EFFECTS OF SINGLE NUCLEOTIDE POLYMORPHISMS IN LEPTIN AND PRO-OPIOMELANOCORTIN ON PERIPHERAL LEUCOCYTE COUNTS IN BEEF CATTLE

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ABSTRACT

Single nucleotide polymorphisms (SNP) in leptin (*LEP*) and pro-opiomelanocortin (*POMC*) have been associated with beef carcass quality and yield respectively. Both hormones also play a role in immune performance. Since both of these genes are pleiotrophic, it was important to determine whether selection based on these SNPs would negatively affect immune cell numbers. A SNP in each of these hormones was assessed for effects on immune cell counts and antibody titres in twenty-seven beef cattle herds (n = 556). A commercial rabies vaccine was administered to these animals. Prior to being vaccinated, the types of lymphocytes evaluated included B cells, gamma delta cells, regular and activated CD_4 and CD_8 cells and numbers of lymphocytes as well as baseline serum antibody titres. On day 21, antibody titres were measured and a booster vaccine was administered. Finally on day 42, antibody titres and lymphocyte types were again counted. Several cell types were significantly associated with the *LEP* genotype however, no consistent pattern of correlation was observed between *LEP* genotype (TT, CT or CC) and peripheral blood lymphocyte populations. The number of different lymphocytes significantly associated with *LEP* genotype increased from two on day 0 to four on day 42. Animals with CT and CC genotypes had significantly higher increased rabies antibody titres in the first 21 days after vaccination than those with TT genotypes. The *POMC* SNP also did not show a clear pattern of association between lymphocyte subtypes and genotype. There was no difference in response to the rabies vaccination associated with the *POMC* genotype. Our results suggested that selection at either of the SNPs examined in this research would not detrimentally impact immune function in beef cattle.

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DEDICATION

To my family who have sacrificed a great deal through out the period of my absence from home

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
B Cell	B-cells
BSA	Bovine Serum Albumin
BVDV	Bovine Viral Diarrhea Virus
CD	Cluster of Differentiation
CD_4	CD_4-regular cells
CD_{4_25}_P4	Interleukin –2 (IL-2)
CD_{4_26}	Activated CD_4
$CD_4CD_{25}CD_{45}$	Leucocyte Common Antigen
CD_4CD_8Ratio	Ratio of CD_4 to CD_8
CD_8	CD_8-regular cells
$CD_8CD_{25}CD_{45}$	Activated CD_8
$CD_8CD_{26}CD_{45}$	Activated CD_8
CD_8gd_P1	CD_8 gamma delta cells
IBR	Infectious Bovine Rhinotracheitis
Igα	Immunoglobulin α
Igβ	Immunoglobulin β
IL	Interleukin
INF-γ	Interferon gamma
LPS	Lipopolysaccharide
MHC	Major Histocompartibility Complex
NK	Natural Killer Cells

POMC	Pro-opiomelanocortin
TCR	T-Cell Receptor
TNF	Tumour Necrosis Factor
W.C. 1	Workshop Cluster 1

1.0. GENERAL INTRODUCTION

Prior to the outbreak of the mad cow disease in May 2003, the beef cattle industry contributed about $13 billion to the economy of Canada (Bird 2001). With this substantial contribution to the nation's economy, it is critical that proper steps are taken to ensure a healthy cattle production system in order to maximize profit. The optimum functioning of the immune system in cattle is imperative since it plays a major role in maintaining the animals' performance and growth efficiencies.

Without the effective protective measures employed by the immune system, it is possible that a majority of living things would perish due to the presence of numerous harmful organisms. These harmful organisms are able to access the body through inhalation, contacting the epithelial surfaces and ingestion among others. The immune system is an assembly of immunoglobulins and lymphocytes, which are cross-linked to eradicate foreign materials that enter the body.

This project was designed to investigate the roles played by leptin and pro-opiomelancortin (POMC) in the immune system of beef cattle. Leptin is the protein product of the obese gene secreted predominantly by the adipose tissue, which was found to be associated with appetite and energy metabolism (Zhang et al. 1994; Houseknecht et al. 1998; Lamas et al. 2004). A single nucleotide polymorphism (SNP) in this gene was found to be associated with carcass quality and milk yield in beef and dairy cattle

respectively (Buchanan et al. 2002; 2003). As a consequence, selection for the beneficial allele or the allele with economic advantage is occurring. Recent publications have indicated leptin's ability to influence immune performance (Lord et al., 1998; Faggioni et al. 1999; Lamas et al. 2004; Matarese et al. 2005).

Similarly, POMC, a hormone produced in the anterior pituitary, also plays a role in appetite regulation (Mori, 2001; Pritchard et al.2002) and a SNP has also been identified (Thue and Buchanan 2003) which is associated with yield in beef cattle (Buchanan et al. 2005). POMC has also been found to modulate some aspects of the immune system (Mechanick et al.1992; Adachi et al. 1999).

2.0. LITERATURE REVIEW

2.1. *Background to the immune function*

The resistance to diseases, especially infectious diseases, is referred to as immunity. The immune system is the basic mechanism by which the body recognizes, fights and eliminates foreign materials that enters it. The result is the survival of the body against the pathogenic organisms to which it is exposed. Without effective protective measures, diseases caused by pathogenic organisms or toxins produced by microorganisms would have denied a huge number of living things their survival and performance. The immune system is a collection of cells, tissues and organs that function together to mediate resistance to infections. Physiologically, the immune system prevents infections as well as fights existing infections (Goldsby et al. 2000).

The efficiency of the immune system is dependent on a number of factors including genetic, nutritional, degree of exposure to immunogenic factors and whether there has been a previous encounter with a particular immunogenic factor. Multiple systems have to be present to be effective against the different pathogens and the different levels at which they can attack the body. For instance, some invaders will act on body surfaces, fluids or in cells. Effective elimination of invaders requires multiple defense mechanisms that can act both on the surface and within cells to destroy organisms (Tizard 2000). Hence the protection of the body depends on a complex system of overlapping and interconnected defense mechanisms that act together to destroy almost all invaders (Tizard 2000).

Basically, there are two main types of immune response. These are innate and adaptive immune responses. Innate immune response is a general non-specific type of response, which acts as the first line of combating diseases (Abbas and Lichtman 2001). Innate immunity is always present in healthy individuals to block the entry of microbes and to rapidly eliminate microbes that are able to enter host tissues. It is also known as native or natural immunity. Adaptive immunity is a specific immune response that is stimulated by microbes. It is also known as specific or acquired immunity. Adaptive immunity usually is a follow up to innate immunity should the mechanisms employed by innate immunity fail to eradicate the disease or infection.

2.1.1 *Innate immunity*

This is the first line of defense of the immune system. It is the non-specific component of immunity hence its mechanisms are not peculiar to a particular pathogen (Goldsby et al. 2000). It is mediated by epithelial barriers, phagocytes, natural killer cells and several plasma proteins including proteins of the complement system. Innate immunity usually occurs within the first few hours (about 0-12 hours) after antigens enter the body by evading the anatomical and physiological barriers (Abbas and Lichtman 2001).

Anatomical barriers include the skin and mucous membranes. The skin acts as a keratinized physical barrier. Sebatious glands, found in the skin secret sebum. Sebum contains lactate and fatty acids. These acids maintain the skin pH between 3 and 5 thus preventing the survival of many organisms (Abbas and Lichtman 2001). The mucous

secretions such as saliva and tears wash away pathogens. Mucous secretions may also contain lysosome, a membrane-bound acidic organelle that contains proteolytic enzymes that degrade proteins derived mainly from the extra cellular environment. The continuous epithelia that line the skin, gastrointestinal and respiratory tracts contain intra-epithelial lymphocytes as well as peptide antibiotics, which prevent or fight the entry of microbes.

Physiological barriers include body temperature; maintained at 37^{o}C for humans, 39^{o}C for cattle and 41^{o}C for poultry, skin pH (3-5); and stomach pH (1.5-3.5). Survival of microorganisms at such temperatures and pH levels is limited (Abbas and Lichtman 2001). For example, the body temperature of chickens prevents the growth of the anthrax bacteria (Goldsby et al. 2000). Interferons secreted by virally infected cells induce a non-viral state in neighboring cells. During infection, the secretion of acute phase protein such as serum amyloid A and C-reactive protein is increased.

Although physical barriers are very useful, they are sometimes unable to clear the invaders due to the persistence of the invaders (Tizard 2000). The mechanism employed by innate immunity is that it recognizes and responds to microbes but does not react against non-microbial substances (Abbas and Lichtman 2001). For example, phagocytes have receptors for bacterial lipopolysaccharides (LPS) also called endotoxins, terminal mannose residues on glycoproteins, double stranded RNA and unmethylated CpG nucleotides all of which are components of bacteria and/or viruses.

