protein. The second exonic SNP will lead to an Ala to Val change of the 59th amino acid (A59V) and is located at the between-species conserved region of the B-helix of the leptin protein.

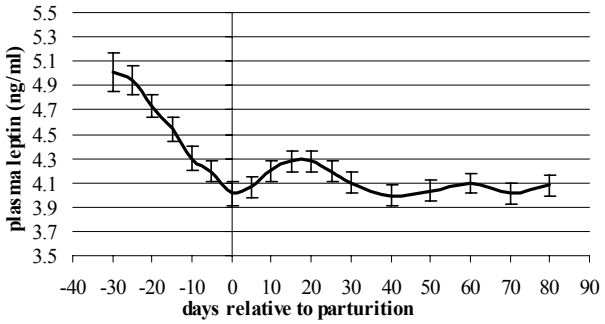


**Figure 1.** *The lactation curve for leptin (±SE). The concentration of leptin was measured by a RIA (Delavaud et al., 2000). The spline function used gives mean leptin concentrations and standard errors for any DIM, taking into account fixed and random effects included in the model.*

Leptin concentrations were high during late pregnancy and declined rapidly to a nadir at parturition (Figure 1). Shortly after parturition, leptin concentrations seemed to rise, but they soon declined again to the nadir level at calving. This appeared to remain constant until at least 70 days after parturition.

The four polymorphisms were in linkage disequilibrium (LD). This indicates that pairwise genotype combinations are observed more than expected on the basis of random mating. Table 2 displays the observed minus expected percentages of the pairwise genotype combinations. For example, the homozygous genotype combinations R4C-*CC* / A59V-*TT*, R4C-*CC* / BM1500-*144*144*, and A59V-*TT* / BM1500-*144*144* were observed more than expected.

**Table 2.** *Observed minus expected percentages of pairwise genotype combinations for R4C, RFLP1, A59V, and BM1500. The pairwise genotype combinations R4C-CC / A59V-TT, R4C-CC / BM1500-144*144 and A59V-TT / BM1500-144*144 are observed more than expected, and these three genotypes separately show higher leptin concentrations during pregnancy*

| | | R4C | | | RFLP1 | | A59V | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CC | CT | TT | CC | CT | CC | CT | TT |
| | Frequency | 0.46 | 0.42 | 0.12 | 0.81 | 0.09 | 0.58 | 0.34 | 0.08 |
| RFLP1 | | | | | | | | | |
| CC | 0.81 | -3.1 | 1.2 | 1.9 | | | | | |
| CT | 0.09 | 3.1 | -1.2 | -1.9 | | | | | |
| A59V | | | | | | | | | |
| CC | 0.58 | -6.6 | 3.3 | 3.3 | -2.7 | 2.7 | | | |
| CT | 0.34 | 3 | -2.6 | -0.4 | 1.5 | -1.5 | | | |
| TT | 0.08 | 3.6 | -0.7 | -2.9 | 1.2 | -1.2 | | | |
| BM1500[a] | | | | | | | | | |
| 136*136 | 0.18 | -7 | 7.1 | -0.1 | 3.4 | -3.4 | 5.7 | -4.5 | -1.2 |
| 136*144 | 0.29 | -9.1 | -2.2 | 11.3 | -1.6 | 1.6 | -0.9 | 2.7 | -1.8 |
| 144*144 | 0.25 | 11.1 | -2 | -9.1 | -2.5 | 2.5 | -4.9 | 0.6 | 4.3 |
| 144*146 | 0.11 | 5.5 | -1.2 | -4.3 | -0.2 | 0.2 | -0.9 | 1.1 | -0.2 |
| 146*146 | 0.05 | 2.6 | -0.6 | -2 | -0.3 | 0.3 | -1.2 | 1.4 | -0.2 |
| 136*146 | 0.12 | -3.1 | -1.1 | 4.2 | 1.2 | -1.2 | 2.2 | -1.3 | -0.9 |

[a]Allele frequencies: 136: 0.41; 144: 0.45; 146: 0.14.

Genotype effects on leptin concentrations during late pregnancy were found for all four polymorphisms (Figure 2A-2E). The R4C-*CC* genotype showed significantly higher serum leptin concentrations during late pregnancy and the first 5 days of lactation compared with the R4C-*TT* genotype (Figure 2A). The A59V-*TT* genotype showed significantly higher

leptin concentrations during late pregnancy than individuals with the A59V-*CC* genotype (Figure 2B). For RFLP1, the intronic SNP, the *CT* genotype showed significantly higher leptin concentrations between 30 and 20 days before parturition than the RFLP1-*CC* genotype (Figure 2C). In the case of the BM1500 microsatellite, two analyses were performed. There were no significant differences in leptin concentrations between the six BM1500 genotypes. However, the analysis with the presence of a specific allele (*136, 144*, or *146*) in the genotype showed significant differences. The presence of one or two *136*-bp alleles resulted in lower leptin concentrations during late pregnancy and the first 5 days of lactation (Figure 2D). The presence of one or two *144*-bp alleles resulted in significantly higher leptin concentrations until 40 days after parturition compared with genotypes without a *144*-bp allele (Figure 2E). Finally, the 146-bp allele did not show any significant effect on leptin concentrations.

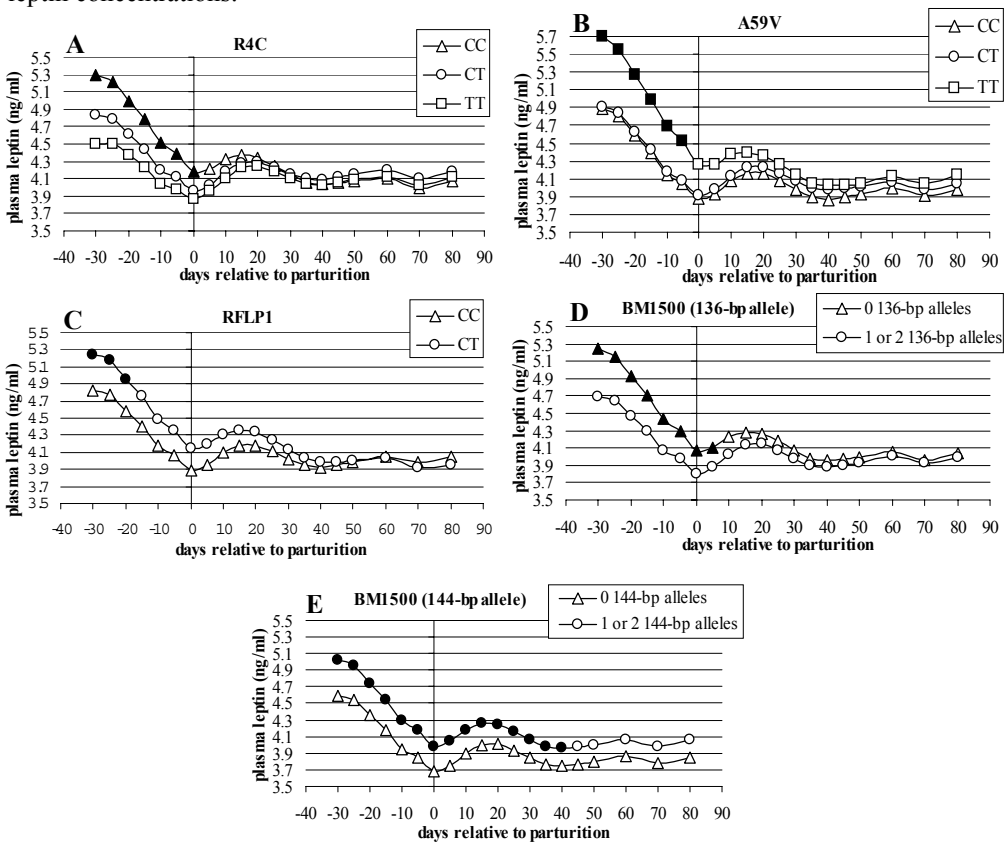


**Figure 2.** *Effect of genotypes of the four polymorphisms (A: R4C, B: A59V, C: RFLP1, D and E: BM1500; D: presence of the BM1500 136-bp allele, E: presence of the BM1500 144-bp allele) on leptin concentrations during late pregnancy and lactation. ●, ■ and ▲ = significant difference p<0.05), ○, □ and Δ = no significant difference (p>0.05). For R4C and A59V, significances were calculated for the difference between the homozygous genotypes.*

## DISCUSSION

### Mutations

Of 60 animals, the two exons and their intronic boundaries of the leptin gene were sequenced, and a total of four intronic and two exonic SNPs (missense mutations) were found. Four of these six SNPs were reported earlier by Konfortov et al. (1999) in a comparison of leptin sequences between 22 animals representing a diverse range of breeds within the subspecies *Bos taurus* and *Bos indicus*. The intronic SNP at 46 bp (*AJ512638*) was not detected by Konfortov et al. (1999) because this area was not sequenced, but the intronic SNP at 564 bp (*AJ512638*) was not present in the animals used by this group. Furthermore, this group found four silent mutations at exon 3, which we did not detect. The two exonic mutations (R4C and A59V) both were used in the analysis, together with the intronic SNP (RFLP1) and microsatellite BM1500, which were used in earlier analysis by our group (Liefers et al., 2002). Frequencies are reported in Table 2.

### Leptin Profiles

The concentration of plasma leptin is high during late pregnancy and starts to decline a few weeks before parturition, where it reaches a nadir at parturition. This is in agreement with earlier reports in sheep and cows (Block et al., 2001; Ehrhardt et al., 2001; Ingvartsen and Boisclair, 2001; Liefers et al., 2003a). Several factors could be involved in the observed hyperleptinemia during pregnancy in cows. Ehrhardt et al. (2001) found that the expression of leptin mRNA in white adipose tissue is increased 2.3-fold during pregnancy in ewes. Furthermore, an increase in serum concentrations of the leptin-binding soluble leptin receptor (Ob-Re) in mice accounts for the massive increase of bound leptin during pregnancy (Gavrilova et al., 1997). In women, bound leptin levels are higher during pregnancy than after, even though total circulating levels of Ob-Re do not change (Lewandowski et al., 1999). In ruminants, no clear evidence for the presence or absence of Ob-Re during pregnancy is present today. However, no Ob-Re was detected in serum of prepubertal heifers and mature cycling cows (Garcia et al., 2002). Furthermore, in mice and humans, the placenta synthesizes leptin and contributes to circulating leptin during late pregnancy (Hoggard et al., 1997; Bodner et al., 1999). However, the ruminant placenta has negligible levels of leptin mRNA throughout gestation, and thus it is not likely that placental leptin is involved (Block et al., 2001; Ehrhardt et al., 2001; Thomas et al., 2001). The decline in leptin levels towards parturition in dairy cows may be caused by a decrease of leptin mRNA production in white adipose tissue, which could be caused by a decrease in energy balance (Block et al., 2001).

**Association between Genotype and Circulating Leptin Concentrations**

BM1500, RFLP1, and the two exonic SNPs, R4C and A59V, are associated with leptin concentrations mainly during pregnancy. These polymorphisms are in linkage disequilibrium, so they do not act independently of each other. Because R4C adds an unpaired cysteine to the leptin protein, which may have an effect on its tertiary structure, the effects found might be ascribed to the R4C polymorphism.

As mentioned before for rodents (Gavrilova et al., 1997), during pregnancy more Ob-Re could also be present in bovine species. However, plasma leptin was increased ca. 50-fold in late-pregnant mice, whereas the increase is about twofold in bovine (Block et al., 2001 and Figure 1), which indicates that differences in Ob-Re concentrations during pregnancy and lactation are not as large as in mice. The structural change of R4C leptin could lead to a sub-optimal binding stringency to Ob-Re. The half-life of free and bound plasma leptin has not yet been determined in any farm animal species, but kinetic studies in the rat indicate that the half-lives of free and bound leptin are 3.4 min and 71 min, respectively (Hill et al., 1998). This suggests that bound leptin may be protected from proteolytic degradation. As a result, plasma leptin levels would be lower in R4C animals because more leptin would circulate in its free form and would be cleared sooner from the circulation.

The chicken leptin sequence, in contrast to mammalian leptin, contains an unpaired Cys at position 3 of the original cDNA. Dridi et al. (2000) showed that leptin with this unpaired Cys does not have a different biological function in stimulating proliferation of BAF/3 cells transfected with the human leptin receptor (Ob-R) compared with chicken leptin without this extra Cys. Also, the presence or absence of the Cys amino acid did not influence leptin's actions on inhibiting feed intake in chicken. But chicken leptin showed a less stringent binding to human Ob-R than did ovine leptin. There is only 80% amino acid sequence homology between chicken and bovine leptin, and the amino acid sequence at the leptin binding domain of chicken Ob-R [amino acids 428-635] (Sandowski et al., 2002) is very different from this domain in humans and rodents (Ohkubo et al., 2000). Taking these findings together, we can not conclude that bovine R4C has the same binding stringency with bovine Ob-Re as wild-type bovine leptin. In addition, the R4C marker in beef cattle seems to be important for leptin mRNA levels and carcass fat content (Buchanan et al., 2002). Clearly, more study in cattle is needed to clarify this issue.

Lammert et al. (2002) reported recently that the size of Ob-Re in non-pregnant mice is 120 kDa, whereas the size of Ob-Re is 150 kDa in pregnant mice as a result of different glycosylation. If R4C has a lower stringency with the 150 kDa Ob-Re than wild-type leptin and if they both have the same stringency with the 120 kDa protein, this could explain the lower leptin levels for R4C observed during pregnancy but not during lactation. However, until now, no confirming data are available for glycosylation of Ob-Re in bovine.

The promoter activity of human leptin seems to be different in the placenta than in adipocytes (Bi et al., 1997; Ebihara et al., 1997). Thus, a polymorphism can influence only the expression of placental leptin. But, as mentioned earlier, the ruminant placenta has negligible levels of leptin mRNA throughout gestation (Block et al., 2001; Ehrhardt et al., 2001), making this explanation less probable.

Another explanation for the present results would be that an effect of leptin polymorphism on leptin secretion by adipose tissue could be easier to detect during late pregnancy when leptin expression is up-regulated than during lactation when plasma leptin is at its nadir or baseline level (Figure 1).

In summary, in this study we investigated the association of genetic polymorphisms in the bovine leptin gene with circulating leptin concentrations. The associations found might be ascribed to the R4C mutation. However, more study on Ob-Re in ruminants, such as its presence during pregnancy, binding stringencies between R4C and wild-type leptin, and degree of glycosylation during pregnancy, would provide a better understanding of the results found in the present study.

## ACKNOWLEDGEMENTS

We thank Henry van der Gaast for collecting data and Bart Jungerius for his assistance with the SnaPshot Multiplex method. We also thank Leo Kruijt for refining the leptin RIA, and Michel Breuer and Joop Testerink for their excellent laboratory work in performing the RIA. This work was financially supported by CR-Delta, Arnhem; the Dutch Ministry of Economical Affairs (grant number BTS 98194); and the Dutch Ministry of Agriculture, Nature Management and Fisheries.

# CHAPTER 6

SHORT COMMUNICATION

# A Missense Mutation in the Bovine Leptin Receptor Gene is Associated with Leptin Concentrations during Late Pregnancy

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**ABSTRACT**

Parts of the bovine leptin receptor (*Ob-R*) gene were sequenced to search for polymorphisms. The gene consists of 20 exons divided over 1.75 Mb. Parts of exon 4 (79 bp), exon 11 (95 bp) and exon 20 (513 bp) of 20 heifers (Holstein Friesian) were sequenced *(AJ580799; AJ580800; AJ580801*). In exons 4 and 11 no SNPs were found. In exon 20, a *T* to *C* missense mutation was found at nucleotide 115, which causes an amino acid substitution at residue 945 (T945M). Frequencies for allele *C* and *T* were 0.93 and 0.07 in a population of 323 Holstein Friesian heifers and *TT* animals were not detected. Using genotypes of these cows an association study was performed for leptin concentrations during the periparturient period. Leptin concentrations were determined by radioimmunoassay (RIA). The T945M mutation showed an association with circulating leptin concentrations only during late pregnancy ($p<0.05$) but not during lactation ($p>0.05$). The *CC* genotype had higher concentrations during this period than the *CT* genotype. A combined effect with previously described leptin polymorphisms on prepartum leptin concentrations was observed, giving one genotype combination with significantly lower levels of leptin up to 50 days, but interaction effects were not significant. The T945M polymorphism may have induced a structural change in the intracellular domain of the Ob-R, which may have influenced the signal transduction pathway. However, the effect was found for the heterozygous genotype since the *TT* genotype was not detected in this population of 323 Holstein Friesian cows.