Collectively, these structures shared by classes of microbes are referred to as molecular patterns (Abbas and Lichtman 2001). Receptors for innate immunity have the ability to recognize molecular patterns. Host cells that have been damaged by microbes may also trigger an innate immune response. Innate immunity attacks microbial substances needed for the survival of the microbes. For this reason, mutation of the microbes does not allow the microbes to elude innate immunity. Microbial recognition receptors of innate immunity are encoded in the germ line. This results in limited diversity in the receptors of innate immunity. Receptors of innate immunity are non clonal, and are identical on all cells of the same lineage.

This is different from adaptive immune receptors, which are clonal. This means that in adaptive immunity, clones of lymphocytes with distinct specificities express different receptors. Innate immune response is capable of discriminating between self and non-self antigens. Host cells are not recognized or they may express molecules that prevent innate immune reactions.

The principal cells of innate immunity are the phagocytes (neutrophils and monocytes/ macrophages) and the Natural Killer (NK) cells. Neutrophils, the most abundant blood phagocytes number about 4,000 to 10,000 per cubic millimeter of blood (Abbas and Lichtman 2001). Production of neutrophils in the bone marrow increases in response to infection to about 20,000 per cubic millimeter of blood. The role of the neutrophils is to ingest microbes in blood and tissues. Monocytes differentiate into macrophages in extravascular tissues. Macrophages secrete the cytokines interleukin - 1 (IL-1) and tumor necrosis factor (TNF), which act on the endothelium to release two adhesion molecules, E-selectin and P-selectin to bind carbohydrates expressed by

neutrophils and monocytes. Together with integrins, selectin mediates the rolling of cells on the endothelium. The endothelial cells produce cytokines known as chemokines, which bind to endothelial membrane proteins and stimulate the motility of leucocytes.

As a result, the leucocytes begin to migrate through the vessel wall and along the chemokine concentration gradient to the site of infection. NK cells produce interferon gamma (INF-γ), which is a cytokine that activates macrophages to secret more of IL-1 and TNF-α. INF-γ plays important physiological roles to back up innate and adaptive immune responses. In its absence, cellular responsiveness in humans and experimental animals significantly predisposes the host to microbial infection, a result that affirms the physiologic importance of this cytokine in preventing infectious diseases (Ikeda et al. 2002). NK cells are responsible for killing infected host cells. The production of IL-1 and TNF-α as a result of the activation of macrophages by INF-γ allows the cycle to go on to eliminate infected host cells.

2.1.2 *Adaptive immunity*

Adaptive or specific immunity is the type of host defense that is stimulated by microbes that invade tissues (Abbas and Lichtman 2001). This component of immunity is able to recognize and selectively eliminate specific foreign microorganisms and molecules (Goldsby et al. 2000). Cells employed by adaptive immune response are mainly the B and T lymphocytes produced in the bone marrow by hematopoiesis (the development of mature blood cells in the bone marrow and fetal liver). B-lymphocytes mature in the bone marrow. T lymphocytes migrate to the thymus where they undergo

T-cell education and maturation. This further development of T cells in the thymus is to enable the T cells to recognize and eliminate foreign antigens whilst being able to tolerate or ignore self-antigens (Abbas and Lichtman 2001).

T lymphocytes are differentiated into T cytotoxic or T cytolytic (T_C) and T helper cells (T_H). T cytotoxic cells express the membrane glycoprotein called CD_8^+ that kill cells harboring intracellular microbes by lysis of these cells. T helper cells express membrane glycoprotein called CD_4^+ cells. T_H cells activate B cells to produce antibodies and help phagocytes to destroy ingested microbes hence the name T helper cells. There are two types of T helper cells namely T_H1 and T_H2 cells. T_H1 cells produce cytokines such as IL-2, which is required for T cell proliferation, and interferon gamma (INF-γ), which is responsible for stimulating phagocytes that function in the elimination of intracellular microbes.

T_H2 cells on the other hand mediate anti-helminthic and hypersensitive responses by producing IL-4 and IL-5, which stimulate immunoglobulin E (Ig E) and eosinophils. In addition to these cytokines that function in antihelminthic responses, T_H2 cells also produce IL-6 and IL-13. IL-6 stimulates the synthesis of acute phase proteins by hepatocytes (Abbas and Lichtman 2001).

T_H1 and T_H2 cytokines work differently and T_H2 down regulates T_H1 responses. The effectiveness of cell-mediated immune response against microbes is dependent on the balance between the productions of $T_H1$and T_H2 cells in response to that microbe (Abbas and Lichtman 2001). Eradication of an infectious microbe depends on the

relative activation of T_H1 and T_H2. The possibility of using T_H1-type cytokine genes in the treatment of cancer has been explored by Roth and Cristiano (1997) whereas T_H2 cytokines have been used effectively to inhibit autoimmune diseases (Prud'homme 2000)

Adaptive immunity displays a high degree of specificity as well as the remarkable property of memory. Specificity is a means by which specific lymphocytes are produced to combat specific antigens. This is important because different lymphocytes bearing antigen receptors are required for different antigens. When activated, T cytotoxic cells differentiate into effector cells that can destroy infected cells. The rearrangement to produce specific lymphocyte receptors enable all kinds of antigens to be combated except the host's own potentially antigenic substance known as self antigens. After the elimination of the microbes some of the lymphocytes remain in the body. These are referred to as memory cells. The purpose of the memory cells is to give a rapid and higher response to the same antigen for which it was produced upon a second encounter. Adaptive immune response employs lymphocytes and their products, mainly cytokines and antibodies, to eliminate the antigen. Adaptive immune responses against antigens occur within five or six days after the initial exposure to that antigen.

There are two types of adaptive immunity namely humoral and cell-mediated immune responses. Humoral adaptive response occurs in body fluids in circulation. It is mediated by antibody production by the B-lymphocytes and combats microbes that enter blood circulation. It is primarily directed against exogenous/extra cellular invaders. Antibodies can recognize a wide variety of macromolecules including proteins, polysaccharides, lipids and nucleic acids. Antigen recognition by antibodies begins with the coming together (cross-linking) of two or more of receptor molecules. The receptors

are noncovalently attached to protein molecules namely immunoglobulin α (Igα) and immunoglobulin β (Igβ) to form the B cell receptor complex (BCR). Igα and Igβ transmit signals of antigen recognition to evoke immune response.

Cell-mediated immune response occurs in the cells by the action of Tc (CD_8^+) lymphocytes. Cell mediated immune response is responsible for combating microbes that are found in the body cells. Invaders cause cellular abnormalities. Cell mediated immunity is primarily directed against endogenous/intracellular invaders. Specialized cytotoxic cells destroy the abnormal cells. Whereas B cells recognize a wide variety of molecules without the need for an intermediary host presentation, most T cells can recognize only peptides. Specialized host presentation molecules must present the peptides recognized by T cells.

The specialized host presenting molecules are known as major histocompatibility (MHC) molecules. This property of T cells is called MHC restriction. There are two types of MHCs namely MHC class 1 and MHC class 2. MHC Class 1 molecules are expressed on all nucleated cells, but MHC class 2 molecules are expressed mainly on professional antigen presenting cells (APCs) such as dendritic cells, macrophages and B-lymphocytes. MHC Class 1 molecules have a peptide-binding cleft that can accommodate 8 to 11 amino acids (Abbas and Lichtman 2001). MHC Class 2 molecules have a peptide-binding cleft that is large enough to accommodate peptides of 10 to 30 residues (Abbas and Lichtman 2001).

The variability in the MHC receptors is determined by the genetic constitution and differs greatly among individuals except for identical twins. Due to such great diversity in MHC molecules, susceptibility to diseases varies among individuals (Abbas and

Lichtman 2001). MHC also regulates the choice of mating partners in individuals (Tizard 2000). For instance, under controlled conditions, mice would mate with MHC-incompatible partners. The results of such matings are MHC-heterozygous progeny, which are better able to resist diseases (Tizard 2000).

The T cell receptor (TCR) recognizes antigens by means of α and β chains attached to the receptor. After the TCR recognizes antigens, it requires a transmission of biochemical signals to the interior of the cell in order to trigger an immune response. Two molecules, namely, CD3 and ζ that are non-covalently attached to the TCR, perform this signaling role. The TCR, CD3 and ζ together form the TCR complex.

When a microbe breaches the epithelium and enters the sub-epithelial tissue, macrophages respond by producing cytokines. Two of these cytokines, interleukin 1 (IL-1) and tumor necrosis factor (TNF-α) stimulate the endothelium to produce two adhesion molecules namely E-selectin and P-selectin. Selectin binds loosely to circulating neutrophils and monocytes. Flowing blood disrupts this bond, but the bond reforms. This results in the rolling of the leucocytes on the endothelial surface aided by integrins-another set of adhesion molecules expressed by activated leucocytes, which causes a firm adhesion of leucocytes to endothelial surfaces. Another cytokine, named chemokine is produced both by the tissue macrophage that encountered the microbe and by endothelial cells responding to macrophage-derived IL-1 and TNF- α. Chemokines stimulate the leucocytes to move through the vessel wall along the chemokine concentration gradient to the site of infection.

The sequence of selectin-mediated rolling, integrin-mediated firm adhesion, and chemokine mediated motility leads to the migration of blood leucocytes to an extravascular site of infection within minutes after the infection. Leucocytes accumulate at the site of infection. Vascular dilation occurs and there is the increased permeability of the vessels. These three processes are together referred to as inflammation. Inflammation involves the accumulation and activation of leucocytes and plasma proteins at the site of infection, toxin exposure or cell injury.

2.1.3. *Cytokines*

Cytokines are secreted proteins (in the range of 5-20kD) that function as mediators of immune response (Abbas and Lichtman 2001). They act as hormones of the immune system that facilitate immune response. They either act in an autocrine (acting on cells that they are produced from) or paracrine (acting on cells other than the ones that they are produced from) manner. Structurally defined cytokines are referred to as interleukins. In innate immunity cytokines are produced by macrophages and NK cells and by mainly T lymphocytes in adaptive immunity.

Cytokines produced by macrophages include TNF-α, IL-1 and IL-12. TNF-α stimulates the recruitment of neutrophils and kills microbes. It stimulates vascular endothelial cells to secret chemokines. Excessive production of TNF-α in severe infections results in septic shock. IL-1 mediates host inflammatory response in innate immunity, induces endothelial cell adhesion molecules and stimulates chemokine production by endothelial cells and macrophages (Abbas and Lichtman 2001).

IL-12 is a key inducer of cell-mediated immune responses to intracellular microbes. IL-12 activates NK cells, enhances cytotoxic activity of NK cells and cytotoxic T lymphocytes and promotes the proliferation of $T_H1$cells. Macrophages therefore function in both innate and adaptive immunity (Abbas and Lichtman 2001).

NK cells are a proportion of peripheral blood lymphocytes, which are neither T nor B cells but belong to a distinct population of cytotoxic lymphocytes (Tizard 2000). NK cells produce INF-γ to activate phagocytes in both innate and adaptive immune responses. NK cells form about 10 percent of the total lymphocyte count in blood and peripheral lymphoid organs (Abbas and Lichtman 2001). They respond to microbes by killing infected cells and by producing INF-γ, which is a macrophage activating cytokine. Macrophages in turn produce IL-12, which functions to activate NK cells. NK cells do not express immunoglobulins or T-cell receptors but have abundant cytoplasmic granules and are likely to have receptors for structures found on host cells infected with viruses and on phagocytes harboring viruses and intracellular bacteria. NK cells also express receptors for the fragment crystalline (Fc) portions of some immunoglobulin G antibodies and use these receptors to bind to cells coated with antibodies. Fc is that portion of an immunoglobulin molecule that binds to a cell when the antibodies are occupied or the antibody is aggregated.

When NK are activated, they exhibit two major responses. They kill infected host cells by discharging of their proteins contained within their cytoplasmic granules towards the infected cells. Secondly, INF-γ synthesized by activated NK cells activates

macrophages to become more effective at killing phagocytosed microbes. T lymphocytes produce the cytokines INF-γ, TNF-α and chemokines that act as assessory molecules in the mechanism by which T cells eradicate external agents that enter the body.

2.1.4 *Immune cell types relevant to this project*

Other immune cell types that have been mentioned in this project include CD_4, CD_5, CD_8, CD_{21}, CD_{25}, CD_{26}, and CD_{45}.

CD refers to cluster of differentiation and are cell surface molecules expressed on various cell types in the immune system (Abbas and Lichtman 2001).

CD_4: -Originally called T4 is a specific receptor for MHC class 2 molecules (Abbas and Lichtman 2001). CD_4 plays a key role in the recognition of processed antigen by helper T cells. It is a 59-kDa glycoprotein found on helper T cells, thymocytes and monocytes. It is also a cell-binding receptor for HIV.

CD_5: -A 67-kDa molecule whose ligand is CD_{72}. It is found on a subset of B cells and on all T cells. If CD_5 is blocked, T cells will no longer respond to antigens. Its expression on B cells varies among species. Thus it is found on all rabbit B cells and on a subpopulation of B cells in most species including mice and humans, but it is not found on B cells in rats or dogs.

CD_8: -It is a 32kDa dimeric glycoprotein that used to be called T8. It is a specific receptor for MHC class 1 found on cytotoxic T cells. CD_8 plays a key role in the recognition of endogenous antigens by these cells.

CD_{21}: A complement receptor also called CR2. It is a glycoprotein of 145 kDa found on B cells, some T cells and dendritic cells. Its several ligands include CD_{19}, CD_{23} and C3d. Like its major ligands, CD_{21} plays a key role in regulating B cell responses (Abbas and Lichtman 2001).

CD_{25} is the alpha chain of the IL-2 receptor. It is a glycoprotein of 55kDa that is expressed on activated T cells, B cells and monocytes. When CD_{25} binds IL-2, it activates these cells (Abbas and Lichtman 2001). Thus, CD_{25} is commonly considered a marker for activated T and B cells.

CD_{26} is involved in signaling in T cells.

CD_{45} is a family of 190 to 220kDa glycoproteins found on all cells of hematopoietic origin except red cells. Alternative splicing of three exons generates various isoforms of CD_{45}. They are all phosphotyrosine phosphatases, some of which are required for signaling through the TCR.

W.C.1 are a subset of gamma delta T cells, which are not homologous among species such as humans or mice. The CD molecules have homology among domestic mammals as well as in humans and mice (Tizard 2000).

2.2 *Leptin*

Leptin is a hormone product of the obese gene originally identified in mice (Zhang et al. 1994). It is a 16-kDa protein that is secreted almost exclusively by white adipocytes and then transported through blood and the brain barrier to the hypothalamus (Houseknecht et al 1998). Leptin plays a central role in appetite regulation and energy expenditure (Zhang et al. 1994; Houseknecht et al. 1998; Buchanan et al. 2002). Mice that are homozygous for the recessive mutation (ob/ob) in the obese gene do not express leptin. Another fat mouse (db/db) homozygous for the recessive mutation is diabetic and has a defective receptor but is capable of producing leptin. Both ob/ob and db/db mice are phenotypically similar with each weighing three times more than a normal mouse and having a fivefold increase in body fat (Friedman and Halaas 1998)

Leptin possesses a four-α-helix bundle structure that has identified it as a member of the haemopoietic cytokine family (Rock et al. 1996). The structure of leptin bears close resemblance with the cytokine IL-6 (Tartaglia et al. 1996) that makes it an important factor in the regulation of immune response (Matarese et al. 2005). In support of this, leptin receptors are not only found in the central nervous system but also in peripheral tissues such as hematopoietic and immune systems (Sanchez-Margalet et al. 2003). The cell types connected with immune and inflammatory response express the long form of leptin receptor, OB-R that allows leptin to adjust their response to diverse

stimuli. Gainsford et al. (1996) also found that the CD_4 and CD_8 T -lymphocytes express the long form of the ob receptor. Leptin also plays a role in reproduction. The ob/ob mouse is infertile (Zhang et al. 1994) however administration of recombinant leptin to infertile ob/ob mice restores fertility (Chehab et al. 1996).

2.2.1. *Leptin and appetite regulation*

Leptin regulates appetite and energy metabolism by decreasing food intake and increasing energy expenditure and physical activity (Houseknecht et al. 1998). A higher body fat content results in an increased hypothalamic regulation of the secretion of leptin and vice versa. Leptin is produced in the white adipose tissue and transported through the blood and brain barrier to the hypothalamus. In the hypothalamus leptin binds to the long form of its receptors to elicit a neural response (Houseknecht et al. 1998). The result of the neural response is an increased physical activity and energy expenditure and a decreased food intake. This causes a reduction in the size of the adipose tissue, which later results in a negative feedback to inhibit further expression of the leptin gene (Houseknecht et al. 1998). The expression of the ob gene is dependent on the level of energy stores and also on the size of the adipocyte. It increases after food intake and decreases rapidly with food restriction (Waelput et al. 2002). The leptin receptor (Ob-R) has five isotypes namely Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re (Caprio et al. 2001). The Ob-Rb receptor, which is the longest form, is responsible for regulating appetite. It is located in the hypothalamus (Caprio et al. 2001).

Deficiency in leptin results in an obese phenotype with an increased appetite and a decreased metabolism. The increase in appetite results partially from the inability of leptin to induce the secretion of alpha melanocyte stimulating hormone (α-MSH) from the hypothalamus. One of the functions of α-MSH is to bind to central melanocortin-4-receptor (MC4R) bearing neurons, which in turn reduces appetite (Figure 2.1). This allows a diffusion of α-MSH to the periphery where upon binding to MC-Rs on adipocytes causes a mobilization of fat stores (Forbes et al. 2001). There are five ways in which leptin reduces appetite. This is illustrated in Figure 2.1.