Leptin, the *ob* gene product, is synthesized in adipose tissue and plays a role in body weight homeostasis (Zhang et al., 1994) and reproduction (Chehab et al., 1996). The leptin receptor (Ob-R), a class I cytokine receptor, is produced in at least six alternately spliced forms both in rodents (Cioffi et al., 1996) and humans (Tartaglia et al., 1995). Some spontaneous mutations in leptin or in its receptor result in obesity in mice (Zhang et al., 1994; Chen et al., 1996), rats (Chua et al., 1996) and humans (Montague et al., 1997; Clement et al., 1998).

Ob-R is a glycoprotein with a single transmembrane-spanning region. The isoforms that share identical extracellular and transmembrane domains are characterized by intracellular domains of variable length (Tartaglia et al., 1995). Ob-Rb is the longest form and is thought to play an important role in signal transduction in the hypothalamus (Vaisse et al., 1996). Ob-Rb consists of 1142 amino acids of which the first 816 form the extracellular part, the next 23 amino acids form the transmembrane region and the last 303 amino acids are located intracellularly which play a role in signal transduction (Tartaglia, 1997). Amino acids 428-635 are important for leptin-binding (Fong et al., 1998). Leptin-binding to the Ob-R results in the formation of a receptor complex leading to phosphorylation and activation of the JAK/STAT (Janus Kinases/Signal Transducers and Activators of Transcription) signalling pathway. Two conserved tyrosines in the cytoplasmic domain of Ob-R are important for the activation of this signalling pathway (White et al., 1997).

During pregnancy many physiological processes take place and the leptin / leptin receptor complex might play an important role during this time. In previous studies, mutations in the leptin gene were associated with leptin concentrations in pregnant cows (Liefers et al., 2003a). The purpose of this study was to find polymorphisms through sequencing parts of the *Ob-R* gene and to study associations between these polymorphisms together with previously described leptin polymorphisms (Liefers et al., 2003a) and leptin concentrations during the periparturient period.

The bovine *Ob-R* gene is located on chromosome 3q33 (Pfister-Genskow et al., 1997). Only two exonic parts of the bovine *Ob-R* gene were sequenced until now (*U83512,* exon 4 and *U62385*, exon 20) and no mutation sin these exons have been reported yet. Sequencing all exons of the bovine *Ob-R* gene using DNA isolated from blood samples of 20 unrelated cows and primers obtained from known sequences of different species (human, sheep, cow, waterbuffalo) succeeded only for parts of exons 4, 11, and 20. Primer sequences for these three polymerase chain reaction (PCR) products were respectively: Exon 4 (*U83512*): forward 5'-*GAATGGACGTTATGAGGCAG*-3', reverse: 5'-*CAGAGCAGTTTTTATCTTC CGC*-3'; Exon 11 (*U43168*): forward 5'-*ATTTGCAGAGTGATGGTTTTTATG*-3', reverse: 5'-*ACCACAGAATCAGGAAGGACACA*-3'; Exon 20 (*U62124*): forward: 5'-*GACCTT TGGCCCTCTTCTTTT*-3', reverse: 5'-*GACAGGCCTTTCATTATTATTTTC*-3'. The PCR products were purified using the Qiagen QIAquick PCR purification column and 100 ng of the PCR product was used for the sequence reaction. Products were sequenced with the

capillary sequencer CEQ8000 (Beckman Coulter Inc., Fullerton, CA, USA) and analyses were performed on the Lasergene software (DNAstar inc, Madison, WI, USA). Sequences found are available in the EMBL database, respectively: exon 4, *AJ580799* (59 bp); exon 11, *AJ580800* (95 bp); exon 20, *AJ580801* (513 bp). In these sequences, a total of 667 bp, which is 17% of the total coding region of the *Ob-R* gene, one non-conservative polymorphism was found in exon 20 (*AJ580801*). A nucleotide substitution at position 115 (*C* => *T*) causes an amino acid substitution at residue 945 (Thr => Met, T945M). This amino acid is located in the intracellular region of the Ob-R molecule. For this mutation, two genotypes (*CC* and *CT*) were observed in the group of 20 cows.

Of 323 Holstein Friesian cows of known pedigree, 243 cows participated in the breeding program of CR-Delta (Arnhem, The Netherlands), and 80 cows originated from the ID-Lelystad farm ('t Gen) (Liefers et al., 2003b). The 323 cows were daughters of 54 sires. The SNP-Primer Extension Kit (Beckman Coulter Inc.) was used for genotyping. One primer was used with its 3'-end 1 bp before the polymorphism: *5'-TGCCAG CAACTACAGATGCTCTACTTTTGA*-3'. After the PCR product was purified, labelled ddNTPs, SNP-Primer Extension Premix and primer were added and this mix was submitted to a reaction of 25 cycles of 10 s at 96˚C, 5 s at 50˚C and 30 s at 72˚C. The products were purified and separated on the CEQ8000 (Beckman Coulter Inc.). Results were analysed with the software CEQ8000 Genetic Analysis System, Fragment Analysis (Beckman Coulter Inc.). Amongst the 323 cows genotyped for T945M, 300 had genotype CC (92.9%) and 23 genotype *CT* (7.1%). No *TT* animals were observed, but according to the Hardy-Weinberg equilibrium only 1.5 cows were expected to be *TT*.

To determine leptin concentrations for the 323 cows, from 30 days before up to 80 days after calving, blood samples were taken every 2 weeks at a fixed time of the day after milking and before feeding. This means that six to eight samples per animal were taken. Leptin concentrations were determined by radioimmunoassay (RIA) essentially as described by Delavaud et al. (2000) but with three slight modifications. First, antibody 8172 was used instead of antibody 7137 because this antibody showed higher titers, second a final dilution of 1:45,000 was used instead of 1:15,000 and third, bound and free ligands were separated using antirabbit-SACCEL (IDS Ltd., Boldon, England) instead of antirabbit ram plasma. The effects of Ob-R genotypes on leptin concentrations were estimated by fitting a spline function in ASREML (Gilmour et al., 2001) describing leptin concentrations as a function of days in milk (DIM) as described before (Liefers et al., 2003b). Fixed effects were sire, date of sampling, genetic group (CR-Delta or 't Gen) and age at calving (linear and quadratic). Animal was fitted as a random effect including the additive genetic relationship between animals to account for background genes. To present the results, leptin levels and the SE were predicted at different DIM (from -30 to 80 days with 10 day intervals) for each genotype and the Students *t*-test was performed to test statistical significance between the genotypes at each DIM. The T945M polymorphism showed a significant ($p<0.05$)

association with circulating leptin concentrations during late pregnancy but not during lactation, with the *CC*-genotype showing higher leptin levels than the *CT*-genotype. A similar effect on leptin during late pregnancy was found with the leptin gene polymorphisms R4C, RFLP1, A59V and BM1500 (Liefers et al. 2003a). To identify possible combined effects on prepartum leptin concentrations all 5 SNPs (4 on the leptin gene and 1 on the *Ob-R* gene) and their interactions were combined, in a backward, forward and all possible subset regression analysis in Genstat 6.1 using the same fixed effects as mentioned before. R4C and A59V, both located in exonic regions of the leptin gene, together explained 8.6% (over and above the variance explained by the fixed effects) of the variance in prepartum leptin concentrations. T945M added 0.45% to this variance. The interactions between the genotype-effects were not significant for prepartum leptin concentrations. Subsequently, the interaction between the three (exonic) polymorphisms T945M, R4C and A59V were fitted in a spline function describing leptin concentrations during the periparturient period as described before for the *Ob-R* genotypes. Figure 1 presents the splines for the genotype combinations of T945M/R4C/A59V with 10 or more cows. Average SED was 0.3 ng/ml. Significant differences were observed in late pregnancy between genotype combinations *CC/CC/*** and *CT/CT/AA, CC/CT/AB, CC/TT/AB* and between *CC/CC/BB* and *CC/**/AA*, where ** means that every genotype can be filled in for this polymorphism. Furthermore, the *CT*-genotype of T945M in combination with R4C-*CT* and A59V-*AA* had significantly lower leptin levels until 50 days after parturition in comparison with the genotype combination *CC/CT/AA*.

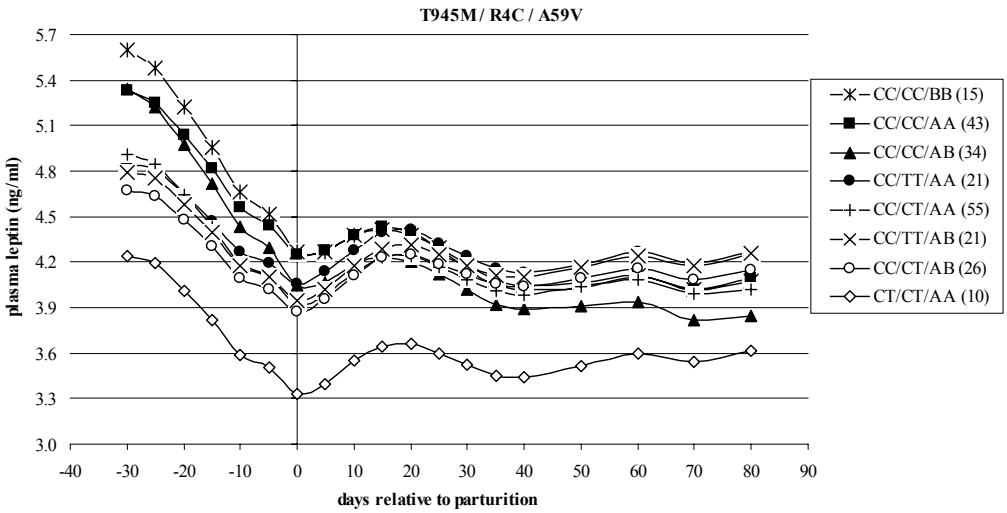


**Figure 1.** *Effect of T945M/R4C/A59V genotype combinations on leptin concentrations during the periparturient period. R4C and A59V are exonic polymorphisms in the leptin gene (Liefers et al. 2003b). T945M is an exonic polymorphism in the leptin receptor gene. Only genotype combinations with 10 or more animals are presented, number of animals are listed in brackets. Average SED was 0.3 ng/ml.*

The hyperleptinemia during pregnancy in rodents was associated with the appearance of the circulating short form of the leptin receptor (Ob-Re) (Gavrilova et al., 1997; Garcia et al., 2000). As the T945M polymorphism is only present in Ob-Rb, the association with leptin concentrations during pregnancy found here can not be ascribed to the appearance of Ob-Re during pregnancy.

The two conserved tyrosines in the intracellular domain of the Ob-R that are required for signal transduction are located at positions 986 and 1079 (White et al., 1997). T945M is located 39 amino acids before the first important tyrosine residue and might induce a structural change in the intracellular domain, which might influence the conformation of the tyrosine residues and as a result, the efficiency of the JAK/STAT pathway. If the pathway is more efficient due to the presence of the *T*-allele, the system might signal to the adipose tissue to produce less leptin. As leptin concentrations are very high during late pregnancy, this polymorphism could have more influence on the downregulation of leptin expression during this period than later, during lactation. However, the effect was only observed for the heterozygous genotype since the *TT*-genotype was not detected in this dataset of 323 cows. Further study is needed to reveal the functions of the polymorphism, whether *TT*-genotypes are present in the population and if this genotype has even lower leptin levels during late pregnancy.

## ACKNOWLEDGEMENTS

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# CHAPTER 7

# Leptin Promoter Mutations affect Leptin Levels and Performance Traits in Dairy Cows

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**ABSTRACT**

Leptin concentrations in body fluids and tissues undergo dynamic changes during the periparturient period. Polymorphisms in the leptin gene have shown to be associated with differences in leptin concentration during late pregnancy but not during lactation. As the promoter of leptin regulates the expression of leptin, polymorphisms at this region could play an important role in the found differences in leptin expression during the periparturient period. We sequenced the leptin promoter and analyzed the sequence for transcription factor binding sites. We discovered 20 SNPs in a 1.6 kb region of the bovine leptin promoter. The 14 SNPs that were genotyped for 613 Holstein Friesian heifers all were found to be associated with leptin concentrations during late pregnancy but not during lactation. Because none of these SNPs are located on important known transcription factor binding domains on the bovine leptin promoter, the associations found with leptin concentrations during late pregnancy might not be ascribed to the SNPs found on the promoter region. More likely, the earlier described R4C mutation on exon 2 of the leptin gene is the functional mutation causing this difference in leptin concentrations during late pregnancy. In the association study of the SNPs with dairy traits three SNPs were found to be associated with fertility, energy balance and protein yield. These might serve as markers for future breeding programs for better fertility and energy balance without significantly influencing milk yield in dairy cattle.

## INTRODUCTION

Leptin is expressed in a variety of tissues including adipose tissue, placenta (Hoggard et al., 1997; Masuzaki et al., 1997), mammary gland (Bonnet et al., 2002), skeletal muscle (Wang et al., 1998), gastric mucosa (Bado et al., 1998) and brain and hypophysis (Jin et al., 2000). The expression is regulated by mass and cell size of adipose tissue and by hormones such as insulin, growth hormone and glucocorticoids (Houseknecht et al., 1998; Leury et al., 2003). To date, several important transcription factor binding domains have been identified in the promoter region of the leptin gene in humans, rodents and ruminants. These include domains for CAAT/enhancer binding proteins (C/EBP) (Hwang et al., 1996; Miller et al., 1996; Taniguchi et al., 2002), SP1 (Fukuda and Iritani, 1999), LP1 (Mason et al., 1998), ADD1/SREBP1 (Kim et al., 1998), peroxisome proliferator activated receptor γ (PPARγ) (Hollenberg et al., 1997), and HIF-1 (Grosfeld et al., 2002; Meissner et al., 2003).

Leptin concentrations in body fluids and tissues undergo dynamic changes during pregnancy and lactation. In mice and humans, leptin is expressed in the placenta and contributes partly to the high levels of circulating leptin during late pregnancy (Hoggard et al., 1997; Bodner et al., 1999). Adipose tissue and placenta have similar sized leptin mRNA transcripts and the same promoter is involved in placental and adipose tissue leptin expression. Two placenta-selective binding domains in a 100 base-pair region from -1946 to -1847 were identified as placental leptin enhancers (Bi et al., 1997). The ruminant placenta has negligible levels of leptin mRNA throughout gestation (Bispham et al., 2003) but other factors like the gestational increase in leptin expression in adipose tissue could be involved in the hyperleptinemia during pregnancy (Ehrhardt et al., 2001). Towards parturition leptin concentrations decline (Liefers et al., 2003b) which could be due in part to changes in energy metabolism before the onset of lactation (Block et al., 2001). However, plasma leptin remains low during at least 3 months after calving, despite the sharp increase in energy balance which occurs from week 2 or 3 post-partum (Holtenius et al., 2003; Reist et al., 2003).

Polymorphisms in the leptin gene are associated with differences in leptin concentration during pregnancy but not during lactation (Liefers et al., 2003a). Also associations with dairy traits like milk yield (Liefers et al., 2002; Buchanan et al., 2003) and feed intake (Lagonigro et al., 2003) were reported.

Mutations in the promoter of leptin might play an important role in differences in leptin expression during the periparturient period. The objective of this study was to identify polymorphisms in the leptin promoter and to investigate whether these play a role in the regulation of leptin levels during pregnancy and lactation and if they were associated with dairy traits like energy balance, milk yield and fertility. Therefore we sequenced and analyzed 1.6 kb of the bovine leptin promoter in search for SNPs and related these to differences in leptin concentration during the periparturient period in dairy cattle and with

several dairy traits. Another objective was to determine putative transcription factor binding domains and conserved regions in the leptin promoter sequence in order to find out whether the detected SNPs were within (potentially) important regulatory domains.

## MATERIALS AND METHODS

### Animals and Traits

Between 1990 and 1997 a total of 613 Holstein-Friesian heifers with known pedigree were followed from 4 weeks before until 15 weeks after parturition. A total of 450 cows participated in the breeding program of CR-Delta (Arnhem, The Netherlands) and 163 cows originated from the ID-Lelystad farm ('t Gen). Cows were fed ad libitum and feed intake was recorded daily using automated feed intake units.

During the first 15 weeks of lactation live weight, milk yield and feed intake were measured for 565 of these cows. Milk samples were taken at a fixed day of the week for measurement of fat, protein and lactose yields. Energy balance (MJ/day) was calculated as the difference between energy intake and calculated energy requirements for milk, fat and protein yields and maintenance costs as a function of live weight. Milk progesterone was measured twice a week for the first 100 days of lactation to determine the interval between calving and first postpartum luteal activity (FPLA) for 323 cows. Independently of FPLA, first observed estrus (FE) was recorded by farm staff until 250 days after parturition.

Traits analyzed were average yields of milk (MY), fat (FY), protein (PY) and lactose (LY), percentages fat (F%), protein (P%) and lactose (L%) in milk, dry matter intake (DMI), feed intake (FI), energy balance (EB) and mean live weight over the first 15 weeks of lactation (LW). Additionally, live weights in week 1 (LW1), week 15 (LW15) and minimum live weight (LWM) during these 15 weeks were analyzed. Live weight change was analyzed as the difference between LW15 and LW1 (ΔLW). The differences between LWM and LW1 (ΔLW1M) and between LWM and LW15 (ΔLW15M) were also included in the analyses.

### Sequencing

PCR primers were developed on the published sequence of the bovine leptin promoter (*AB070368*) (Taniguchi et al., 2002) and amplifications were carried out for 20 animals in 20-µl reactions containing 50 ng of genomic DNA, a standard reaction-mixture containing 1.5 nM $MgCl_2$, 200 µM dNTP, 0.3 µM of each primer (Table 1), 10 mM Tris HCl, 50 mM KCl and 0.5 U Taq DNA Polymerase. For PCR 4 a total concentration of 10% DMSO was used to optimize the PCR reaction. PCR products were purified using the QIAquick PCR purification column (Qiagen GmbH, Germany) and DNA concentrations were determined by spectrophotometer. The sequence reaction contained 100 ng of purified PCR product (5 µl), 4 µl of CEQ DTCS Quick Start Kit (Beckman Coulter Inc., Fullerton, CA, USA), and

10 pmol primer (1µl). Sequences from both forward and reverse primers were obtained. The sequence reaction required 30 cycles at 96˚C for 20s, 50˚C for 20s and 60˚C for 4 min. The product was purified with ethanol absolute and resuspended in 40 µl of Sample Loading Solution (Beckman Coulter Inc.). The CEQ Separation Gel LPA-1 (Beckman Coulter Inc.) was used to separate for 85 minutes on the CEQ8000 (Beckman Coulter, Inc.) and analyses were performed on the Lasergene software program Seqman (DNAstar Inc, Madison, WI, USA).

**Table 1.** *Primers that were used to amplify 1.6 kb of the bovine leptin gene 5'-flanking DNA sequence. Primer sequences were based on the published sequence of Taniguchi et al. (2002) (EMBL AB070368). Primerset 3b was used for genotyping SNP -578*

| Name | Sequence | Position on *AB070368* |
|---|---|---|
| PCR1 | 5'-GGGGGAGGCGGAGAGGAG-3' | -1600 … -1583 |
| | 5'-TACATGGCCACTAAAAAGGTTG-3' | -1035 … -1056 |
| PCR2 | 5'-AAGTCCCCTGTAGATGTTTTTATG-3' | -1358 … -1335 |
| | 5'-GCCTGGTTGTTTTGCTTTTA-3' | -931 … -950 |
| PCR3 | 5'-TAGTACAATATCCTTCCTTTCTT-3' | -987 … -965 |
| | 5'-CCTGCCTTGATGATGGTGTGG-3' | -444 … -464 |
| PCR4 | 5'-CTTACCCCTCCACACCATCATCAA-3' | -473 … -450 |
| | 5'-GAGCCGGGCACTTACCT-3' | 49 … 33 |
| PCR3b | 5'-GCAACGCACGGGGCTATCAATG-3' | -630 … -609 |
| | 5'-GCCTGGCCTGGAAAATCACACCT-3' | -424 … -446 |

**Genotyping of Polymorphisms**

For 20 cows the promoter region (-1600 to 0) of the leptin gene was sequenced**.** We tried to design primers for all 20 SNPs to genotype these simultaneously on the pyrosequencer but this failed because of the presence of too much hairpins and loops in the sequence for a good primer-design. Therefore we decided to genotype only a few SNPs between the genotypes -1600 and -415 with the primer extension method. The SNPs located between -415 and -105 were all typed by sequencing because they were located close to each other and on one single PCR product (PCR4). We used the comparison with *AB070368* to divide the sequence (-1600 to -415) into small blocks (-1457 to -1446, -1392 to -901, -578 to -282) (see Table 2). From each block one or two SNPs were chosen randomly to be genotyped for all 613 animals. The SNPs chosen were respectively -1457, -1238, -963, and -578 and these were genotyped using the SNP-Primer Extension Kit (Beckman Coulter Inc.). This method uses primers of different lengths, which are located just before the SNPs (Table 3). PCR1 and PCR2 were used to genotype the SNPs -1457, -1238 and -963.

**Table 2.** *Polymorphisms found in the 1.6 kb 5'-flanking promoter region of the leptin gene and a comparison with the published sequence of Taniguchi et al. (2002) (AB070368). Locations -1198, -498 and -483 were different compared to AB070368. Bold SNPs were genotyped for 613 animals and allele frequencies are based on these data*

| | *AJ571671* | SNP | Allele frequency SNP | *AB070368* |
|---|---|---|---|---|
| **-1457** | A | G | 0.46 | G |
| -1452 | A | G | | G |
| -1446 | T | C | | C |
| -1392 | G | A | | G |
| -1255 | AG | DEL*AG | | AG |
| **-1238** | G | C | 0.33 | G |
| -1198 | G | No SNP | | DEL*G |
| -1066 | T | A | | T |
| **-963** | C | T | 0.33 | C |
| -901 | A | T | | A |
| **-578** | C | G | 0.44 | G |
| -498 | DEL*C | No SNP | | C |
| -483 | G | No SNP | | A |
| **-415** | G | DEL*G | 0.27 | G |
| **-292** | T | C | 0.47 | C |
| **-282** | G | T | 0.10 | T |
| **-272** | G | A | 0.37 | G |
| **-211** | A | G | 0.10 | G |
| **-201** | C | T | 0.12 | T |
| **-197** | A | C | 0.19 | A |
| **-170** | C | T | 0.40 | C |
| **-147** | C | T | 0.47 | T |
| **-105** | C | G | 0.42 | G |

*DEL = deletion of one or two basepairs.

To optimize PCR3 for genotyping SNP -578, a new primer-pair was developed (PCR3b) (Table 1) and 10% DMSO was added to the PCR reaction mixture. The PCR products were purified with 2U Shrimp Alkaline Phosphatase (SAP) and 1U Exonuclease I (USB Corporation) for 1 hr at 37˚C. Enzymes were inactivated at 75˚C for 15 min. Labeled ddNTPs were added together with the SNP-Primer Extension Premix and the primers (Table 3) to the purified PCR products in a reaction of 25 cycles of 10s at 96˚C, 5s at 50˚C and 30s at 72˚C. The product was purified once more with 1U SAP (USB Corporation) and

separated on a CEQ Separation Gel LPA-1 (Beckman Coulter Inc.). Results were analyzed with the program CEQ8000 Genetic Analysis System, Fragment Analysis (Beckman Coulter Inc.).

Because the product of PCR4 contained 10 SNPs, we decided to sequence this PCR product for all 613 animals. PCR products were purified using a Fine Sephadex column, 15 µl PCR product and 10 µl distilled water. The plate was centrifuged at 15˚C, for 5 min at 910g. The sequence reaction contained 1 µl two-times diluted purified PCR product, 4 µl BigDye Terminatormix, 4 µl distilled water and 10 pmol primer. For the sequence reaction the reverse primer of PCR4 was used. The sequence reaction required 30 cycles at 96˚C for 20s and 60˚C for 2 min. A second purification took place using a Superfine Sephadex column and centrifugation for 5 min at 15˚C at 910g. Sequencing was performed by commercial sequencing (Greenomics, Wageningen, The Netherlands). Analyzes were performed on the Lasergene software program Seqman.

**Table 3.** *Primers used for genotyping of the 4 SNPs located between -415 and -1600 bp. The SNP-Primer Extension Kit (Beckman Coulter Inc.) was used with primers of different lengths which are located just before the SNPs*

| Name | Sequence | Position on AJ571671 |
|---|---|---|
| P54 | 5'-TTTTTTTTTTTTTTTTCTATCAATGTGGGATAC | -616 … -579 |
| SNP -578 | AGATGTGAACAAAACGGACCC-3' | |
| P37 | 5'-ACCTCACAAACATATAGTACAATATCCTTC | -1000 … -964 |
| SNP -963 | CTTTCTT-3' | |
| P42 Rev | 5'-AACGGATTATAAAATGGTATGTGTTTTCTG | -1196 … -1237 |
| SNP -1238 | ATCACACACATT-3' | |
| P49 | 5'-TTTTTTTTTTTTTTAGGCAGGATGTTTAGT | -1492 … -1458 |
| SNP -1457 | CGCAGCATGAGAACTCTT-3' | |

**Transcription Factor Binding Domains**

Putative functional transcription factor binding domains in the leptin promoter (C/EBP, SP1, LP1 and HIF-1) were identified by comparison of published data of different species (Hwang et al., 1996; Miller et al., 1996; Mason et al., 1998; Fukuda and Iritani, 1999; Grosfeld et al., 2002; Taniguchi et al., 2002; Meissner et al., 2003). The TRANSFAC database version 4.0 (Wingender et al., 2001) (http://transfac.gbf.de/TRANSFAC) was used to search for transcription binding domains in the region of the 20 SNPs found (Table 4).

**Table 4.** *Putative transcription factor binding domains and the number of matches in the leptin promoter sequence found by the TRANSFAC database. Transcription factors, which bind at the site of found SNPs, are presented in bold*

| Nr of Matches | Transcription Site | Nr of Matches | Transcription Site | Nr of Matches | Transcription Site |
|---|---|---|---|---|---|
| 1 | AHRARNT | 44 | **GATA1 (-197)** | 1 | NMYC |
| 6 | **AP1 (-292)** | 7 | GATA2 | 1 | OCT |
| 12 | AP2 | 15 | GATA3 | 25 | OCT1 |
| 11 | AP4 | 5 | GC | 1 | OLF1 |
| 1 | ARNT | 10 | GFI1 | 4 | PADS |
| 3 | BARBIE | 3 | GKLF | 3 | **RFX1 (-415)** |
| 11 | BRN2 | 3 | HFH1 | 2 | RORA1 |
| 4 | **CAAT (-1238)** | 4 | HFH2 | 14 | S8 |
| 4 | CDP | 7 | HFH3 | 3 | SOX5 |
| 18 | CEBPB | 8 | HFH8 | 6 | SP1 |
| 8 | CETS | 1 | HLF | 9 | SRY |
| 1 | CHOP | 8 | HNF3B | 1 | STAT |
| 1 | CLOX | 5 | IK1 | 1 | T3R |
| 5 | **CMYB (-1457, -211)** | 17 | IK2 | 1 | TAL1A |
| 1 | CP2 | 1 | IK3 | 2 | TAL1B |
| 2 | CREB | 2 | IRF1 | 12 | TATA |
| 3 | CREL | 18 | **LMO2COM (-197)** | 17 | TCF11 |
| 6 | DELTAEF1 | 1 | **MYCMAX (-1446)** | 1 | TH1E47 |
| 1 | E2F | 6 | MYOD | 4 | TST1 |
| 5 | E47 | 4 | MZF1 | 14 | **USF (-1446)** |
| 1 | EGR2 | 4 | NF1 | 3 | VBP |
| 2 | ER | 11 | **NFAT (-415)** | 12 | **VMYB (-1457, -282)** |
| 2 | EVI1 | 10 | NFKB | 3 | XFD1 |
| 5 | FREAC | 8 | **NFY (-1238)** | 2 | XFD2 |
| 13 | GATA | 9 | NKX25 | 1 | XFD3 |
| | | | | 1 | ZID |

**Leptin RIA**

For 323 animals, two to eight blood samples per animal were taken biweekly from 30 days prepartum until 80 days postpartum at a fixed time of the day after milking but before feeding. Leptin concentrations were determined by RIA essentially as described by Delavaud et al. (2000) but with three slight modifications: (1) antibody 8172 was used instead of antibody 7137 because this antibody showed higher titers; (2) a final dilution of

1:45,000 was used instead of 1:15,000; and (3) bound and free ligands were separated by using anti-rabbit-SACCEL (IDS Ltd., Boldon, England) instead of anti-rabbit ram plasma. The coefficients of variation were 11% within assay and 8.5% between assays.

**Statistical Analysis SNP - Leptin Concentrations**

The effect of genotypes on leptin concentrations was modeled by fitting a spline in ASREML (Gilmour et al., 2001) describing leptin concentrations as a function of days in milk (DIM) for each genotype. Fixed effects were date of sampling, genetic group, age at calving (linear and quadratic), DIM, the interaction of genotype with DIM, and sire. Animal was fitted as a random effect including the additive genetic relationship between animals to account for background genes. Rather than presenting function parameters, the ASREML predict function was used to predict leptin levels (±SED) at different DIM for each genotype. To indicate significant differences between the genotypes or alleles the Student's t-test was performed for a range of DIM.

**Statistical Analysis SNP - Traits**

The effects of genotypes on the production and reproduction traits measured were estimated using ASREML (Gilmour et al., 2001). Apart from the genotype effects, fixed effects were year-season, genetic group (CR-Delta or 't Gen), age at calving (linear and quadratic). The additive genetic relationship between animals (pedigree) was fitted as a random effect to account for background genes. To ensure that leptin genotype effects were not confounded with selection in the sires, sire was included as a fixed effect as well. Therefore most information for the genotype effects came from a within sire halfsib comparison. Significance of the genotype effects were estimated using the approximated F-statistic provided by ASREML.

## RESULTS

**Sequencing and Genotyping**

In this study 1.6 kb of the bovine leptin promoter were sequenced (*AJ571671*) and 20 SNPs were found. Allele frequencies of the 14 polymorphisms that were genotyped for all animals are listed in Table 2, together with the comparison of the earlier published sequence *AB070368* (Taniguchi et al., 2002). In Figure 1 the sequence (*AJ571671*) with the found polymorphisms and putative functional binding domains is presented.