Leptin reduces appetite by decreasing the levels of neuropeptide Y (NPY), an appetite stimulant (Houseknecht et al. 1998). It increases the production of POMC, which then causes increased α MSH production (Pritchard et al. 2002). Alpha MSH decreases appetite when bound to MC4R (Forbes et al. 2001). Leptin also reduces the production of agouti related protein (AGRP), which functions to suppress MC4R (Pritchard et al. 2002). This ability of leptin to increase the agonist (α-MSH) and reduce the antagonist (AGRP) results in α MSH possessing an advantage over AGRP for the receptor hence reducing appetite. The production of leptin is also stimulated by glucocorticoids (Houseknecht et al. 1998).

In human obese subjects, this homeostasis is out of balance. Possibilities are that adipocytes release large quantities of leptin, however, no α-MSH is released in response to it to bring about a reduced appetite (Forbes et al. 2001). In the absence of central or

peripheral signaling of α-MSH, appetite increases. The second possible reason for the obese subjects is that there is no functional leptin and this causes a less than sufficient amount of α-MSH to be released, hence contributing to high food intake (Forbes et al. 2001).

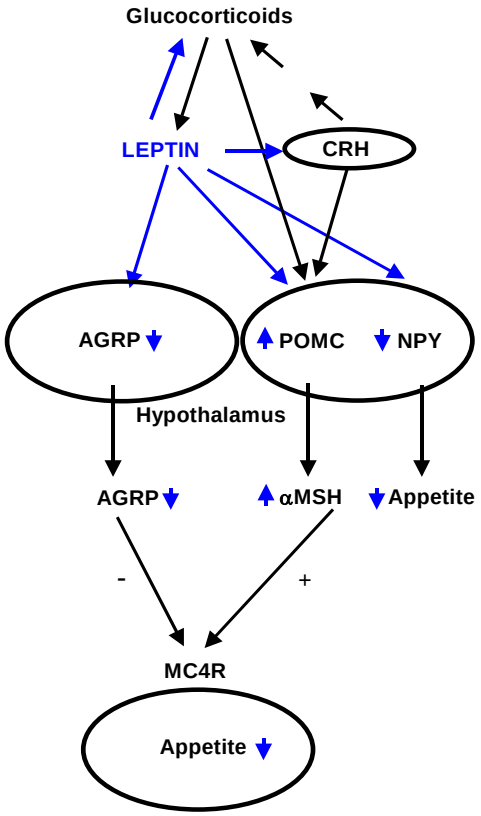

Figure 2.1 Interaction between leptin and other hormones to reduce appetite. CRH-Corticotrophin Releasing Hormone, AGRP-Agouti Related Protein NPY-Neuropeptide Y, MC4R-Melanocortin 4 Receptor

Glucocorticoids stimulate the production of leptin (Houseknecht et al. 1998) and together with leptin they boost the production of POMC leading to the release of α-MSH. When leptin is administered to leptin deficient (ob/ob) mice, peripheral α-MSH levels rise

leading to a high metabolic rate and an increase in weight loss through reduced food intake. This indicates that α-MSH plays an important role in mediating appetite regulation in response to leptin (Forbes et al. 2001).

Leptin has been associated with fat deposition in cattle (Buchanan et al. 2002). In cattle a single nucleotide polymorphism (SNP) i.e. a cytosine to thymine transition results in an amino acid change from arginine to cysteine was responsible for the increased carcass fat deposition (Buchanan et al. 2002). The amino acid change is found on the fourth position from the N-terminus of the mature leptin protein molecule (Buchanan et al. 2002). This is a beneficial non-conserved mutation for animal production since in dairy cattle a high milk and protein yielding animal increases profit. In beef cattle, a premium is paid for marbling of muscle.

2.2.2. Leptin and reproduction

Administration of leptin in both male and female ob/ob mice restored fertility but not in the db/db mice due to the defect in the receptor that led to the conclusion that obesity is not the cause of infertility but rather leptin deficiency directly modifies reproductive capacity (Chehab et al. 1996). Leptin may directly affect the reproductive system or indirectly act on the reproductive system by regulating the secretion of growth hormone (GH) or prolactin (PRL), which then acts on the gonads as indicated in Figure 2.2 (Clarke and Henry 1999; Adashi 1995; Linzer and Arey 1995). Leptin affects

reproduction based on changes that occur in the amount of body fat (Clarke and Henry, 1999). In order to maintain a good reproductive function, normal levels of leptin are required and in humans this is also necessary for sustaining menstrual cycles as well as the prevention of amenorrhea (Mantzoros 2000).

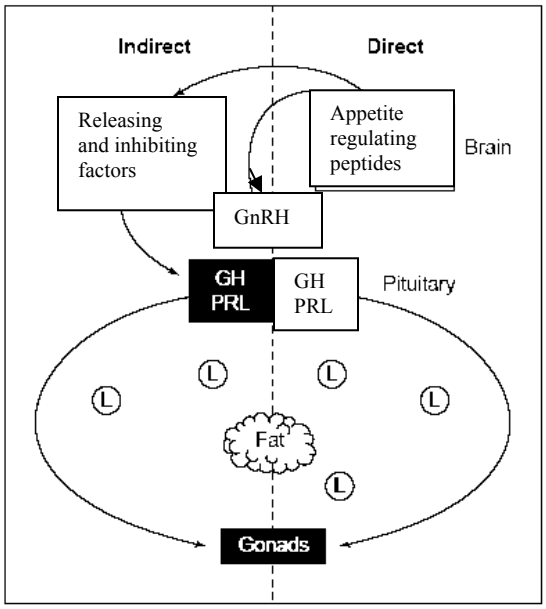

Figure 2.2 Effects of leptin on the reproductive system (Adopted from Clarke and Henry, 1999). GH-Growth Hormone, GnRH-Growth Hormone Releasing Hormone, L-Leptin, PRL-Prolactin

The peptide NPY is of importance to reproduction due to its effect on the gonads (Clarke and Henry, 1999). NPY neurons have estrogen receptors that affect gonadotropin-releasing hormone (GnRH) and hence NPY suppresses luteinizing hormone (LH) secretion. Plasma levels of leptin affect hypothalamic stimulation of the gonadotropic axis and hence sexual behavior (Aubert et al. 2002). The secretion of Gonadotrophin Releasing Hormone-Luteinizing Hormone (GnRH-LH) in rats is

modulated by leptin. Leptin affects reproduction by the release of luteinizing hormone (LH) in a pulsatile fashion similar to the release of the leptin hormone (Caprio et al. 2001). Aubert et al. (2002) concluded that in mice, leptin promotes sexual maturation but did not have a definite conclusion on experiments performed using other species such as rats, monkeys and humans.

Leptin must be above a critical threshold to induce sexual maturation, vaginal opening and increase in ovarian weight which results from the activation of the gonadotropic axis (Gruaz et al. 1998). To initiate puberty leptin acts on the hypothalamus, hence leptin offers a permissive role with respect to puberty (Clarke and Henry 1999). Leptin accelerates estrus and mating (Chehab 1997).

The concentration of leptin in the body increases during pregnancy but reduces during the latter stages of pregnancy and reaches its lowest level during lactation (Ingvartsen and Boisclair 2001). In rats, leptin levels were highest between day 9 and 19 of pregnancy-a two-fold difference compared to non pregnant mice and fell sharply to levels lower than non pregnant rats on day 21 just before parturition (Gruaz et al. 1998). The placenta produces leptin (Casabiell et al. 2001) hence leptin levels are higher in females than in males. On the contrary, Gruaz et al. (1998) reported that the placenta does not produce leptin but is rather a target organ for leptin since it has a number of leptin receptors. Maternal plasma leptin levels increases as pregnancy progresses due to over expression of leptin mRNA in both adipose tissue and placenta (Garcia et al. 2000).

2.2.3. *Leptin and immune response*

Leptin also regulates immunity (Ingvartsen and Boisclair 2001). Leptin is secreted almost exclusively by white adipocytes (Houseknecht et al. 1998). Leptin secretion is induced by lipopolysaccharides and cytokines (Faggioni et al. 1999). Animals that either lack leptin (ob/ob) or are deficient of leptin receptors (db/db) have limitations in their immune and inflammatory reponses (Fantuzzi and Faggioni 2000). Lord et al. (1998) found ob/ob mice to have defective cell mediated immunity. Busso et al. (2002) observed that the ob/ob mice lymph node cells had a decreased interferon gamma (INF-γ) production. ob/ob mice are more affected by the lethal effect of LPS compared to normal mice (Faggioni et al. 1999). These limitations are also present in starved and malnourished animals, which are marked by low circulating leptin levels (Fantuzzi and Faggioni 2000).