-1599 gggg aggcg gagag gagga aagat tttct tcaaa atgta atttc attgt agaca cttct ttaaa agaaa cattt
-1525 cttta tttga cagtt ccagg cctta gtttc agcag gcagg atgtt tagtc gcagc atgag aactc tta**a**c tgc**a**g
A/G A/G
-1450 catg**t** gggac ccagt tcagt tccct gacca gatat cgaac ctggg gcccc tgcat ttgga a**g**cag ggagt
T/C G/A
-1380 cttag ccact ggacc accag ggaag tcccc tgtag atgtt tttat gaaaa gcaga aaagc acaaa gaaga
-1310 gctta aagat tcctg atcct actcc caata gtgat aatgt atatt ttggt gtgag **ag**tgt gtgta ttgat tg**g**aa
del AG G/C
-1235 tgtgt gtgat cagaa aacac atacc atttt ataat ccggt tcttt ccagc tcaca aaata aagtt atttt cctac
-1160 atcat taaat attac tttac aacat aattt ttaat gtgtg catat tgctg ctatg tgatt ttcaa taact tacta atttc
-1080 ctatg ctgaa catt**t** agttg ttgtc caacc ttttt agtgg ccatg taatt ataaa tcatg gtcaa tgcta acaat
T/A
-1005 ttctg acctc acaaa catat agtac aatat ccttc ctttc tt**c**aa tagat aatta ttaaa agcaa aacaa ccagg
C/T
-930 ctcaa acaaa gcaat tataa aatat ctt**a** aaaag acatt gggta aaatt caaat gcaga ctagc tcatg atgtt
A/T
-855 aaaga attac tcttg tgtgg taatg gtctt gtgat agaga tagaa atgct tcctt atttt tcaga taaac actta
-780 agtat ttaag gatga aacgc cctga tgttt gtaat ttgct ttaga atatt ttagc caaaa gaatt aatga tgcaa
-705 atatg caaaa agagt acgtt aaacc taaat ttgcg atttt cattt aaaaa tatat cttaa aaatg aaaat cttcg
-630 tgcaa cgcac ggggc tatca atgtg ggata cagat gtgaa caaaa cggac cc**t**g tggga ctcgg cggag
C/G
-560 cacac agatt ttgcg ggagc acgtt cccgt tagga agtct ctgat gcaat acgac cggtg ccttc aggac
-490 ctgtg aggct gactt tcctt acccc tccac accat catca aggca ggtgt gattt tccag gccag gccta
-420 cggcc **g**gttt ccccg ggggc ccaga gccgt cgggt cttgc cgccc agcgg agctg gctgc tccgg
ins G
-355 cctca ctgtc ggggc gccac cgccc ccagc cggct cagag gaacc cctca ccgcc accct gtc**t**c
T/C
-290 aggcg gcc**g**t tcccc gag**g**c ccgag ggtca gatcc tgggg ccacc tcgag gattt ctcac acctg
G/T G/A
-225 cccag ccacc ccca**a** ctttt cagg**c** gat**a**c cggag ggtgg gcgtg gggct cctgg **c**gcat ccgag
A/G C/T A/C C/T
-160 tccct ccctg gag**c**c cccga ccgcg gccgc ccggc ccgac gctgc cccgc cgccc **c**gcag ggcgg
C/T HIF-1 C/G Sp1
-95 gagcc ggcgc tgcgg gtgcg ccccg gccag ccggg cagtt gcgca agttg tgctt cggcg gctat aagag
LP1 C/EBP TATA
-25 gggcg ggcag gcatg gagcc ccgga
Sp1

**Figure 1.** *Nucleotide sequence upstream from the first (untranslated) exon of the bovine leptin gene (AJ571671). Nucleotide numbering starts with the putative transcription start site (+1), and proceeds as indicated in the left margin. Found polymorphisms and putative functional HIF-1, SP1, LP1, C/EBP and TATA sequences are underlined.*

**Transcription Factor Binding Domains**

In the TRANSFAC database several transcription factors were located that might bind at the site of found SNPs (Table 4). This database locates putative binding domains on the base of sequence similarity. All known domains are listed in Table 4, whereas Figure 1 only shows the binding domains that have been earlier described to have a possible function in leptin expression. SNP -1457 is located at binding sites for CMYB and VMYB, SNP -282 for VMYB and SNP -211 for CMYB. SNP -1446 is located at a binding site for MYCMAX and USF and SNP -1238 is a binding site for NFY and CAAT whereas SNP -415 binds NFAT and RFX1. SNP -292 is a binding site for AP1 and SNP -197 for GATA1 and LMO2COM.

**Statistical Analysis SNP - Leptin Concentrations**

Almost all 14 genotyped SNPs were associated with leptin concentrations mainly during late pregnancy. Genotype SNP -282 *TT* had higher leptin concentrations during the whole period and SNP -201 *TT* only during the lactation period. SNP -211 showed no significant differences in leptin concentrations between the genotypes. Figure 2 represents a typical result for the other 11 SNPs. For this Figure SNP -578 was taken. The genotypes associated with higher and lower leptin concentrations are listed in Table 5.

**Statistical Analysis SNP - Dairy Traits**

A total of 14 SNPs were chosen to be genotyped for all 613 animals. An overview of the associations (*p*-values) between dairy traits and polymorphisms are listed in Table 6. Despite the close proximity between the polymorphisms, we found effects ($p<0.05$) on dairy traits for only one SNP per trait. SNP -1457 was associated with FPLA and ΔLW1M, SNP -963 was associated with FI, DMI, EB and FE and SNP -578 was associated with P%. The effect of the genotypes on the different traits are listed in Table 7. For SNP -1457 the *G* allele positively influences FPLA and also seems to increase weight loss from week 1 to the minimum weight. However, the heterozygous genotype *AG* seemed to be more favorable than the *GG* genotype. The *T* allele of SNP -963 had a positive effect on feed intake and energy balance, but a negative effect on first observed estrus, and the SNP -578 *G* allele had a positive effect on P%.

# DISCUSSION

**Sequence of the Bovine Leptin Promoter**

The bovine leptin promoter was sequenced for 1600 5'-flanking basepairs of the first untranslated exon of the leptin gene. Differences between *AB070368* (Taniguchi et al., 2002) and our sequence are probably caused by the different cattle breeds that were used. A total of 11 nucleotides were different between the two sequences. This is the first report of polymorphisms in the bovine leptin promoter. A total of 20 SNPs were found in the 1600 basepairs upstream of the first (untranslated) exon of the leptin gene. This means 1 SNP per 80 bp. These data are comparable with the results of Konfortov et al. (1999) who reported 1 SNP per 89 bp in the leptin gene.

**Table 5.** *Genotypes of SNPs with higher leptin concentrations compared to other genotypes during late pregnancy. The alleles of these genotypes are linked because of their close proximity and this might be an explanation of the uniform association with leptin concentrations during late pregnancy*

| SNP / genotype effect | -1457 | -1238 | -963 | -578 | -415 | -292 | -282 |
|---|---|---|---|---|---|---|---|
| □ High | AA | GG | CC | CC | Del G | TT | TT |
| X Medium | AG | GC | CT | CG | X | TC | GT |
| ○ Low | GG | CC | TT | GG | G | CC | GG |

| SNP / genotype effect | -272 | -211 | -201 | -197 | -170 | -147 | -105 |
|---|---|---|---|---|---|---|---|
| □ High | GG | GG | TT | X | CC | CC | CC |
| X Medium | GA | AG | CT | AC | CT | CT | CG |
| ○ Low | AA | AA | CC | AA | TT | TT | GG |

**Alignment with Mouse and Human Sequences**

Alignments with published promoter sequences of mouse (*U36238*) and human (*U43589*) revealed several conserved regions. These matched with the earlier described binding domains for TATA (-28 to -33), C/EBP (-49 to -58), LP1 (-85 to -90) and Sp1 (-93 to -101) (Hwang et al., 1996; Miller et al., 1996; Taniguchi et al., 2002). The second Sp1 site (-19 to -25) that was found by using TRANSFAC was not conserved between bovine, human and mouse, and according to Mason et al. (1998) and Fukuda and Iritani (1999), this is not the functional Sp1 site. The HIF-1 site (-118 to -122, *U43589*) in the human leptin promoter reported by Grosfeld et al. (2002) was not found in bovine or in mouse. Furthermore a conserved region at -211 to -217 (*CCCCCAA*) was seen, where SNP -211 is

located. This putative binding domain matches that of the transcription factor CMYB. This is a proto-oncogene involved in hematopoiesis, growth regulation and differentiation (Mucenski et al., 1991). As leptin regulates the amount and size of adipocytes and thus controls growth of fat mass, the CMYB binding site might have a function in the regulation of leptin expression.

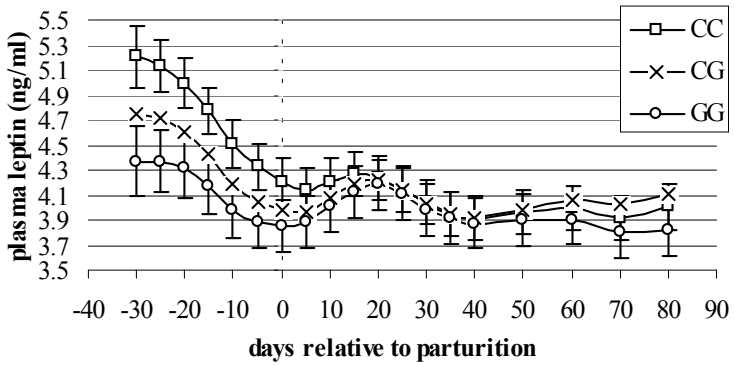


**Figure 2.** *Leptin concentrations during the periparturient period of SNP -578, which is a representative figure for all SNPs. Similar figures were found for 10 other SNPs. In Table 5 genotypes associated with higher and lower leptin concentrations during late pregnancy are listed.*

**Transcription Factor Binding Domains**

Several studies reported the transcriptional regulation of leptin by different binding domains. Miller et al. (1996) demonstrated that 217 bp of DNA upstream of the transcription start site controls basal human leptin gene expression. Several transcription factor binding domains are located at this conserved region and in our study using the TRANSFAC database these transcription factors were also found on the bovine leptin promoter.

C/EBP is an important factor for the transcription of most adipocyte genes and of other genes involved in energy metabolism (Darlington et al., 1995). Several groups mutated the C/EBP site of the leptin promoter in rat and mouse and demonstrated that this site was functional in mediating the effect on leptin gene expression (Hwang et al., 1996; Mason et al., 1998). Taniguchi et al. (2002) showed in a transfection study that C/EBP activates the promoter of the bovine leptin gene.

In another study using a leptin promoter/luciferase gene construct the Sp1 and LP1 binding domains were mutated and showed a respectively 2.5-fold and a 2-fold drop in luciferase activity. Also binding studies with adipocyte nuclear extract and mutated Sp1 and LP1 sites were performed. Sp1 is a zinc finger protein involved in the stimulation of leptin transcription by glucose and insulin, which in turn is inhibited by addition of

polyunsaturated fatty acids (Fukuda and Iritani, 1999). The factor that binds to LP1 and activates the leptin promoter has still to be discovered, however, it seems to be present in pre-adipocytes and adipocytes but not in other cell types (Mason et al., 1998). Thus, the LP1 factor may be important for fat-specific leptin expression.

ADD1/SREBP1 is a key transcription factor linking changes in nutritional status and insulin levels to the expression of certain genes that regulate energy metabolism. In mice this transcription factor strongly activats the leptin promoter (Kim et al., 1998). The sequence in mice is located at -52 to -47 (*CANNTG*) and this sequence is not present in bovine (*AB070368* and *AJ571671*) or human (*U43589*) at this site.

**Table 6.** *p-values of all associations of SNPs with traits. Significant (p<0.05) traits are listed in bold and trends (0.05<p<0.10) are underlined*

| SNP | -1457 | -1238 | -963 | -578 | -415 | -292 | -282 | -272 | -211 | -201 | -197 | -170 | -147 | -105 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LW1 (kg) | 0.543 | 0.259 | 0.304 | 0.436 | 0.446 | 0.086 | 0.278 | 0.103 | 0.449 | 0.313 | 1.000 | 0.083 | 0.176 | 0.192 |
| LW15 (kg) | 0.482 | 0.368 | 0.186 | 0.482 | 0.498 | 0.419 | 0.407 | 0.657 | 0.684 | 0.571 | 0.480 | 0.748 | 0.763 | 0.811 |
| ΔLW (kg) | 0.275 | 0.990 | 0.595 | 0.249 | 0.888 | 0.449 | 0.554 | 0.262 | 0.631 | 0.878 | 0.294 | 0.131 | 0.179 | 0.196 |
| LW (kg) | 0.176 | 0.144 | 0.164 | 0.212 | 0.480 | 0.154 | 0.454 | 0.270 | 0.741 | 0.383 | 0.624 | 0.310 | 0.387 | 0.543 |
| LWM (kg) | 0.114 | 0.110 | 0.372 | 0.179 | 0.458 | 0.278 | 0.379 | 0.391 | 0.705 | 0.330 | 0.920 | 0.407 | 0.512 | 0.571 |
| ΔLW1M (kg) | **0.027** | 0.533 | 0.340 | 0.065 | 0.888 | 0.533 | 0.852 | 0.463 | 0.741 | 0.923 | 0.888 | 0.270 | 0.463 | 0.458 |
| ΔLW15M (kg) | 0.468 | 0.403 | 0.657 | 0.543 | 1.000 | 0.779 | 0.217 | 0.583 | 0.533 | 0.827 | 0.108 | 0.368 | 0.383 | 0.415 |
| FI (kg/d) | 0.275 | 0.638 | **0.027** | 0.340 | 0.791 | 0.835 | 0.093 | 0.733 | 0.137 | 0.583 | 0.249 | 0.631 | 0.942 | 0.644 |
| DMI (kg/d) | 0.247 | 0.549 | **0.030** | 0.298 | 1.000 | 0.844 | 0.100 | 0.748 | 0.142 | 0.619 | 0.348 | 0.664 | 0.970 | 0.712 |
| EB (MJ/d) | 0.071 | 0.063 | **0.015** | 0.093 | 0.603 | 0.445 | 0.270 | 0.638 | 0.221 | 0.487 | 0.279 | 0.497 | 0.323 | 0.527 |
| MY (kg/d) | 0.289 | 0.502 | 0.748 | 0.154 | 0.920 | 0.664 | 0.748 | 0.932 | 0.142 | 0.270 | 1.000 | 0.852 | 0.298 | 0.771 |
| FY (g) | 0.252 | 0.264 | 0.852 | 0.217 | 0.632 | 0.440 | 0.878 | 0.803 | 0.126 | 0.174 | 0.340 | 0.638 | 0.383 | 0.861 |
| PY (g) | 0.307 | 0.905 | 0.990 | 0.270 | 0.920 | 0.748 | 0.583 | 0.914 | 0.081 | 0.252 | 1.000 | 0.763 | 0.454 | 0.819 |
| LY (g) | 0.208 | 0.432 | 0.625 | 0.076 | 0.752 | 0.454 | 0.819 | 0.869 | 0.125 | 0.350 | 0.920 | 0.684 | 0.183 | 0.651 |
| F% (g/kg) | 0.795 | 0.887 | 0.566 | 0.980 | 0.560 | 0.795 | 0.961 | 0.795 | 0.533 | 0.852 | 0.252 | 0.619 | 0.990 | 0.961 |
| P% (g/kg) | 0.094 | 0.116 | 0.317 | **0.014** | 0.647 | 0.482 | 0.346 | 0.395 | 0.914 | 0.844 | 1.000 | 0.264 | 0.235 | 0.206 |
| L% (g/kg) | 0.304 | 0.497 | 0.192 | 0.141 | 0.294 | 0.194 | 0.407 | 0.242 | 0.861 | 0.244 | 0.920 | 0.185 | 0.014 | 0.527 |
| FPLA (d) | **0.017** | 0.060 | 0.477 | 0.543 | 0.517 | 0.522 | 0.170 | 0.202 | 0.069 | 0.273 | 0.841 | 0.130 | 0.387 | 0.333 |
| FE (d) | 0.057 | 0.157 | **0.007** | 0.055 | 0.488 | 0.122 | 0.566 | 0.340 | 0.333 | 0.560 | 0.920 | 0.326 | 0.098 | 0.307 |

*Abbreviations:* LW1: live weights in week 1 of lactation, LW15: live weights in week 15 of lactation, ΔLW: the difference between LW15 and LW1, LW: mean live weight over the first 15 weeks of lactation, LWM: minimum live weight in the first 15 weeks of lactation, ΔLW1M: the difference between LWM and LW1, ΔLW15M: the difference between LWM and LW15, FI: feed intake, DMI: dry matter intake, EB: energy balance, MY: milk yield, FY: fat yield, PY: protein yield, LY: lactose yield, F%: fat percentage, P%: protein percentage, L%: lactose percentage, FPLA: first postpartum luteal activity, FE: first observed estrus.

PPARγ, induced by C/EBP, is a steroid superfamily transcription factor, which promoted differentiation of preadipocytes into adipocytes (Kliewer et al., 1995) and inhibited leptin expression in rodents (Zhang et al., 1996). PPARγ and C/EBP are antagonists, probably mediated by competition for a critical co-factor (Hollenberg et al., 1997). The DR+1 binding domain for PPARγ is located 4 kb upstream of the first untranslated exon of leptin in rats and was not found in this study because only 1.6 kb upstream the first exon of leptin was sequenced.

The 20 SNPs we found on the leptin promoter were not located in one of the known transcription factor binding domains important for the regulation of expression of leptin. However, the TRANSFAC database showed that several SNPs are located at core sites of transcription factor binding domains. For example SNP -1457 is located at a CMYB and VMYB binding domain. CMYB is a product of a proto-oncogene involved in hematopoiesis, growth regulation and differentiation (Mucenski et al., 1991) and VMYB, which also is a product of a proto-oncogene and interacts with CEBP (Mink et al., 1996). Further study will be needed to determine the possible function of these transcription factor binding domains in the leptin promoter region. Other putative transcription factor binding sites were detected at the location of found SNPs (AP1, CAAT, GATA1, LMO2COM, MYCMAX, NFAT, NFY, RFX1 and USF) but the functions of these binding sites are not known yet (Table 4).

In the first 104 basepairs before the start site where the most important regulation sites like Sp1, C/EBP and TATA are located no SNPs were found. Furthermore, this region was conserved over species. This confirms the importance of the transcription factor binding domains in this region. Furthermore, between basepairs -578 and -901 no SNPs were found in the bovine leptin promoter region, which might indicate an important region for leptin expression in cattle. However, no similarity of this region with human and mouse sequences was found, which suggests that if this region is important for leptin expression in cattle, this might be species dependent.

**Association between Genotype and Leptin Concentrations**

Due to linkage between all 14 SNPs, associations were found with leptin concentrations during late pregnancy for almost all typed polymorphisms. These effects were also found with SNPs in the coding sequence of the leptin gene (Liefers et al., 2003b). There we suggested a functional mutation in exon 2 (R4C) that may induce a structural change in the leptin molecule. This conformational change could lead to a different binding stringency with the soluble form of the leptin receptor, which is mainly present during pregnancy. Because the SNPs at the leptin promoter are not located on known important transcription factor binding domains, it is not obvious that one of the promoter SNPs is the functional mutation for differences in expression of leptin during pregnancy. The SNPs at

the leptin promoter region are probably strongly linked with R4C, which could be the functional mutation.

A second possibility to clarify the association of the SNPs with leptin concentrations was also mentioned earlier (Liefers et al., 2003b). The presence of a placental enhancer (PLE) located 1.9 kb upstream the leptin gene in human (Bi et al., 1997; Ebihara et al., 1997) could play an important role as the effect on leptin concentration is only present during pregnancy. We only sequenced 1.6 kb upstream the leptin gene, so could not detect possible SNPs in this enhancer. However, in the alignment of the bovine, human, and mouse sequences, no clear conserved region could be detected in the human PLE region nor identical PLE sequences could be detected in bovine or mouse, indicating that the placental enhancer PLE is only present in human. Furthermore, several research groups mentioned that the ruminant placenta has negligible levels of leptin mRNA throughout gestation, making this explanation less probable (Ehrhardt et al., 2001; Bispham et al., 2003). Therefore the first explanation of R4C being the functional mutation seems to be more probable.

**Table 7.** *SNPs with a significant effect on different traits. The genotype with the highest frequency was denoted as zero and the deviation with SE of the other two are listed in this table*

| SNP | Trait | Genotype | Effect | ± | SE |
|---|---|---|---|---|---|
| -1457 | FPLA (d) | AG | -7.1 | ± | 2.5 |
| | | GG | -4.8 | ± | 3.2 |
| -1457 | ΔLW1M (kg) | AG | 7.0 | ± | 2.6 |
| | | GG | 3.9 | ± | 3.4 |
| -963 | FI (kg) | CT | 0.7 | ± | 0.3 |
| | | TT | 0.8 | ± | 0.5 |
| -963 | DMI (kg) | CT | 0.4 | ± | 0.2 |
| | | TT | 0.5 | ± | 0.3 |
| -963 | EB (MJ/d) | CT | 3.4 | ± | 1.2 |
| | | TT | 3.0 | ± | 2.3 |
| -963 | FE (d) | CT | 16.1 | ± | 7.6 |
| | | TT | 40.5 | ± | 14.1 |
| -578 | P% (g/kg) | CG | 0.7 | ± | 0.3 |
| | | GG | 0.6 | ± | 0.2 |

*Abbreviations:* FPLA: first postpartum luteal activity, ΔLW1M: the difference between the minimum live weight and live weight in week 1, FI: feed intake, DMI: dry matter intake, EB: energy balance, FE: first observed estrus, P%: protein percentage in milk.

**Association between Genotype and Dairy Traits**

First luteal activity for the SNP -1457 heterozygous genotype (*AG*) was found 7.1 days earlier than for the homozygous genotype *AA*, and 4.8 days earlier for the *GG* genotype. For FPLA the standard deviation is 17.5 days (Liefers et al., 2003a) thus the improvement of FPLA due to the SNP -1457 *AG*-genotype is about 0.4 standard deviation. This SNP might be used as marker for FPLA in future breeding programs to improve fertility in high yielding dairy cattle. The SNP -1457 *AG*-genotype also shows a decrease in weight loss between week 1 and its minimum weight and a tendency ($0.05<p<0.10$) for a higher P%, an improved EB, and an earlier FE. Furthermore, SNP -1457 is located at a putative transcription factor-binding site of CMYB and VMYB. These might have a role in the associations found. Further study will be needed to determine if SNP -1457 is a functional mutation or a mutation in linkage disequilibrium with the causal mutation. Taking this into account, SNP -1457 might be a good marker to use in future breeding programs.

The *T*-allele of SNP -963 positively influences EB, probably because it also causes a higher DMI. However, it seems that FE is negatively influenced. The *G*-allele of SNP -578 is associated with a higher P%.

Several studies were performed in search of QTLs for dairy traits on Chr 4, where the leptin gene is located (82.8 cM). Lindersson et al. (1998) reported QTLs for milk production traits close to the leptin gene. They found QTLs for milk, fat, and protein yield at 65 and 85 cM, and for F% and P% at 75 and 95 cM respectively. However, when these authors tested for an effect of the obese locus using BM1500 and BM1501 microsatellite no associations were found with milk production traits. Spelman et al. (1999) published an association of marker TGLA116 on Chr 4 with overall opinion of the cow by the dairy farmer and Ashwell et al. (1999) detected in a genome scan several loci affecting milk, health, and type traits, but only an association for teat length was found on BTA 4 (marker BL21, 63.9 cM). Schrooten et al. (2000) reported an association of gestation length between markers TGLA159 and TGLA420 (17 cM) but found no associations for other functional traits.

Overall, we can conclude that the bovine leptin promoter has 3 polymorphisms located between -1600 and 0 upstream the transcription start site which are associated with dairy traits like fertility, energy balance, and protein percentage. These SNPs are not located on important known transcription factor binding domains on the bovine leptin promoter and therefore might not be the functional mutations but SNPs in close linkage disequilibrium with functional mutations on genes in close vicinity of the leptin locus.

In summary, in this study we investigated the association of genetic polymorphisms in the bovine leptin promoter with leptin concentrations during the periparturient period and with dairy traits. The associations found with leptin concentrations during late pregnancy might not be ascribed to the SNPs found on the promoter region but rather to the R4C mutation on exon 2. Associations with several SNPs were found for dairy traits like fertility and energy balance. After SNPs -1457 and -963 have been validated with a larger dataset

these might be good candidates for marker assisted breeding for better fertility and energy balance without significantly influencing milk yield in dairy cattle.

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# CHAPTER 8

# General Discussion

# GENERAL DISCUSSION

The study described in this thesis was performed to obtain a better understanding of the role of leptin in the periparturient cow on physiologic and genetic level and to investigate opportunities to use information on the leptin and leptin receptor gene to improve selection for production and reproduction traits in dairy cows. In this chapter results of this study will be evaluated and discussed in relation to recently published literature. Also results of some additional calculations that were performed recently, in order to answer questions that rose during the process of writing this discussion, will be described.

## HIGH LEPTIN CONCENTRATIONS DURING PREGNANCY

### The Reason of High Leptin Concentrations during Pregnancy

In Chapter 4 we described that during pregnancy leptin concentrations in plasma were higher than during early lactation. Pregnant cows have a normal feed intake and this might indicate that during pregnancy a state of leptin resistance at hypothalamic level exists. In some cases of obesity, food intake is not inhibited even if blood leptin levels increase and this is thought to be due to the failure of leptin to enter the hypothalamus (Kastin et al., 1999). As a result no negative feedback signal to the adipocytes is present and leptin expression will not be inhibited.

Several mechanisms can explain this leptin resistance state. First, the decreased hypothalamic expression of the signaling form of the leptin receptor (Ob-Rb), which was investigated in pregnant rats by Garcia et al. (2000) and Seeber et al. (2002). However, these results were in conflict. In ruminants, no studies on hypothalamic leptin receptor expression during pregnancy has been performed yet. Second, changes in leptin permeability at the blood brain barrier (BBB) during pregnancy might play a role. Recent studies in rats show that leptin levels in the brain, but not in plasma, were linked to the time of feeding in rats, which might direct to differences in leptin transport across the BBB (Asakuma et al., 2003). Impairment of the transport of leptin across the BBB might be responsible for the leptin resistance during pregnancy. Due to this leptin resistance during pregnancy, the negative feedback signal is not present which results in an increased leptin expression. This was confirmed in studies with rodents (Kawai et al., 1997; Tomimatsu et al., 1997) and sheep (Ehrhardt et al., 2001).

Furthermore, other studies showed that not only the lack of a negative feedback was responsible for the high leptin levels during pregnancy. The higher adiposity level during pregnancy (Ehrhardt et al., 2001) might also contribute to the increase in leptin levels and we showed that prepartum leptin concentrations in cows were related to live weight (Chapter 4). In humans, placental leptin seemed to play an important role (Masuzaki et al., 1997; Bodner

et al., 1999). However, in rodents and ruminants the placenta only produced negligible levels of leptin (Kawai et al., 1997; Amico et al., 1998; Kronfeld-Schor et al., 2000; Thomas et al., 2001; Ehrhardt et al., 2002; Bispham et al., 2003). The loss of the placenta did not have effect on leptin levels in cows (Block et al., 2001) and leptin levels already started to decline 30 days before parturition (Liefers et al., 2003a). The increase of the soluble form of the leptin receptor (Ob-Re), which binds to circulating leptin and extend its half-life, contributed to elevated plasma leptin during pregnancy in mice (Gavrilova et al., 1997), but in ruminants leptin binding activity of plasma from pregnant cows is negligible (Ehrhardt and Boisclair, unpublished results cited in: Leury et al., 2003). Finally, Kronfeld-Schor et al. (2000) showed that pregnancy-associated hormones also stimulated leptin expression.

In conclusion, the explanations for the elevated plasma leptin during pregnancy in ruminants seem to be the increase in adipose tissue leptin mRNA due to the lack of a negative feedback and the increase in adiposity, whereas placental leptin does not seem to play an important role. In ruminants, no studies have been performed yet to investigate the changes in expression of circulating Ob-Re and the long form of the leptin receptor in the hypothalamus (Ob-Rb) during pregnancy.

**The Function of High Leptin Concentrations during Pregnancy**

Several studies have been performed to explain the function of pregnancy-induced high leptin concentrations. Malik et al. (2001) performed a study in normal and *ob/ob* mice to investigate the role of leptin during pregnancy and lactation. They reported that leptin was essential for normal pre-implantation and/or implantation processes during early pregnancy. It was also essential for normal development of the mammary glands during the last weeks of pregnancy, but was not required for pregnancy and parturition once implantation has been established. Leptin in humans seemed to be important for maintenance of pregnancy as studies have shown low concentrations of leptin in women who subsequently miscarry (Laird et al., 2001). During late pregnancy transplacental passage of maternal leptin to the fetus is a source of fetal leptin (Smith and Waddell, 2003) and may provide a growth promoting signal for fetal development (Schulz et al., 2000). Maternal leptin might be a key player in the hormonal regulation of early fetal growth also because fetal adipose tissue did not synthesize leptin until late gestation (Ehrhardt et al., 2002). The soluble form of the leptin receptor (Ob-Re) and one of the short forms of the leptin receptor, Ob-Ra, might be responsible for the leptin transport across the placenta (Schulz et al., 2000; Smith and Waddell, 2003). However, in ruminants leptin binding activity of plasma from pregnant cows is negligible (Ehrhardt and Boisclair, unpublished results cited in: Leury et al., 2003) and therefore no clear evidence of the presence of Ob-Re in ruminants is present yet.

## THE DECLINE IN LEPTIN LEVELS TOWARDS PARTURITION

In Chapter 4 we showed that plasma leptin concentrations were elevated during late pregnancy and declined towards parturition. These results supported earlier reports about periparturient leptin concentrations in dairy cows (Block et al., 2001; Kadokawa et al., 2000). Several hypotheses have been proposed for the reasons of this decline towards parturition, which will be discussed in this section.

In Chapter 4 we suggested that leptin levels reflect the energy balance of the cow. Towards lactation the negative energy balance is already initiated due to a decline in DMI together with an increased energy demand of the fetus (Hayirli et al., 2002). As a result adiposity decreases, which also decreases leptin expression. Block et al. (2001) showed that there was a large similarity between the periparturient energy balance and plasma leptin curves. This suggests that a possible explanation of the decline in leptin levels towards parturition is the mobilization of adipose tissue. Also the inhibition of leptin expression by adipose tissue in early lactation, which was demonstrated by Block et al. (2001) and Sorensen et al. (2002) could be one of the reasons of the low leptin levels during lactation.

Another option is the regulation of leptin expression by insulin and growth hormone (GH). The onset of negative energy balance around parturition was also associated with decreased plasma insulin and increased plasma GH (Block et al., 2001), suggesting that both hormones could mediate a portion of the effect of energy balance on plasma leptin. Evidence supporting this idea exists in rodents and humans, with insulin increasing and GH decreasing plasma leptin (Ahima and Flier, 2000). Recently, Leury et al. (2003) performed a study to investigate the effect of insulin and GH on periparturient leptin levels in cows. Administration of insulin increased circulating leptin in both late pregnant (5 and 2 weeks prepartum) and early lactating (1 and 5 weeks postpartum) cows whereas GH treatment did not reduce plasma leptin, which suggested that GH has no direct effect on leptin concentrations during the periparturient period. Also during lactation, insulin regulates plasma leptin but GH treatment has no effect on plasma leptin (Block et al., 2003b). Sauerwein et al. (2003) investigated early pregnant (10 weeks) and non-pregnant (100 days postpartum) cows and found that only during early pregnancy GH treatment reduced leptin concentrations.

Recapitulating these options, we might conclude that the decline in plasma leptin levels towards parturition is probably caused by several mechanisms. These are the decline in adiposity, the decreased expression of leptin in adipose cells and the decline in insulin concentrations. GH only seems to play a role in regulating leptin levels during early pregnancy.

Several investigations have been performed to elucidate the possible biological function of the low leptin levels during lactation. It was proposed that the increase in feed intake (hyperphagia) during lactation could be driven by the decrease in leptin levels (Pickavance et

al., 1998; Woodside et al., 2000; Sorensen et al., 2002) but others suggested that the lactational hypoleptinemia only acts together with other signals to induce lactational feed intake (Vernon et al., 2002; Denis et al., 2003).

## LEPTIN CONCENTRATIONS AND DAIRY TRAITS

We found that leptin concentrations during lactation reflect the energy balance state of the cow (Chapter 4, Figure 4) A high leptin concentration means that energy reserves have accumulated and that the new adipose tissue that has been formed produces leptin. Block et al. (2001) measured NEFA, GH, glucose and insulin during lactation and showed a positive correlation of leptin with glucose and insulin and a negative correlation with NEFA and GH. This also proves that a negative energy balance is associated with low leptin levels. These low concentrations stimulate feed intake to recover from the negative energy balance and rebuild fat reserves.

No relation of leptin levels with first postpartum luteal activity was found, although leptin concentrations tended to increase towards first ovulation (Chapter 4 Figure 6, and Huszenicza et al. (1999)). As the level of leptin did not seem to influence the induction of first ovulation, other regulatory mechanisms in hypothalamus, hypophysis and ovary might play a role in the onset of first postpartum luteal activity. During pregnancy a leptin resistance state is present, which prevents a decrease in feed intake, but also might prevent ovulation. After parturition this resistance state is decreased because more leptin receptors, both long (signalling) and short (transport) forms, are expressed at hypothalamic level (Garcia et al., 2000; Sorensen et al., 2002) which results in a higher sensitivity to leptin at hypothalamic level. A possibility is that a certain level of hypothalamic sensitivity to leptin has to be reached before GnRH neurons are activated and able to stimulate gonadotrophin secretion from the hypophysis. However, as leptin also directly stimulates the hypophysis (LH and FSH secretion) and ovary (steroidogenesis), postpartum changes in sensitivity to leptin as a result of high or low leptin receptor expression might also be present in these organs.