A reduction in leptin level contributes to the adaptive response to preserve energy for the immediate vital functions of the body, which are required for survival (Waelput et al. 2002). Significantly low leptin levels associated with restricted feed intake and low energy levels reduce the mRNA expression of the leptin gene in rat splenocytes (Lamas et al. 2004). Reduced appetite and energy balance are linked with depressed immune function (Grunfeld et al. 1996). This finding is in agreement with work done by Lord et al. (1998) in mice where the immunosuppressive effects of acute starvation have been overcome by supplying leptin.

Leptin replacement restores normal immune response in ob/ob mice. The restoration of the proper immune function in mice that had previously been starved is an affirmation of the interrelation between immune function and energy balance (Lord et al. 1998). Human leptin deficiency also causes a marked immune deficiency during starvation, which results in the suppression of a T-lymphocyte response. This is a possible explanation for the increased sensitivity to infections when starved (Cason et al. 1986). Inherent leptin deficiencies in humans are causes of childhood infections and early mortality (Ozata et al. 1999).

Leptin mediates both innate and adaptive immunity. In innate immunity, leptin has effect on monocytes and macrophages by up regulating their phagocytic role and also promotes the secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 (Matarese et al. 2005). Leptin has a proliferating effect on human circulating monocytes *in vitro.* The effect of leptin on adaptive immunity is through the production of lymphocytes that respond to peptide-major histocompatibility complexes as well as the production of survival signals for CD_4 and CD_8 lymphocytes during T lymphocyte maturation (Howard et al. 1999). Leptin also increases the expression of activation markers such as CD_{25} (α-chain of IL-2 receptor) among others (Matarese et al. 2005). Leptin stimulates hematopoesis, T-cell immunity, phagocytosis and cytokine production, and also reduces susceptibility to infections (Ingvartsen and Boisclair 2001; Busso et al. 2002).

Busso et al. (2002) found leptin to stimulate the growth of CD_4 lymphocytes *in vitro*. Leptin affects cytokine production as well as having proliferative and anti-apoptotic effects on a diverse range of cell types including T lymphocytes, leukemia

cells, and hematopoietic progenitors (Fantuzzi and Faggioni 2000). Leptin production is stimulated during infection and inflammation to activate monocytes and macrophages that function in innate immune response (Fantuzzi and Faggioni 2000). Wound healing, angiogenesis (the formation of new blood vessels) and hematopoiesis are all characteristic of, and affected by leptin secretion (Fantuzzi and Faggioni 2000).

Leptin increases T_H1 and decreases T_H2 lymphocytes in mice (Ingvartsen and Boisclair 2001). Similar results were obtained by Lord et al. (1998) who found ob/ob mice to have a shift toward T_H2 cytokine production as well as having a defective cell mediated immunity. Whilst leptin increases IL-2 and INF-γ production, it decreases IL-4 production (Waelput et al. 2002). This shows leptin's role in regulating the T_H1/T_H2 balance. Large quantities of T_H1 cytokines such as IL-2 and INF- γ are present in inflammatory lesions whereas T_H2 cytokines, for example IL-4, are not. In their study using mice with antibody-induced arthritis, Busso et al. (2002) found that the ob/ob mice lymph node cells had decreased INF- γ production, a T_H1 cytokine, and an increased secretion of IL-10, a T_H2 cytokine after being stimulated with methylated BSA *in vitro*. T_H1 is a T-helper lymphocyte subset of CD_4^+ cells, which produces IL-2, INF-γ and TNF-α. T_H1 cells are very important in mediating phagocytotic killing of microbes (Abbas and Lichtman 2001). Faggioni et al. (2001) also suggested that a defect in leptin production as found in the ob/ob mice might be associated with a shift of the immune response toward pro-inflammatory cytokines and a reduction in anti-inflammatory cytokines. The expression of class II MHC molecules on macrophages is stimulated by INF- γ. This action of INF- γ increases T-cell responses. Leptin regulates the growth and rapid increase of naïve and memory T cells (Lord et al. 1998).

Leptin activates monocytes to produce cytokines such as TNFα and IL-6 (Santos-Alvarez et al. 1999). The level of production of these cytokines depends on the quantity of leptin supplied. In mice, Agnello et al. (1998) found that at doses higher than that required to cause body weight loss, leptin had the ability to induce IL-1 to stimulate the production of IL-6 and serum corticosterone *in vivo*. They indicted that it is only at these high doses that leptin shows some of the *in vivo* activities associated with the IL-6 family of cytokines. Leptin's role as a pro-inflammatory cytokine has been illustrated in Figure 2.3. Leptin has the ability to activate T cells thereby leading to the production of IL-2, the T cell growth factor. This results in further proliferation of T cells that attack infected body cells to kill them and remove the source of the infection.

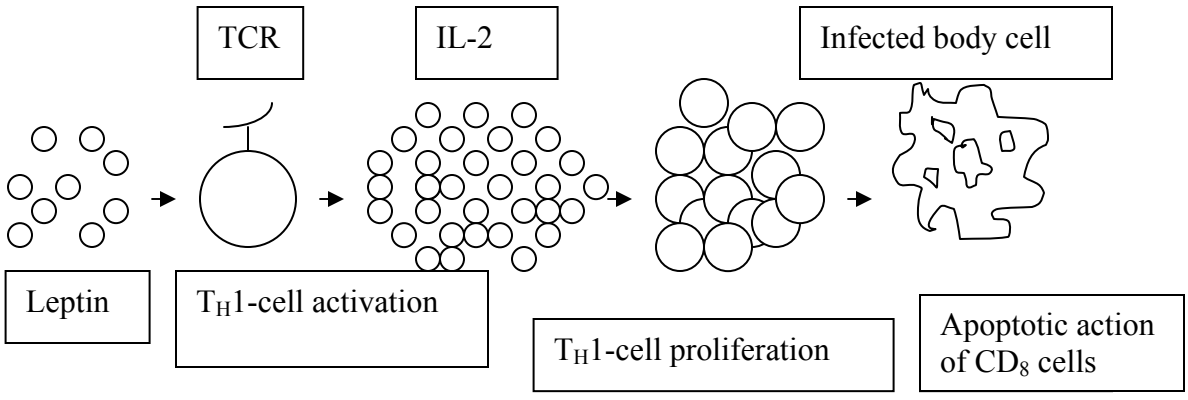

Figure 2.3 The role of leptin as a pro-inflammatory cytokine

The effect of leptin on CD_4^+ proliferation has been tested in mice and it was observed that the cell population that had been preincubated with leptin for 12 hours had a marked increase in proliferation in the subsequent mixed-lymphocyte reaction (Lord et al. 1998). The mixed-lymphocyte reaction was found to cause a proliferative response by both naïve and memory T cells.

Santos-Alvarez et al. (1999) found leptin to induce proliferation, differentiation and functional activation of hematopoietic cells. The cytokine (TNF and IL-6) production by activated monocytes was measured. The outcome showed that leptin administration produced an increased number of activated monocytes depending on its dose. This indicates that leptin is a potent stimulatory hormone on human peripheral blood monocytes and suggests that it may also have a role as a proinflammatory cytokine (Santos-Alvarez et al. 1999; Sanchez-Margalet et al. 2003). Faggioni et al. (1997; 1999) established that when leptin from an external source is supplied to leptin deficient mice, LPS and TNF-α induced lethality is forestalled. In previous experiments, Gainsford et al. (1996) and Houseknecht et al. (1998) had indicated the ability of leptin to stimulate mature macrophages and to regulate hematopoiesis. Leptin stimulates the growth of antibody-producing B-lymphocytes (Busso et al. 2002). On the contrary, Agnello et al. (1998) suggested that the affect of leptin is more pronounced in the control of body weight but not in inflammatory or hematopoitic processes.

The effect of starvation on immune function is not always suppressive. There seems to be an increased monocyte response because the activity of blood monocyte bacteria and natural killer cell cytolytic activity are enhanced by fasting (Wing et al. 1983). This was in agreement with the work done by Moriguchi et al. (1989) who

observed that macrophage function was enhanced by day two of starvation in mice but was suppressed at a longer term. The number of peripheral blood monocytes increased in starved mice compared to fed mice. This suggests that the proliferating population of monocytes could be the reasons for the resistance of the starved mice against infectious agents (Moriguchi et al. 1989).

Leptin has been shown to induce proliferative responses *in vitro* in mice (Waelput et al. 2002). These responses are made possible using peripheral blood lymphocytes and highly purified CD_4^+ T lymphocytes as responders. This observation is not present in db/db mice (Waelput et al. 2002). Grunfeld et al. (1996) observed an increased leptin level and increased ob gene expression in adipose tissue following the administration of inflammatory stimuli, for example LPS or turpentine to hamsters.