## THE LEPTIN GENE AND TRAITS IN DAIRY AND BEEF CATTLE

### The Leptin Gene

Interest in the bovine leptin gene has increased during the last few years. In 1999, Konfortov et al. reported a study to investigate DNA sequence diversity in two genes (leptin and APP) in a diverse panel of cattle. Both introns and exons were sequenced and a total of 20 polymorphisms in the leptin gene were detected. The frequency of polymorphisms in the

leptin gene was 1 per 84 base pairs in exonic sequences whereas the APP gene had a frequency of 1 per 156 base pairs in exonic sequences. This indicates that the leptin exons have a high mutation frequency. In this section an overview of the SNPs around the two coding exons of the leptin gene will be given and the results of association studies will be discussed. Main findings are summarized in Table 1 and Figure 1.

**Table 1.** *Overview of association studies that have been performed with polymorphisms in the leptin gene and important traits in dairy and beef cattle. Number of animals used in each study are presented*

| 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) |
| R4C* | Carcass Traits | Beef | 154 | Buchanan et al. (2002) |
| | No | Dairy | 623 | Liefers et al. (2003c) |
| | No | Beef/Dairy | 246 | Lagonigro et al. (2003) |
| | Milk yield<br>Protein yield | Dairy | 416 | Buchanan et al. (2003) |
| RFLP1 | Milk yield<br>Protein yield | Dairy | 623 | Liefers et al. (2002) |
| LEP*Sau3AI* | Calving Interval<br>Weight at first Calving | Beef | 149 | Almeida et al. (2003) |
| LEP*BsaAI* | No | Beef | 96 | Almeida et al. (2003) |
| A59V* | No | Dairy | 623 | Liefers et al. (2002) |
| | No | Beef | 100 | Almeida et al. (2003) |
| BM1500 | No | Dairy | 623 | Liefers et al. (2002) |
| | Fat Deposition | Beef | 158 | Fitzsimmons et al. (1998) |
| | No | Beef | 102 | Almeida et al. (2003) |

*exonic polymorphisms.

We genotyped a total of 623 cows for 3 SNPs and one microsatellite (R4C, RFLP1, A59V and BM1500) and performed an association study on dairy traits (Chapter 3). Because of the putative conformational change in the leptin molecule, the R4C polymorphism received a lot of attention by several research groups. We and others (Lagonigro et al., 2003) did not find any association of R4C with dairy and beef traits (Holstein Friesian n=613; British Friesian, Aberdeen Angus, Hereford, Highland and Charolais n=245) which is in contrast with results of Buchanan et al. (2002, 2003) who found associations for carcass content in beef cattle (Angus, Charolais, Hereford, Simmental, n=154) and milk and protein yield in dairy cattle (Holstein, n=416). In our study RFLP1 turned out to be associated with milk yield. Almeida et al. (2003) studied the RFLP1 SNP for reproductive performance in beef cattle (Brangus Ibagé; 5/8 Aberdeen Angus x 3/8 Nelore) and did not find associations for weight at first calving or calving interval. Next to our study where no associations were

found with A59V, no other studies were performed on A59V in dairy or beef cattle. Also for the BM1500 microsatellite we found no association confirming the results of Lindersson et al. (1998) who also found no associations of BM1500 with milk, fat, and protein yields.

Recently, Lagonigro et al. (2003) reported a new non-conservative mutation in exon 2 of the leptin gene. They used 5 different breeds (British Friesian n=49, Aberdeen Angus n=42, Hereford n=50, Highland n=48 and Charolais n=56) and the frequency of the rare allele ranged from 0 (Highland) to 15.2% (Charolais). Frequencies of British Friesian, Aberdeen Angus and Hereford were 4.1, 3.6 and 12% respectively. This polymorphism (252; Figure 1) caused a change in amino acid in the signalling sequence of the leptin protein. Animals with one rare allele had 19% higher mean feed intake than individuals with no rare allele. Only a few individuals with two rare alleles were detected and thus this genotype could not be included in the analysis. Next to Lagonigro et al. (2003), also Buchanan et al. (2003) and Konfortov et al. (1999) sequenced the two coding exons of the leptin gene for different breeds, and they also did not detect this polymorphism probably because of the low number of animals that were sequenced in these studies. A total of 60 Holstein Friesian cows were sequenced for exon 2 and 3 of the leptin gene (Chapter 5) and this polymorphism was not found either. Supposedly Holstein Friesian cows do not have this polymorphism, just like the Highland breed. Lagonigro et al. (2003) also genotyped SNPs 103, 126, R4C and A59V (Figure 1) but did not find associations with beef traits.

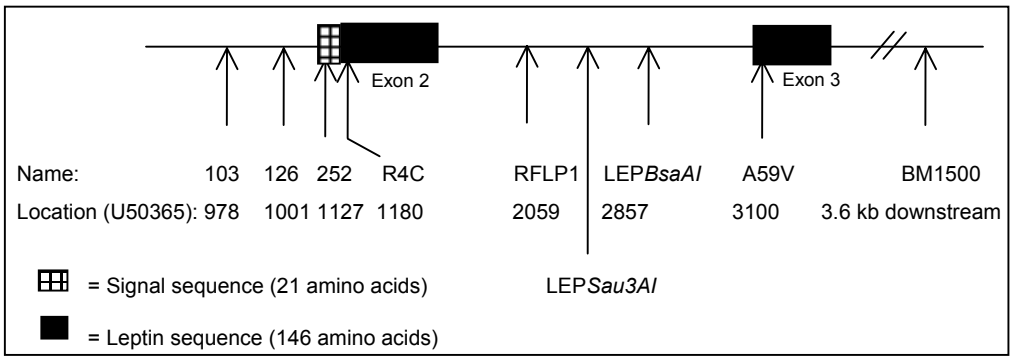


**Figure 1.** *Organization of the bovine leptin gene and the names and locations of the polymorphisms used in association studies. Exon 1 is located approximately 17 kb before exon 2 and is not transcribed into protein . The exact location of the Sau3AI RFLP is not known. Location numbers refer to Genbank Accession U50365.*

Another association with a leptin SNP was described by Almeida et al. (2003). The PCR-based polymorphism as described by Pomp et al. (1997) showed a second rare polymorphism. The restriction enzyme *Sau3AI* digested the PCR product four times instead of three times. According to the information given by Pomp et al. (1997), this *Sau3AI* polymorphism is probably located between polymorphisms RFLP1 and LEP*BsaAI*. Almeida

et al. (2003) genotyped a total of 149 Brangus Ibagé and found a frequency of 0.06 for the rare allele, which was associated with a longer calving interval and a higher weight at first calving (Table 1). However, a frequency of 0.06 in 149 animals means that approximately 9 animals had the polymorphism and thus the power of this experiment was low.

Candidate gene tests must be interpreted with caution because spurious results can occur because of linkage disequilibrium to the causative gene. Recently Ovilo et al. (2002) showed that the analysis of the effects of candidate genes were dependent on the model used. When the candidate gene model was fitted, both candidate genes showed significant effects on the traits of interest, but when the candidate gene effect was included in a QTL regression analysis these associations were not observed. This suggests that the found polymorphisms were not the causal mutations responsible for the effects found. The main difference between the QTL model and the candidate gene model concerns the inclusion of the probabilities of the parental origin of the QTL alleles, which is ignored in the candidate gene model. However, the QTL regression model with the effect of the candidate gene included can present problems when the candidate gene and the QTL are closely linked. Also gene frequencies being higher in certain subsets of the data can be a problem, but in our analysis we included sire as fixed effect next to the pedigree to avoid within family effects.

Lindersson et al. (1998) reported QTLs for milk production traits on BTA 4, close to the leptin gene, which is located at 82.8 cM. They found QTLs for milk, fat, and protein yield at 65 and 85 cM, and for fat and protein percentage at 75 and 95 cM respectively. However, when these authors tested for a direct effect of the obese locus using BM1500 and BM1501 microsatellite no associations were found with milk production traits.

Overall, we can conclude that the leptin gene was studied intensively the last few years and this gene seems to be important for beef and dairy traits. There are three non-conservative exonic polymorphisms on the leptin gene (252, R4C, A59V) and a study using a set of all (seven) possible leptin molecules would be useful to get insight in the function of these three non-conservative polymorphisms. For example, *in vitro* binding studies to investigate differences in binding capacity to the leptin receptor would be useful to get insight in the functionality of (a combination of) the three non-conservative polymorphisms.

**The Leptin Promoter Region**

In Chapter 7 the first report of SNPs in the promoter region of the leptin gene is presented. A total of 20 polymorphisms were found in the sequenced region of 1600 bp, thus there is frequency of polymorphisms of 1 per 80 bp in the leptin promoter region, which is comparable with the polymorphism frequency in the leptin gene (1 per 84 bp). A total of 14 polymorphisms in the leptin promoter region were genotyped for 613 animals and one SNP was associated with the fertility trait FPLA (SNP -1457) and one SNP was associated with EB and DMI (SNP -963). Putative leptin transcription factor binding sites being important for

leptin expression in bovine have been reported previously to be located within the first 250 5'-flanking basepairs before the transcription start site (Taniguchi et al., 2002). However, possible enhancers further upstream the transcription start site, which are not discovered yet, could be important in the regulation of leptin expression and this could have influence on FPLA, EB, and DMI.

We found three SNPs at the leptin gene and its promoter region to be associated with milk yield, energy balance and first postpartum luteal activity. All these traits could be used for selection and therefore we tried to find an optimal combination of these genotypes. If a combination of genotypes would give us a cow with both a good fertility, a good energy balance and a good milk yield, this combination would be preferable above three separate genotypes. To obtain this optimal genotype combination we used ASREML to predict first postpartum luteal activity, energy balance and milk yield for a combination of SNPs -1457, -963, and RFLP1. Results are shown in Figure 2. The three genotype combinations with a good MY were *AA/CC/AA*, *AA/CC/AB*, and *AG/CT/AB*. For EB these three were *GG/CC/AA*, *AG/CT/AA*, and *GG/CT/AA*. For FPLA the three best genotype combinations were *AG/CC/AA*, *GG/CC/AA* and *AG/CT/AB*. So the two genotype combinations *GG/CC/AA* and *AG/CT/AB* seemed to be the best to use in a study with a larger dataset of cows to confirm the associations found. Then one of these two genotype combinations could turn out to be useful in future selection programs. However, the real importance for breeding is the economical value of the different traits. For example the difference in economic value of the gain of 1 kg milk per day and an 1 day earlier FPLA has to be calculated and by using these economic values the best genotype combination can be defined.

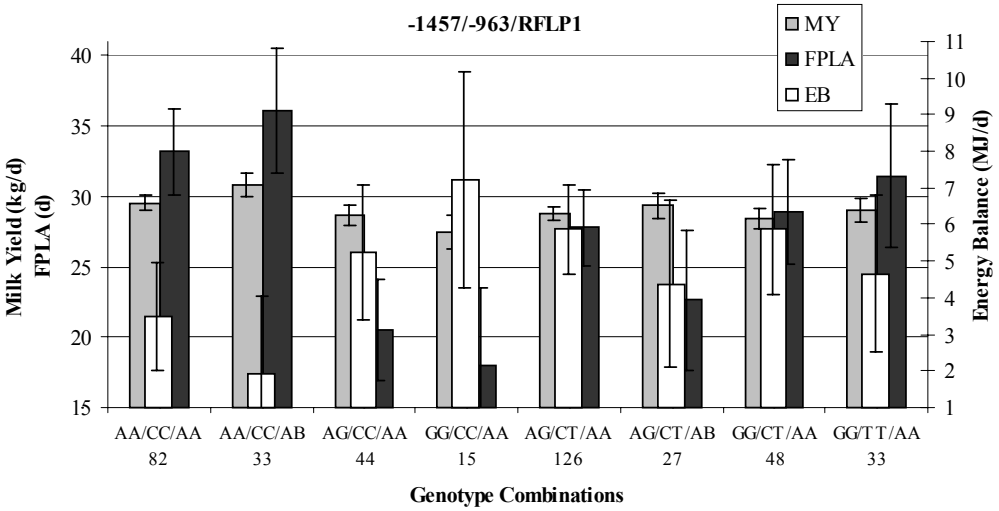


**Figure 2.** *Effects of genotype combination -1457/-963/RFLP1 on milk yield (MY), energy balance (EB) and first postpartum luteal activity (FPLA). MY and FPLA are read from the left axis, EB from the right axis. Numbers of animals in each group are mentioned below the genotype combination on the x-axis.*

In the previous section about the high leptin concentrations during pregnancy, we suggested that further research is necessary to reveal the function of the high leptin concentrations during pregnancy. To do this kind of research it might be useful to select cows with a high and low leptin concentration during pregnancy. In Chapters 5 and 6 we showed that a relation exists between prepartum leptin concentrations and polymorphisms on the leptin gene and its promoter region and on the leptin receptor.

**Table 2.** *Percentage of the variance that is explained by the different genotype combinations*

| Genotype Combination | % Variance |
|---|---|
| -147 | 10.0 |
| -147 and -282 | 12.4 |
| -147 and -282 and -197 | 14.3 |
| -105 | 10.4 |
| -105 and -197 | 12.3 |
| -105 and -197 and A59V | 13.5 |

In order to find out which of these SNPs explained most of the variance in prepartum leptin concentrations, we used the backward and forward analysis of the statistical program Genstat 6.1 (unpublished results). All 19 SNPs (14 on the leptin promoter region, 4 on the leptin gene and 1 on the leptin receptor gene) were added in the analysis together with the fixed effects sire, genetic group, sample date, age at calving (polynomial) and days before parturition (polynomial). It turned out that SNP -105 and SNP -147, located on the leptin promoter, explained respectively 10.4% and 10.0% of the variance in prepartum leptin concentrations (Table 2), whereas the other 17 individual SNPs each explained 7.4% or less (over and above the variance explained by the fixed effects). The difference in leptin concentrations between the SNP -147 genotypes *TT* and *CC* was 1.1 ng/ml. Together with SNPs -197 and -282 14.3% of the variance was explained. Here the difference between the highest and lowest leptin concentration was 2.1 ng/ml. The three found SNPs (-147, -197, and -282) are all located on a small region of the leptin promoter, which indicates that this region seems to be important for pregnancy-induced leptin synthesis. The leptin gene itself and the leptin receptor gene seemed to be less important. SNP -105 explained 10.4% of the variance in prepartum leptin concentrations individually, and a combination with two other SNPs did explain 13.5% of the variance (Table 2).

The combination of the three SNPs -147, -197, and -282 could be used to reveal the function of leptin concentrations during pregnancy. Figure 3 shows the most apparent genotype combinations (with 10 or more animals per group) with their prepartum leptin

concentrations. The two extreme combinations *TT/AA/GG* (low) and *CT/AA/GT* (high) could be used to investigate the function of leptin concentrations in pregnant cows.

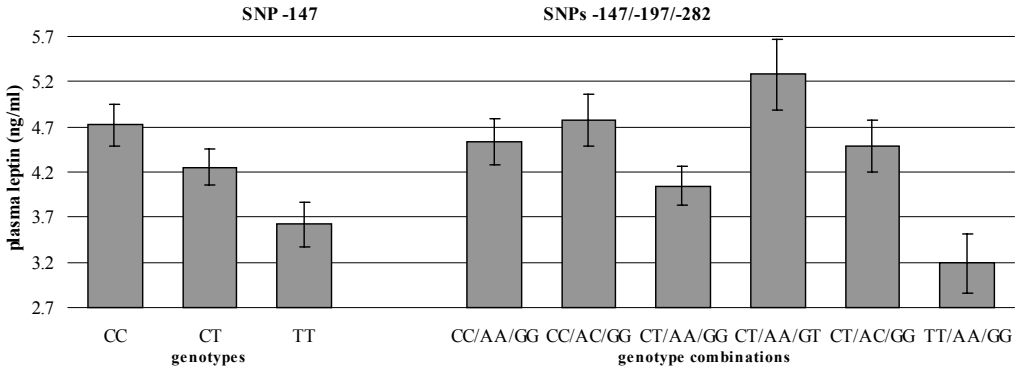


**Figure 3.** *Prepartum leptin concentrations for SNP -147 and for the best explaining combination of SNPs: -147/-197/-282. Only genotype combinations represented by 10 or more animals are shown. Number of animals per combination: CC/AA/GG 52; CC/AC/GG 31; CT/AA/GG 69; CT/AA/GT 13; CT/AC/GG 32; TT/AA/GG 22.*

We hypothesize that on this region of 135 bp, in the bracket between SNP -282 and SNP -147, a pregnancy-dependent enhancer exists in cattle. The promoter of the leptin gene in humans seems to have a special enhancer to produce leptin in the placenta (Bi et al., 1997), whereas in ruminants negligible expression of placental leptin was found. So maybe ruminants have instead of a placental enhancer a pregnancy-dependent enhancer on the promoter site of leptin. This might explain the differences found in prepartum leptin concentrations between different genotypes. In Chapter 7 we determined putative transcription binding domains on the leptin promoter region and mentioned that around SNP -197 a GATA1 and a LMO2COM binding site were located. Around SNP -282 a VMYB binding site is present. GATA1 interacts with CEBP in erythroid cells and affects differentiation and expression of erythroid genes (Blobel et al., 1998). CEBP, which is located at position -58 on the bovine leptin promoter and regulates leptin gene expression in mouse, rat and bovine (Hwang et al., 1996; Mason et al., 1998; Taniguchi et al., 2002). It could be hypothesized that during pregnancy an interaction between GATA1 and CEBP might be important for leptin expression. If GATA1 is expressed in adipose tissue, this might explain the differences in prepartum leptin concentrations found with SNP -197.