Tumor Necrosis Factor is an important factor in the regulation of obesity by decreasing energy intake or inducing thermogenesis (Coombes et al. 1987, Rothwell 1988). Adipocytes of obese and morbidly obese patients over expressed TNF-α, which was proportional to the extent to which they were obese (Bullo et al. 2000). This was also true in the work done by Hotamisligil et al. (1995) who found that the adipose tissue expression of TNF-α was positively related to BMI and decreased in proportion to the loss in body weight.

2.3. *Pro-opiomelanocortin*

Pro-opiomelanocortin (POMC) is a hormone derived in the hypothalamus (Arends et al. 1998) that is also processed in the cortitrophs of the anterior pituitary (Adachi et al. 1999). The POMC peptide which has a molecular weight of 32 kDa and serves as a precursor to several other biologically active peptides such as adrenocorticotropin (ACTH), beta-lipotropin, alpha-melanocyte stimulating hormone (α-MSH), beta-melanocyte stimulating hormone (β-MSH) and beta-endorphin, which are released by the pituitary glands of fish and mammals (Arends et al. 1998; Ali et al. 2005). The basic function of POMC is to stimulate adrenal glands to release cortisol (Arends et al. 1998). In humans, the POMC gene maps to chromosome 2p23.3 (Chen et al. 2005) and to chromosome 11 in cattle (Thue and Buchanan 2003).

Pritchard et al. (2002) describe POMC's role in the regulation of appetite (Figure 2.1). This role of POMC is linked with the bioactive peptides derived from pro-hormones such as α-MSH and ACTH (Pritchard et al. 2002). α-MSH regulates appetite and energy expenditure by binding to the melanocortin-4 receptor (MC4R) that is expressed mainly in the hypothalamus (Mori, 2001; Pritchard et al. 2002). α-MSH or its analogues inhibits appetite whilst the MC4R antagonist agouti-related peptide (AGRP) stimulates appetite.

When MC4R is removed from adult mice, these mice tend to have a reduced energy utilization leading to obesity (Mori, 2001). Humans and mice lacking POMC are obese and present hyperphagia as well as having adrenal insufficiency (Krude and Gruters 2000). POMC neurons serve as important mediators in regulating feeding

behavior, insulin levels and body weight (Boston 2001). This is consistent with the fact that POMC has a close link with appetite and obesity and hence qualified as a positional candidate gene for a growth quantitative trait locus in beef cattle (Thue and Buchanan 2003). Buchanan et al. (2005) have subsequently found an association between POMC and shipping weight, hot carcass weight and average daily gain in beef cattle.

2.3.1. *POMC and immune response*

POMC derived α-MSH modulates some aspects of inflammation through direct effects on T cells, B cells and monocytes (Adachi et al. 1999). The production of POMC peptides and POMC gene expression is under the stimulation of pro-inflammatory cytokines such as IL-1, IL-2, IL-6, INF-γ and TNF-α (Besedovsky et al. 1996). However, Adachi et al. (1999) found that although the activation of bone marrow cultured murine mast cells induced the expression of mRNAs for the inflammatory cytokines IL-1β, IL-4, IL-6, TNF-α, and the chemokine lymphotactin; mRNAs for IL-1 β, TNF-α and lymphotactin were down-modulated in the presence of α-MSH. This was consistent with the finding of Blalock (1999) in which the POMC product α-MSH functionally acts as an IL-1 antagonist. Again, Getting et al. (1999) observed that in mice ACTH prevents neutrophil accumulation while peripheral blood cell count remained unchanged. There was a similar occurrence with both α and β melanocyte stimulating hormones. ACTH also significantly reduces chemokine release *in vivo*. However, MC3R and MC4R both prevented this repressive effect of ACTH (Getting et al. 1999).

The expression levels of peptides derived from POMC depend on immune cytokine release (Slominski et al. 2000). Expression and release of POMC in the immune system is stimulated by CRH, but is inhibited by glucocorticoids (Bertagna et al. 1994). Despite the inhibitory action of glucocorticoids on the pituitary gland, glucocorticoids increase POMC gene expression in the hypothalamus (Pritchard et al. 2002). In rats, Mechanick et al. (1992) reported their inability to find POMC expression in either tissue-derived lymphocytes or peripheral blood mononuclear cells *in vivo*.

OBJECTIVE

- To genotype 556 yearling beef cattle with single nucleotide polymorphisms (SNPs) in leptin and POMC in order to determine the correlation between genotypes with the numbers of B and T-lymphocytes in these animals and their immune response to rabies vaccination.

HYPOTHESES

- SNPs in both leptin and POMC may have an influence on immune response in cattle since they have been speculated to be pro-inflammatory cytokines.

- There would be an association between the different genotypes (CC, CT, TT) of leptin and POMC, their lymphocyte population and the manner in which animals possessing these genotypes are able to respond to immune challenges. Based on a previous study with leptin (Buchanan et al. 2002) we expect that the T allele may be linked with a better immune response.

3.0. MATERIALS AND METHODS

3.1. *Blood Collection and Vaccination*

The blood samples, lymphocyte counts as well as rabies antibody concentrations were made available by the Western College of Veterinary Medicine at the University of Saskatchewan from a previous experiment in which a licensed commercial rabies vaccine had been administered to 556 healthy mixed breed yearling beef cattle. These animals were selected from 27 beef herds in Alberta and Saskatchewan. Animals had been vaccinated intramuscularly with two separate I M doses (2ml) of a killed, commercial rabies vaccine (IMRAB Bovine Plus, Merial Canada) on days 0 and 21 of the study period. The study period was between March 13 and August 27, 2002. Day 0 ranged between March 13 and May 15 for the various herds while day 42 ranged between April 23 and August 27, 2002. Sixty percent of these animals constituting 16 out of the total of 27 herds received Bovine Viral Diarrhea Virus (BVDV) vaccination within one month prior to the investigation, or between day 0 and day 21. Blood samples were collected just before vaccination on day 0 from each animal by jugular venipuncture to determine baseline serum rabies antibody titres. The same method of blood collection was repeated on day 21 prior to administration of the booster vaccine and also on day 42 of the study. Blood samples were divided into 3; one was used for rabies antibody titre analysis, one

for lymphocyte subtype analysis and one for DNA analysis. Blood samples that were used for DNA analysis were frozen at minus 80 degrees Celsius between the period of blood collection and DNA analysis. Dan Bechtel of the Western College of Veterinary Medicine (WCVM) at the University of Saskatchewan as part of his PhD research performed the entire cell typing and coordinated the assessment of the rabies antibody measurements.

3.2. *Measurement of Rabies Antibody Titres*

Rabies virus neutralizing antibody titres were determined at the Kansas State University Rabies Laboratory (Manhattan, KS, USA) using the rapid fluorescent focus inhibition test (RFFIT; Smith et al. 1973). This test measures antibody directed against the glycoprotein antigen in the viral envelope, which prevents infection by the rabies virus and thus reflects the production of protective neutralizing antibodies.

3.3. *Lymphocyte Subtype Measurements Using Flow Cytometric Analysis*

Flow cytometry was used to measure lymphocyte subtype populations with monoclonal antibodies (mAbs) using subtype-specific cell surface markers, followed by isotype-specific secondary fluorescent antibodies (Davis et al. 1995). Previously determined titred dilutions of mAbs specific for lymphocyte subtype cell surface markers

were placed in triplicate (50µl per mAb dilution/well) into 96-well Falcon 7 U-bottom microtitre plates (Becton Dicinson, Franklin Lakes, NJ). Prepared plates were made up in advance and were stored at 2-8^{o}C for no more than one week. One to two animals were randomly selected each week to act as negative controls in which cells were treated with either the primary mAbs or fluorescent secondary antibodies only, to ensure that background fluorescence, within a single fluorescence channel, remained below 1% of the total cell population.

A flow cytometer (Epics Elite ESP7, Beckman Coulter Inc., Mississauga, ON) was used to obtain data on the labeled cell types. Data from 10,000 events per sample were acquired and subsequently analyzed using Expo327 acquisition and analysis software (v. 1.2, Beckman Coulter Inc., Mississauga, ON). Cell type proportions were converted to absolute cell counts before statistical analysis. Either manual counting with a Neubauer hemocytometer (VWR International) or an automated cell counter (Cell Dyn 3500, Abbott Park, IL) was used to determine total white blood cell (WBC) counts. Absolute cell counts were determined by first identifying neutrophils and monocytes, using the DH59B mAb.

The neutrophil population was gated using the side scatter (SSC) forward scatter (FS) dot plot to determine the granulocyte percentage. Total percent positive cells were multiplied by absolute lymphocyte/monocyte counts as determined by subtracting the percent neutrophils, established with the flow cytometer, from the WBC count. To

monitor differential accuracy, the percent neutrophils obtained from the flow cytometer was compared to the percent neutrophils obtained from manual differential WBC counts derived from blood smears (100 cell counts). The analyzed cell types have been listed in Table 3.1.