It is interesting to note that SNP -282 was significantly associated with leptin concentrations during the whole periparturient period and also close to significant effects ($0.05<p<0.10$) were found in this region (-147 to -282) for DMI (SNP -282, $p=0.10$), FPLA (SNP -211, $p=0.07$), LW1 (SNP -170, $p=0.08$), and FH (SNP -147, $p=0.10$) (Chapter 7). Therefore we investigated the effect of the leptin promoter genotype combinations on leptin

concentrations during the whole periparturient period using the same spline function as described in Chapter 6. Figure 4 presents the splines for the genotype combinations of SNPs -147/-197/-282 with 10 or more cows. Average standard error of the differences was 0.2 ng/ml. Genotype combinations *CT/AA/GT* and *CC/AC/GG* (high leptin concentrations) showed significant differences during late pregnancy with the combinations *CT/AA/GG* and *TT/AA/GG* (low leptin concentrations), which was also observed in Figure 3. Surprisingly, also during lactation significant differences were observed between genotype combinations *CT/AA/GT* and *TT/AA/GG*. This effect during lactation might be a carry-over effect from the pregnancy-induced effect discussed before but other regulating functions can not be excluded. No significant effects were found for the dairy traits of these combinations probably because too many genotype combinations resulted in too few animals available per combination. For example the two extreme genotype combinations contained only 13 and 22 animals.

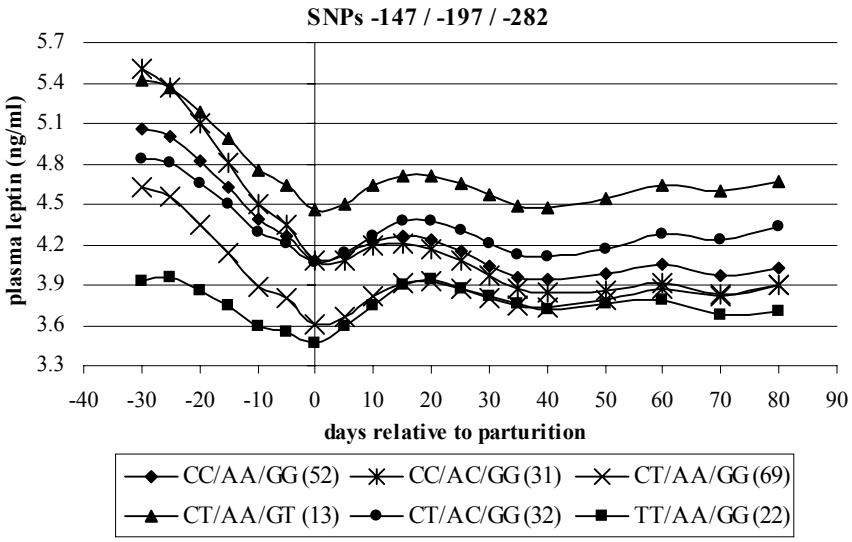


**Figure 4.** *Effect of SNP -147/-197/-282 genotype combinations on leptin concentrations during the periparturient period. Only genotype combinations with 10 or more animals are presented, number of animals are listed between brackets. Average standard error of the differences was 0.2 ng/ml.*

## THE R4C POLYMORPHISM

In Chapter 5 we suggested an important role for the *C* to *T* polymorphism at exon 2 of the leptin gene. This polymorphism results in an arginine to cysteine change of amino acid 4 (R4C) of the leptin molecule. During pregnancy, the *TT* genotype showed significantly lower leptin levels than the *CC* genotype. The *T* allele might cause a structural change in the leptin protein and therefore leptin may bind with less stringency to the soluble leptin receptor, which could lead to a faster clearance-rate of prepartum leptin levels. In this Chapter (8) we showed that three SNPs on the leptin promoter region might also play an important role in regulating prepartum leptin concentrations.

Results of several studies did not provide convincing evidence that the R4C polymorphism influences the function and structure of leptin. We did not find any association of R4C with dairy traits (DMI, $p$=0.482; MY, $p$=0.517; EB, $p$=0.320; FPLA, $p$=0.684) and Lagonigro et al. (2003) did not find an association with feed intake and fat-related traits in 5 cattle breeds. Furthermore Dridi et al. (2000) performed transfection studies with chicken leptin which has an unpaired cysteine on position 3 (instead of position 4) of the leptin protein and with mutated chicken leptin without this unpaired cysteine and this did not lead to any functional difference of leptin in chicken.

However, recently Buchanan et al. (2003) reported an association of R4C with milk and protein yield in dairy cattle. A total of 416 cows in 11 herds were tested, but because sire information was not available the results found could be a sire effect instead of an effect of genotype. Furthermore, this polymorphism was found to be associated with carcass fat content in beef cattle (Buchanan et al., 2002). Referring to these results, recently a commercially available DNA test to determine the R4C genotype for leptin in cattle was developed (http://www.igenity.com/US%20Version/igenity_testing.html). It was suggested that farmers could use this test to make more informed, strategic decisions regarding breeding and management. The *TT* genotype is claimed to result in greater DMI potential, higher peak lactation, improved body condition score and improved energy utilization (a shorter period in negative energy balance). This is a remarkable development considering that we and others (Lagonigro et al., 2003) did not find any relation between R4C and dairy and beef traits. The producer of the commercial test suggested that cows with the *TT* genotype would have higher leptin levels because *TT*-leptin would be largely unrecognized by the normal leptin receptors and this would result in a silencing of the negative feedback. They postulated that, as higher leptin levels reflected a positive energy balance in dairy cows (Chapter 4) and predicted a good carcass composition in beef cattle (Geary et al., 2003), farmers should select for *TT* genotype cows. However our results showed (Chapter 5) that not the *TT* genotype but the *CC* genotype had higher leptin levels. This result can not be ascribed to the different binding stringency of R4C-leptin in the RIA assay because then also during lactation differences would have been observed. Before commercially selling this test

to farmers with the intention to improve breeding values both in dairy and beef cattle, binding studies have to be performed to prove the impaired binding of R4C-leptin to its receptor in comparison with normal leptin. This might provide more insight in the functionality of the R4C-leptin protein. Also pleiotropic effects on fertility and immunity of the R4C polymorphism have to be investigated. We did not find an effect of R4C on FPLA, but did not find an effect on MY either.

## THE LEPTIN RECEPTOR GENE

In Chapter 6 we investigated the role of the leptin receptor gene. This gene consists of 20 exons (4 kb) divided over a region of approximately 170 kb (human). We isolated several BAC clones that contained (pieces of) the leptin receptor gene but because of the large region over which the exons are divided, sequencing these BAC clones was too time-consuming and too expensive. Therefore we tried to isolate hypothalamic mRNA from 17 weeks old bull calves to obtain the cDNA sequence of Ob-Rb, the long form of the leptin receptor. Unfortunately, we could not prove mRNA expression in this tissue at this age, which might be too young to express the leptin receptor. Then we tried to sequence all exons of the bovine leptin receptor gene separately using DNA isolated from blood samples from 20 unrelated cows in order to detect polymorphisms. Primers were based on the known sequence of the leptin receptor gene of different species (human, sheep, cow, waterbuffalo) but PCR reactions only succeeded for parts of exons 4, 11, and 20 (Chapter 6). In these sequences, a total of 667 bp, which is 17% of the total coding region of the leptin receptor gene, one non-conservative polymorphism was found (T945M). This polymorphism was not associated with the dairy traits we measured (unpublished results), but an association was found with prepartum leptin concentrations. The remaining 83% of the leptin receptor exon sequence that was not sequenced is still very interesting to investigate. As sequencing procedures are evolving and becoming faster and cheaper, the BAC clones isolated can now be useful to sequence the whole gene, including intronic sequences. Furthermore, the cow genome is in line to be sequenced entirely, so in the near future the bovine leptin receptor gene will be sequenced and the search for polymorphisms can proceed.

## CONCLUSIONS AND IMPLICATIONS

The aim of this thesis was to obtain a better understanding of the role of leptin in the periparturient cow on physiological and genetic level and to investigate opportunities to use information on the leptin and leptin receptor gene to improve selection for production and reproduction traits in dairy cows.

The first objective was to provide insight into the function of leptin during the periparturient period. In Chapter 4 we concluded that leptin concentrations reflect the energy balance state of the lactating dairy cow and in this Chapter we discussed the possible influence of a region at the leptin promoter on leptin concentrations during pregnancy. Combinations of three leptin promoter genotypes which have high and low prepartum leptin levels were defined and these could be used in future experiments to investigate the function of high and low leptin levels during pregnancy.

The second objective of this study was to detect polymorphisms in the bovine leptin gene and the leptin receptor gene. A total of 25 polymorphisms were detected, four at the leptin gene, twenty at the leptin promoter region (of which 14 were genotyped for all animals), and one at the leptin receptor gene.

The third objective was to obtain more insight in the role of the leptin and leptin receptor gene in dairy traits and to indicate whether these genes might be used for selection. In Chapter 3 we showed that an intronic polymorphism (RFLP1) in the leptin gene explained a part of the variation in milk yield in Holstein-Friesian dairy cows. However, because this polymorphism is located at an intronic region and because the polymorphisms found in the exons of the leptin gene were not associated with milk yield, we can not exclude the effects of closely linked genes. On the promoter region of the leptin gene an SNP was detected that was associated with first postpartum luteal activity and with weight loss from week 1 to the minimum weight (SNP -1457). Fertility traits are considered to be important to select for and here we present a potential candidate marker to be informative for fertility in dairy cows. Another SNP was associated with energy balance and dry matter intake (SNP -963). Two genotype combinations of these three polymorphisms (-1457/-963/RFLP1) which have a good milk yield, energy balance and fertility were defined in this Chapter (8). These combinations could be used in future experiments and economic evaluations to validate if one of these genotype combinations would be a possible candidate to use for selection.

After sequencing 17% of the leptin receptor gene, one non-conservative polymorphism was detected. This polymorphism was not associated with any of the dairy traits studied but differences in prepartum leptin concentrations were seen between leptin receptor genotypes.

In this Chapter (8) we showed that a combination of three polymorphisms located at the leptin promoter region explained most of the variance in prepartum leptin concentrations. During pregnancy significant differences in leptin concentrations were seen for different genotype combination and we hypothesized that a pregnancy-induced enhancer might be present at this region. Furthermore from the combined effect of the three polymorphisms the two extreme genotype combinations were found to affect leptin concentrations also during lactation. Analysis on dairy traits gave no significant effects probably because too many genotype combinations resulted in too few animals available per combination.

# SUMMARY

In dairy cattle, the increase in milk yield has been accompanied by a decrease in fertility and a more negative energy balance. As the hormone leptin is involved in regulation of nutritional status and reproductive function (Chapter 2) this is an interesting protein to investigate during the periparturient period in dairy cattle when many changes take place both in energy metabolism and reproductive physiology. The objectives of this study were to get insight into the function of leptin during the periparturient period and to perform an association study between polymorphisms in the bovine leptin and leptin receptor gene and fertility and production traits. The leptin gene including its promoter region and parts of the leptin receptor gene were sequenced to find polymorphisms, and related to differences in fertility and production traits. Leptin levels were described during the periparturient period in dairy cows and also associations with the genotyped polymorphisms and fertility and production traits were calculated. In Chapter 2 a literature overview regarding leptin and its receptor and their role in metabolic processes and fertility was given. This Chapter also includes recent literature, which was published during the investigations that were described in other Chapters of this thesis.

## PHYSIOLOGY OF LEPTIN

In Chapter 4 leptin levels during the periparturient period in dairy cows were described and these levels were related to differences in production and reproduction traits. Leptin concentrations in the periparturient cow undergo remarkable changes; leptin concentrations were high during late pregnancy and declined to a nadir at parturition.

The reason of elevated plasma leptin during pregnancy in ruminants seems to be the increase in adipose tissue leptin mRNA due to the lack of a negative feedback (leptin resistance state) and the increase in adiposity. We observed an association of live weight during lactation and prepartum leptin concentrations (Chapter 4) but did not investigate the association between prepartum live weight and leptin concentrations.

The reason of the decline in leptin levels towards parturition and during lactation is the mobilization of adipose tissue. Leptin concentrations seem to reflect the state of energy balance during lactation; plasma leptin concentrations were lower in cows with a mean negative energy balance during lactation (Chapter 4).

Further analysis indicated that a combination of three polymorphisms located at the leptin promoter region explained 14.3% of the variance in prepartum leptin concentrations. The two extreme combinations with the highest and lowest prepartum leptin concentrations could be used to investigate the function of leptin concentrations in pregnant cows.

## GENETICS OF LEPTIN

Leptin influences feed intake, energy balance and fertility and therefore the leptin gene and the leptin receptor gene are possible candidate genes to investigate effects on energy balance and fertility in lactating cows. The leptin gene including its promoter region and parts of the leptin receptor gene were sequenced to find polymorphisms, but also to detect putative transcription factor binding sites on the leptin promoter region. An association study was performed with the found polymorphisms. Associations of all genotyped polymorphisms with fertility and production traits measured in dairy cows during the periparturient period (Chapters 3 and 7) and also differences in leptin levels between genotypes of all genotyped polymorphisms were analyzed (Chapters 5, 6, and 7).

An intronic polymorphism (RFLP1) located on intron 2 of the leptin gene explained a significant part of the variation in milk yield, with differences of 0.5 genetic standard deviation between the two extreme genotypes (Chapter 3). However, because this polymorphism is located at an intronic region and because the polymorphisms found in the exons of the leptin gene were not associated with milk yield, we can not exclude that the causative gene is in linkage disequilibrium with this polymorphism. On the promoter region of the leptin gene SNP -1457 was associated with first postpartum luteal activity ($p$=0.017) and with weight loss between week 1 and its minimum weight ($p$=0.027), where more weight loss occurred together with a later first postpartum luteal activity. Fertility traits are considered to be important to select for and this SNP could be a potential candidate marker to be informative for fertility in dairy cows. SNP -963 was associated with energy balance ($p$=0.015) and dry matter intake ($p$ = 0.030), where a higher dry matter intake occurred together with a higher energy balance (Chapter 7).

In order to find an optimal genotype combination with a high milk yield, a good energy balance and fertility some new analysis were performed in Chapter 8. Two genotype combinations of three SNPs were defined in this Chapter and a next experiment and calculations of economical values per trait have to validate if one of these genotype combinations would be a possible candidate to be used in selection.

The R4C polymorphism, located at exon 2 of the leptin gene, received a lot of attention by several research groups because of its putative effect on the leptin structure, but several studies, including our study, did not provide convincing evidence that the R4C polymorphism influences the structure and function of leptin. Other studies showed an effect on milk yield in dairy cattle and carcass fat content in beef cattle. On the basis of these results a commercially available R4C-test was developed to improve breeding values in both dairy and beef cattle.

Polymorphisms in the leptin and leptin receptor gene have shown to be associated with differences in leptin concentration during late pregnancy (Chapters 5 and 6) but, surprisingly, not during lactation. As the promoter of leptin regulates the expression of leptin,

polymorphisms at this region could play an important role in the found differences in plasma leptin levels during late pregnancy. Therefore in Chapter 7 we sequenced the leptin promoter and analyzed the sequence for transcription factor binding sites and polymorphisms. All genotyped polymorphisms were associated with prepartum leptin concentrations but in Chapter 8 we showed that three polymorphisms located on a 135 bp promoter region (282 to 147 bp before the transcription start site) seemed to be important for differences in leptin concentrations during late pregnancy. Also significant differences during the lactation period were found between the two genotype combinations with the highest en lowest leptin concentrations. We proposed a putative pregnancy-dependent enhancer to be located at this site on the leptin promoter.

In conclusion, polymorphisms in the leptin gene and its promoter region are associated with traits in dairy cattle like milk yield (RFLP1), energy balance (SNP -963) and fertility (SNP -1457). Further experiments and calculations are needed to investigate if a combination of these three polymorphisms would be useful in future selection. On the promoter region a pregnancy-dependent enhancer might be located. A combination of polymorphisms located at this region might be useful to investigate the function of high leptin levels during pregnancy.

# SAMENVATTING

In de Nederlandse melkveepopulatie is de gemiddelde melkproductie gedurende de afgelopen 45 jaar toegenomen van gemiddeld 14 tot gemiddeld 26 kilogram melk per dag. Dit is gepaard gegaan met een verminderde vruchtbaarheid van de koe. Omdat de productie van melk veel energie kost, is er minder energie beschikbaar voor andere processen in het lichaam, zoals vruchtbaarheid. Hierdoor zou de voortplantingscyclus van de koe later op gang kunnen komen na de afkalving.

Verschillende studies geven aan dat erfelijkheid meespeelt bij kenmerken zoals melkproductie en vruchtbaarheid. Dat wil zeggen; de stier en koe geven via het DNA erfelijke factoren door aan de nakomelingen, zij erven deze eigenschappen dus over van hun ouders. De fokkerij maakt hier gebruik van door qua erfelijke aanleg goede stieren te selecteren om mee te fokken. Met deze tactiek is de melkproductie de afgelopen jaren sterk gestegen, maar is de vruchtbaarheid als negatief bij-effect gedaald. Tijdens deze selectie is er geselecteerd op kenmerken (melkproductie) en is er niet precies bekend op welke genen op het DNA geselecteerd is.

Genen zijn belangrijke onderdelen van het DNA, ze coderen voor alle eiwitten, hormonen en andere regulerende bestanddelen van het lichaam. In deze genen kunnen mutaties optreden en hierdoor kan de functie van het eiwit dat door het gen gecodeerd wordt, veranderen. De fokkerij kan hier gebruik van maken door varianten van genen te selecteren die een potentiële verbetering kunnen veroorzaken bij een bepaald kenmerk zoals melkproductie of vruchtbaarheid. Bij vruchtbaarheid zullen bijvoorbeeld genen die coderen voor hormonen een belangrijke rol spelen. Een voorbeeld van zo'n hormoon is leptine, dat wordt aangemaakt in vetcellen en na afgifte door deze cellen in de bloedbaan terechtkomt.

Leptine heeft invloed op de voedselopname en op de vruchtbaarheid. Muizen die een onwerkzame variant van het gen dat codeert voor leptine hebben zijn erg vet, eten veel en zijn onvruchtbaar. Dus leptine heeft als functie de voedselopname te remmen en de vruchtbaarheid te stimuleren. Omdat melkkoeien zoveel produceren, zullen zij veel voedsel opnemen, maar meestal is dat niet genoeg om te voldoen aan de energiebehoefte. Daarom verkeren ze, na afkalven, in een negatieve energiebalans. Daardoor hebben ze minder lichaamsreserves in de vorm van vet in vetcellen en produceren ze minder leptine. Aangezien, zoals hiervoor al aangegeven, leptine de voedselopname en de vruchtbaarheid reguleert is het gen dat codeert voor dit hormoon een potentiële kandidaat om te onderzoeken op het voorkomen van varianten bij melkkoeien. De hypothese is dat er tussen koeien verschillen zijn in het DNA, met andere woorden dat er verschillende varianten van het leptine-gen voorkomen in een populatie koeien en dat dit een verschil in voeropname, energiebalans en/of vruchtbaarheid geeft.

Het onderzoek dat in dit proefschrift beschreven is, is uitgevoerd in het kader van een groter project dat als doel had het identificeren van genen die verantwoordelijk zijn voor de verschillen in erfelijke aanleg voor kenmerken zoals melkproductie, energiebalans en vruchtbaarheid. Het leptine- en leptinereceptor-gen zijn onderzocht op mutaties. Daarna is

gekeken of deze mutaties een verschil in functie tot gevolg hebben ten opzichte van dieren die de mutatie niet hebben. Hiervoor hebben wij 623 dieren gebruikt waarvan de kenmerken zoals melkproductie, energiebalans en vruchtbaarheid bekend waren. Ook is de concentratie leptine op verschillende tijdstippen rondom het afkalven in het bloed van een deel van deze dieren gemeten om te kijken naar veranderingen in circulerende leptine concentraties tijdens de late dracht en de vroege lactatie bij koeien met of zonder de mutatie.

Het leptine-gen bestaat uit 2 stukken DNA die coderen voor het leptine hormoon. Daar omheen ligt niet-coderend DNA. Er zijn totaal 4 mutaties in het gen gevonden, waarvan er twee in het coderende deel van het leptine-gen liggen. Een van deze 4 mutaties bleek geassocieerd te zijn met een hogere melkproductie, de andere 3 mutaties waren niet geassocieerd met een van de gemeten kenmerken (Hoofdstuk 3).

De expressie van leptine wordt gereguleerd door DNA dat vóór het coderende deel van het leptine-gen ligt. Dit wordt de promoter van leptine genoemd. Op dit deel van het DNA binden stoffen die de aanmaak van leptine kunnen remmen of stimuleren. Op de promoter van leptine zijn ook diverse mutaties gevonden. Als deze mutaties zich bevinden op een stuk DNA waar een regulerende stof aan bindt, kan dit gevolgen hebben voor de hoeveelheid leptine dat geproduceerd wordt. Van de 20 mutaties die gevonden zijn, zijn er 14 gebruikt om alle 623 dieren te typeren. Twee mutaties bleken geassocieerd te zijn met enerzijds energiebalans en anderzijds vruchtbaarheid (Hoofdstuk 7).

Omdat er drie mutaties gevonden zijn die geassocieerd zijn met belangrijke kenmerken voor melkvee (melkproductie, energiebalans en vruchtbaarheid), hebben wij in Hoofdstuk 8 geprobeerd een combinatie van genvarianten te vinden die samen een goede melkproductie, een goede energiebalans en een goede vruchtbaarheid voorspellen. Twee combinaties van genvarianten bleken potentiële kandidaten te zijn om te gebruiken voor selectie. Uitgebreidere experimenten en bijvoorbeeld berekeningen van de economische waarden per kenmerk zullen moeten aantonen of een van deze genvariant-combinaties een mogelijke kandidaat is om te gebruiken voor het selecteren van stieren voor de fokkerij.

Het leptinereceptor-gen bestaat uit 20 stukken DNA die samen coderen voor het leptinereceptor eiwit. In deze studie zijn drie kleine stukjes van dit gen onderzocht op mutaties en er is 1 mutatie gevonden. Deze bleek niet geassocieerd te zijn met de gemeten kenmerken.

De concentratie van leptine in het bloed is gemeten bij 323 koeien tijdens de late dracht en vroege lactatie. Deze metingen vormen samen een curve die het verloop van de leptine concentratie weergeeft. Het is gebleken dat de concentratie leptine tijdens de dracht hoog is en dat deze daalt vlak voor het afkalven. Tijdens de lactatie blijft de concentratie constant (Hoofdstuk 4). In de Hoofdstukken 5 en 6 hebben we deze concentraties gerelateerd aan de gevonden mutaties en het was opmerkelijk te zien dat alleen tijdens de dracht alle getypeerde mutaties geassocieerd bleken te zijn met leptine concentraties. Omdat de promoter van leptine waarschijnlijk een belangrijke rol speelt bij de regulatie van leptine concentraties in

het bloed, hebben wij ook het DNA van deze promoter onderzocht op mutaties. Ook hier bleken alle getypeerde mutaties op de leptine promoter geassocieerd te zijn met leptine concentraties tijdens de dracht (Hoofdstuk 7). Daarom is in Hoofdstuk 8 een meer geavanceerdere berekening uitgevoerd met alle gevonden mutaties om te kijken welke nu het belangrijkst waren voor de associatie met leptine concentraties tijdens de dracht. Hieruit bleek dat drie mutaties op een klein stukje DNA van de promoter samen de meeste invloed hadden op de regulatie van leptine concentraties tijdens de dracht. Hieruit hebben wij de hypothese geformuleerd dat op dit stukje DNA waarschijnlijk een regulerende plaats ligt die alleen tijdens de dracht een rol speelt.

Naast de genetische aspecten van leptine is er ook gekeken naar de relatie tussen leptine concentraties en de gemeten kenmerken. Zo hebben wij aangetoond dat er een positief verband bestaat tussen leptine concentraties tijdens de dracht en energiebalans. Dat wil zeggen dat koeien die in goede conditie zijn, een hogere leptine concentratie in het bloed hebben dan koeien met een minder goede conditie. Dit zou gevolgen kunnen hebben voor de vruchtbaarheid, omdat een goede conditie meestal samenhangt met een goede vruchtbaarheid. Helaas kon er geen direct verband worden aangetoond tussen leptine concentraties en vruchtbaarheid.

Samenvattend: er bestaat genetische variatie (mutaties in het DNA) in het leptine-gen, het leptinereceptor-gen en de promoter van het leptine-gen. Een mutatie op het niet-coderende deel van het leptine-gen is geassocieerd met melkproductie terwijl twee mutaties op de leptine promoter geassocieerd zijn met vruchtbaarheid en energiebalans. Twee combinaties van deze drie genvarianten zijn mogelijke kandidaten om te gebruiken bij de selectie van stieren voor de fokkerij. Verder zijn er drie mutaties op de leptine promoter gevonden die erg dicht bij elkaar liggen en samen invloed hebben op de leptine concentraties tijdens de dracht. Een combinatie van deze drie mutaties zou gebruikt kunnen worden om de functie van de hoge leptine concentraties in koeien tijdens de dracht te onderzoeken. Voor deze studie zouden de twee extreme combinaties (hoog en laag leptine) gebruikt kunnen worden. Ook zou verder onderzoek uit moeten wijzen of op dit stukje DNA een mogelijke zwangerschap-afhankelijke regulerende plaats ligt. Ook geeft de leptine concentratie tijdens de lactatie de conditie waarin de koe verkeert weer, maar het meten van leptine om deze informatie te verkrijgen is niet haalbaar, mede doordat er tussen koeien grote verschillen bestaan in de zogenaamde basisconcentratie leptine.

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## SUBMITTED

Liefers, Silvia C., Marinus F.W. te Pas, Roel F. Veerkamp, Carole Delavaud, Yves Chiliard, Mariska Platje and Tette van der Lende. Leptin promoter mutations affect leptin levels and performance traits in dairy cows. Submitted to Mammalian Genome.

## PROCEEDINGS

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# CURRICULUM VITAE

Suzanne Christien (Silvia) Liefers werd geboren op 08 augustus 1974 in het stadje Hattem. In 1993 behaalde zij haar VWO diploma aan het Carolus Clusius College in Zwolle. In hetzelfde jaar begon zij met de studie Biomedische Chemie aan de Rijkshogeschool IJselland in Deventer. Haar stage heeft zij uitgevoerd bij afdeling Produktkunde van het Instituut voor Dierhouderij en Diergezondheid, ID-DLO in Beekbergen (bij Frans Schreurs en Jan Waltmann) en haar afstudeervak bij de afdeling Zoogdiervirologie van hetzelfde instituut (bij Roger van der Heijden en Frans Rijsewijk). Na het behalen van haar diploma in 1997 is zij doorgestroomd naar de opleiding Medische Biologie aan de Rijksuniversiteit Groningen. Het eerste afstudeervak in de oriëntatie Immunologie heeft zij uitgevoerd bij de Faculteit der Medische Wetenschappen van de Rijksuniversiteit Groningen, afdeling Klinische Immunologie (bij Sebo Withoff). Het tweede afstudeervak heeft zij uitgevoerd bij de afdeling Immunologie van het Instituut voor Dierhouderij en Diergezondheid, ID-Lelystad (bij Johanna de Groot en Wim Boersma). In februari 2000 heeft zij de studie Medische Biologie afgerond en is een maand later aangesteld als Assistent in Opleiding (AIO) bij de leerstoelgroep Fokkerij en Genetica van Wageningen Universiteit. Gedetacheerd bij de divisie Dier en Omgeving van het Instituut voor Dierhouderij en Diergezondheid, ID-Lelystad (tegenwoording Animal Sciences Group van Wageningen Universiteit en Researchcentrum) heeft zij onder begeleiding van Roel Veerkamp, Marinus te Pas en Tette van der Lende het promotieonderzoek verricht dat staat beschreven in dit proefschrift.

Suzanne Christien (Silvia) Liefers was born in Hattem on the 8th of Augustus 1974. In 1993 she graduated from high school (Carolus Clusius College, Zwolle). In the same year she started the study Biomedical Chemistry at the Statecollege IJselland in Deventer. Her first traineeship she carried out at the department of Productscience of the Institute for Animal Science and Health, ID-DLO in Beekbergen and her second traineeship was executed at the department of Mammalian Virology of the same institute. After her graduation in 1997 she continued her study at the State University Groningen and followed the education Medical Biology. The first main subject was executed at the Faculty of Medical Science of the State University Groningen, at the department of Clinical Immunology and the second main subject was performed at the department of Immunology of the Institue for Animal Science and Health, ID-Lelystad. In February 2000 she finished the study Medical Biology and was engaged as PhD-student at the Animal Breeding and Genetics Group of Wageningen University. Stationed at the division of Animal Sciences of the Institute for Animal Science and Health, ID-Lelystad (nowadays Animal Sciences Group, Wageningen University and Research Centre) she performed the PhD-study described in this thesis under supervision of Dr. ir. R.F. Veerkamp, Dr. M.F.W. te Pas and Dr. ir. T. van der Lende.

# SLOTWOORD

In december 1999 solliciteerde ik naar de vacature Assistent in Opleiding bij de leerstoelgroep Fokkerij en Genetica bij het departement Dierwetenschappen. De rit naar Wageningen beloofde al veel goeds, ik kwam in de buurt van Doetinchem terecht. Geen landkaart bij me, had alleen een routebeschrijving van het internet geplukt. Gelukkig was ik op tijd vertrokken en kwam 5 minuten voor aanvang van het gesprek aan op Zodiac.

Op 13 maart 2000 ben ik bij ID-Lelystad begonnen op de afdeling Fokkerij en Genetica. De afstand tussen mijn bureau (vleugel 19, 2e verdieping) en werkplek op het lab (vleugel 16) was even wennen. Maar na een paar weken op en neer lopen leer je toch snel om twee keer te checken of je alles wel bij je hebt.

Onderzoek doe je niet alleen en om te voorkomen dat ik iemand vergeet wil ik hierbij iedereen bedanken die op een of andere manier heeft bijgedragen tot het gereedkomen van dit proefschrift. Een aantal mensen wil ik specifiek noemen;

Rosemarijn en Mariska, jullie hebben mij veel werk uit handen genomen tijdens jullie afstudeeropdracht en stage. Ik kon mijn concentreren op het schrijfwerk, terwijl julle mij de resultaten aanleverden. Vaak is mij gevraagd of het niet erg veel tijd kostte om stagiaires te begeleiden, maar mijn ervaring is dat je er erg veel voor terug krijgt! Mariska, ik ben je erg dankbaar dat je de voorkant van dit boekje hebt willen ontwerpen. Dankjewel! Je hebt talent en ik hoop dat je er nog veel mee zult doen in de toekomst.

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Wouter, vooral de laatste maanden waarin ik thuis de laatste hoofdstukken aan het schrijven was zullen je nog lang bijblijven. Als je uit je werk kwam, was het altijd een rommeltje van rondslingerende artikelen, telefoonsnoeren om verbinding te krijgen met de computer in Lelystad en gele plak-briefjes, want het leek wel of ik helemaal niks meer kon onthouden. Een terminale AIO heeft toch andere prioriteiten dan een nette woonomgeving maar vanaf nu zal de woonkamer ook weer van jou zijn!

Silvia

# NOTES

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