Table 3.1 Cell Types Analyzed

CD_4	$CD_4CD_{25}CD_{45}$
CD_8	$CD_4CD_{26}CD_{45}$
Ratio of CD_4 to CD_8	$CD_8CD_{25}CD_{45}$
W.C.1 cells	$CD_8CD_{26}CD_{45}$
Gamma delta cells	CD_5CD_{21} & B cells
IL-2	B cells

3.4. *DNA Extraction and Genotyping*

DNA was extracted from frozen whole blood samples (n=556). DNA extraction was initially performed by salt extraction (Montgomery and Sise 1990) for about half of the total number of samples and by a phenol-chloroform method (Sambrook et al. 1989) for the remaining half. The entire set of extracted DNA was purified using the MagNa

pure LC kit (Roche Molecular Biochemicals, Mannheim, Germany). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) tests were run for the SNPs in *LEP* (Buchanan et al. 2002) and *POMC* (Thue and Buchanan 2003).

3.4.1. *Leptin-PCR-RFLP*

Each reaction (20µl) was made up of: 10 pmoles of forward and reverse primers, 1 U *Taq* DNA polymerase (Invitrogen), 200 µM dNTPs, 10X PCR buffer, 1.5 mM $MgCl_2$ (Invitrogen), sterile distilled water and approximately 30 ng of genomic DNA. PCR amplification had an initial denaturation at 94^{o}C for 2 minutes, followed by 35 cycles of 94^{o}C for 45 s, 52^{o}C for 45s, and 72^{o}C for 55s. Final extension was set at 72^{o}C for 3 min.

A test gel (0.7% agarose) was routinely run for each batch of samples after each PCR using 5 µl of PCR product. Each digestion reaction consisted of 15 µl of PCR product, 2 U of *Kpn 2I* (MBI Fermentas), 10X Y+/Tango buffer and sterile distilled water. A total reaction volume of 20 µl was incubated at 55^{o}C overnight and DNA fragments were separated on 3% agarose gel by electrophoresis.

3.4.2. *POMC-PCR-RFLP and Real Time PCR*

Each 20µl reaction contained: 10pmoles of forward and reverse primers, 200 µM dNTPs, Jeffrey's buffer (4.5 mM $MgCl_2$), DMSO, sterile distilled water, 1 U *Taq* DNA polymerase (Invitrogen), and approximately 30 ng of genomic DNA. PCR amplification had an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 94°C for 1min; 52°C for 45s, and 72°C for 1min. Final extension was set at 72°C for 3 min.

A test gel (0.7% agarose) was run after each PCR using 5 µl of PCR product. Each digestion reaction consisted of 15 µl of PCR product, 2 U of *Bts I* restriction endonuclease, 10X NEBuffer 4 and sterile distilled water. A total reaction volume of 20 µl was incubated at 37°C overnight and DNA fragments were separated on 2% agarose gel by electrophoresis.

Samples that failed to amplify for POMC (n=51) were analyzed at Quantum Genetics using real time capillary PCR via a LightCycler 1 (Roche Molecular Biochemicals, Mannheim, Germany). The primer sequences used were: -

Forward primer: - 5'GAT GAG CAG CCG CTG ACT 3'

Reverse primer: - 5'GTC AGC TCC CTC TTG AAT TCG AG 3'

Sensor probe: - 5'CAA CTC CGC TGC TGC TGC 3'

Anchor probe: - ACC ATT CCG ACG GCC GAA GC 3'

The PCR reaction (10.3 µL) comprised distilled water, 2.9 mM $MgCl_2$, 6 pmol of forward primer and reverse primer, 5 pmol per reaction of anchor probe, 5 pmol per reaction of sensor probe and 0.7 µL of LightCycler FastStart DNA master hybridization probes (catalog no. 2 239 272; Roche Molecular Biochemicals, Mannheim, Germany) plus 1 µL

of sample template DNA. The anchor probe was labeled with flourescein as the donor, and the sensor probe was labeled with LightCycler Red 640 (TIB Molbiol LLC, Adelphia, NJ) as the acceptor for the FRET reaction.

The PCR fluorescence resonance energy transfer (FRET) conditions included an amplification program beginning with denaturation at 95^{o}C for 10 min, followed by 45 cycles of 95^{o}C for 2s, 60^{o}C for 10s, and 72^{o}C for 14s. The melting program of the reaction was heated to 95^{o}C and then cooled to 40^{o}C over 120s followed by a continuous increasing temperature transition rate of 0.2^{o}C/s until a temperature of 75^{o}C is reached.

The cooling portion of the reaction is lowering the temperature to 40^{o}C for 5s. Melting temperatures ™ were derived from melting peaks using LightCycler software version 3.5. Each test batch incorporated a maximum of 28 samples plus three positive controls (CC, CT and TT) and a negative control (water).

3.5. *Statistical Analysis*

The primary null hypothesis was that there was no association between the immunological outcome (lymphocyte numbers and change in rabies antibody titres from day 0 to day 42) and genotype. Descriptive statistics were completed using SPSS for Windows (version 11.5.0, SPSS Inc. Chicago, IL, USA). Transformation of the outcome variables was used to improve the normality of the residuals. Dr Cheryl Waldner of the WCVM, University of Saskatchewan performed the statistical analysis while I extracted the relevant information and interpreted the results.

The potential association between genotype and immune function parameter was explored sequentially using a mixed linear model (PROC MIXED, SAS version 8.2, SAS Institute, Cary, N.C., USA). Clustering outcomes at the herd level was accounted for with random intercept for herd. Each association was examined in a model adjusted for herd effects and cell counting method in compliance with rabies vaccination protocol. The adequacy of the models was evaluated using normal probability plots of the residuals and plots of residuals against predicted values to verify that the assumptions of normality and homogeneity of variance had been met.

4.0 RESULTS

4.1 *Leptin*

Results were obtained from a number of cattle used for this research, which were 556 on day 0 and 443 on day 42 for leptin. The subjects for POMC were 519 on day 0 and 415 on day 42. A representative photograph of a leptin PCR-RFLP gel is shown in Figure 4.1. An uncut product is visible at approximately 95 base pairs (bp) indicating a T allele whilst cut products representing a C allele have bands at 75bp and 19bp (not usually visible on gel). Lane 9 is from an animal homozygous for C allele, lane 13 is from an animal homozygous for T allele and the rest of the animals on the gel are heterozygotes.

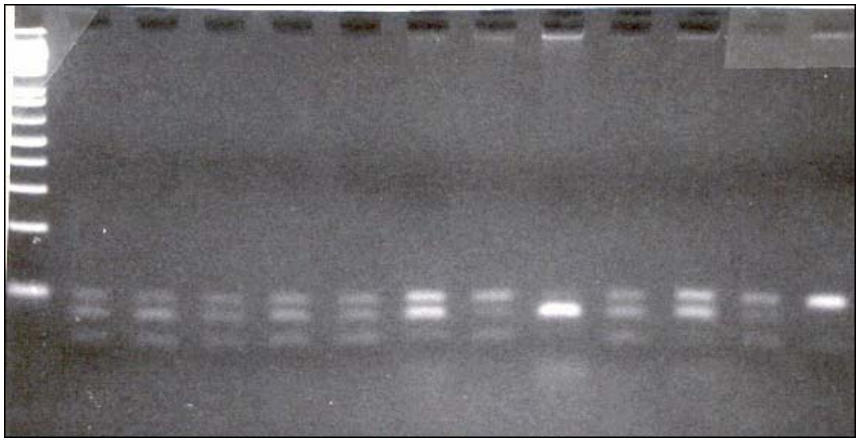

Figure 4.1 PCR-RFLP gel showing genotypes of leptin. Lane 1, 100bp ladder. Bands at 95bp represent the T allele and bands at 75bp represent C allele.

Allele frequencies for leptin on both day 0 and day 42 are shown in Table 4.1. There was not much difference between the allele frequencies for the two visits. The results from the Proc mixed analysis of leptin genotypes with immune data are presented

in Tables 4.2 and 4.3. These represent the outcomes that have probabilities less than or equal to 0.1 ($P \leq 0.1$) on days 0 and 42 respectively. Least square means per genotypes with outcomes ($P \leq 0.05$) are illustrated in Figures 4.2 and 4.3 respectively for day 0 and day 42.

Table 4.1 Allele frequencies for leptin

Allele	C	T	N	Frequency
Day 0	644	468	556	C = 0.58 T = 0.42
Day 42	530	356	443	C = 0.60 T = 0.40

On day 0 (Table 4.2 and Figure. 4.2) there were only two cell types that had significant variations with leptin genotype: the activated CD_4 and the activated CD_8 cells. Cattle with TT genotypes had a significantly higher number of the activated CD_4 cells than those homozygous for the C allele ($P = 0.036$). There was a trend with the CT animals having a lower number of activated CD_4 cells compared to the TT animals ($P = 0.095$). The same association was true for the $CD_{8\_25\_45}$ activated CD_8 cells. Animals homozygous for the C allele had fewer activated CD_8 cells compared to the TT animals ($P = 0.05$). Hence, for day 0, the animals with the TT genotypes seemed to have higher numbers of activated T lymphocytes.

Table 4.2 Relationship between leptin genotypes and numbers of immune cell types On day 0

Cell Type	Genotype	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
Log Activated CD_4 ($CD_{4\_26\_45}$)	CC-TT	-0.063	-0.123	-0.004	**0.036**
	CT-TT	-0.045	-0.098	0.008	0.095
	CC-CT	-0.018	-0.055	0.019	0.330
Log Activated CD_8 ($CD_{8\_25\_45}$)	CC-TT	-0.058	-0.117	-0.000	**0.050**
	CT-TT	-0.043	-0.095	-0.009	0.106
	CC-CT	-0.015	-0.052	0.021	0.401

C.I refers to Confidence Interval

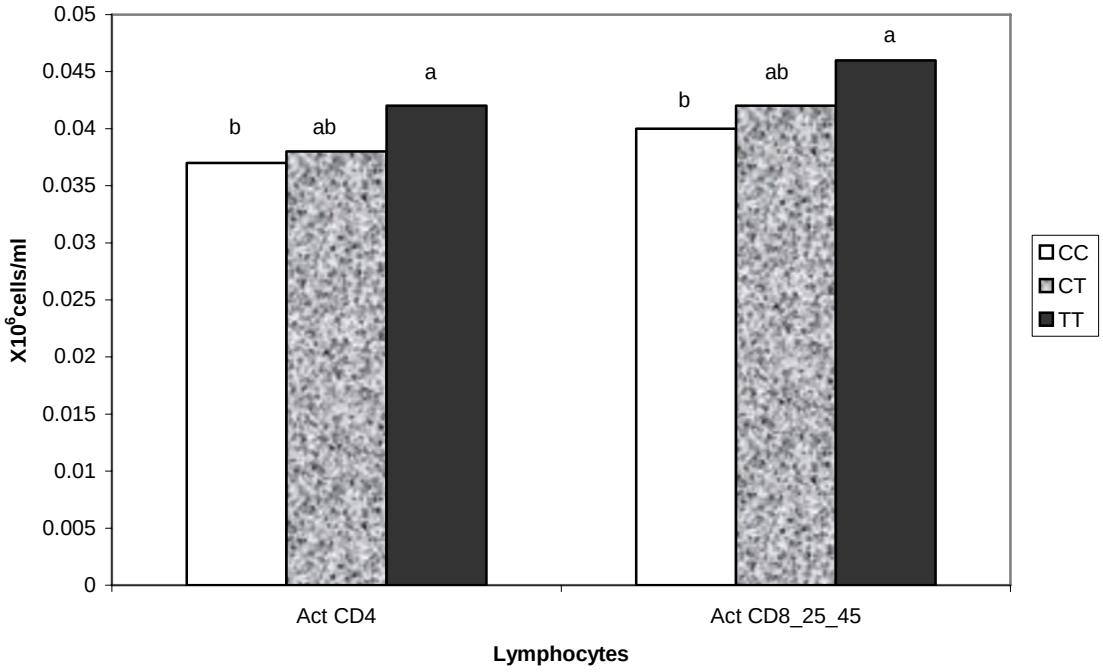

Figure 4.2 Least square means of immune cell types found in peripheral blood in beef cattle having significant variation with leptin genotype on day 0.

Variations in cell counts with respect to leptin genotypes on day 42 (Table 4.3 and Figure 4.3) were seen in seven cell types, namely; regular $CD_{4,}$ activated $CD_{4,}$ $CD_{8\_25\_45}$ activated $CD_{8,}$ $CD_{8\_26\_45}$ activated CD_8 cells, ratio of CD_4 to CD_8 cells, gamma delta cells and W.C. 1 cells. Animals with TT genotype had a trend showing elevated numbers of regular CD_4 cell count compared to both the CT animals and the CC animals.

Table 4.3 Relationship between leptin genotypes and numbers of immune cell types On day 42

Cell Type	Genotype	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
Log regular CD_4 cells	CC-TT	-0.043	-0.094	0.008	0.095
	CT-TT	-0.044	-0.091	0.003	0.067
	CC-CT	0.001	-0.029	0.030	0.967
Log Activated CD_4 ($CD_{4\_26\_45}$)	CC-TT	-0.016	-0.085	0.053	0.651
	CT-TT	-0.054	-0.118	0.010	0.098
	CC-CT	0.038	-0.002	0.078	0.062
Log Activated CD_8 ($CD_{8\_25\_45}$)	CC-TT	-0.022	-0.087	0.043	0.499
	CT-TT	-0.061	-0.121	-0.000	**0.048**
	CC-CT	0.038	0.001	0.076	**0.046**
Log Activated CD_8 ($CD_{8\_26\_45}$)	CC-TT	0.002	-0.069	0.072	0.961
	CT-TT	-0.042	-0.107	0.023	0.202
	CC-CT	0.044	0.004	0.085	**0.033**
Log Ratio of $CD_4$to CD_8	CC-TT	-0.026	-0.070	0.018	0.245
	CT-TT	-0.003	-0.043	0.037	0.884
	CC-CT	-0.023	-0.048	0.002	0.075
Log CD_8 gamma delta cells	CC-TT	0.009	-0.052	0.069	0.780
	CT-TT	-0.039	-0.095	0.017	0.173
	CC-CT	0.048	0.013	0.083	**0.007**
Log W.C.1 cells	CC-TT	-0.096	-0.179	-0.014	**0.023**
	CT-TT	-0.054	-0.130	0.023	0.169
	CC-CT	-0.043	-0.090	0.005	0.079

C.I refers to Confidence Interval

With respect to the activated CD_4 cells, the animals homozygous for the T allele again had a higher cell count compared to the CT animals. However, the homozygous C animals also had higher activated CD_4 counts than the CT animals. A similar pattern was seen in the $CD_{8\_25\_45}$ activated CD_8 cells. Here again, the TT animals had significantly higher number of cells compared to the CT animals and the homozygous C animals had higher cell numbers than the CT animals (P = 0.048; P = 0.046) respectively. With the $CD_{8\_26\_45}$ activated CD_8 cells the CC animals again had a higher cell count than their CT counterparts.

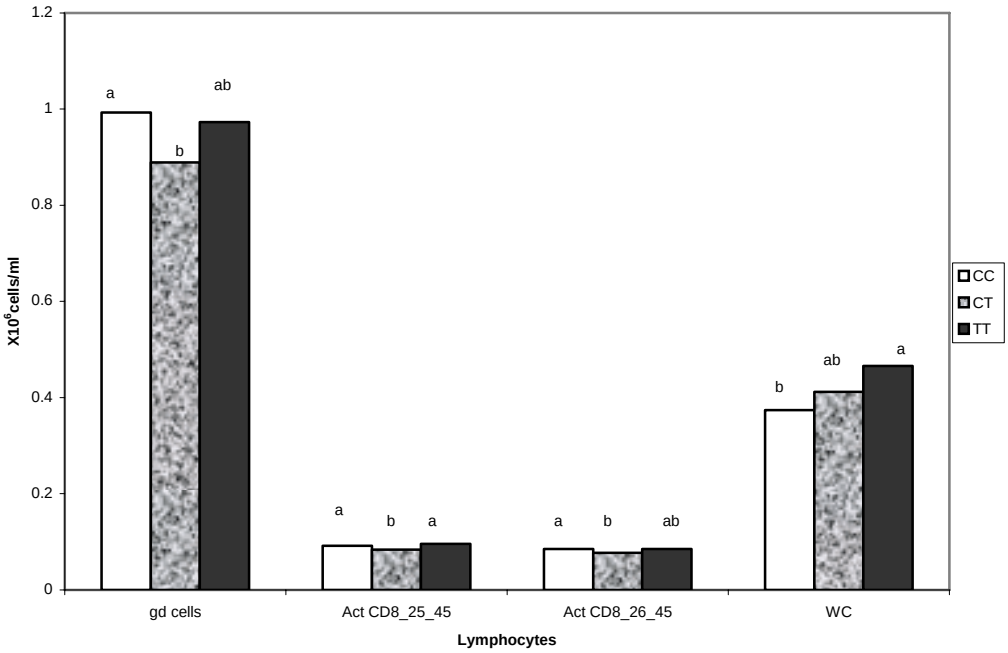

Figure 4.3 Least square means of immune cell types found in peripheral blood in beef cattle having significant variation with leptin genotype on day 42
gd cells = gamma delta cells

The ratio of CD_4 to CD_8 cells was higher in CT animals than in animals homozygous for the C allele. Animals homozygous for the C allele had a higher gamma delta cell count relative to the CT animals. Animals homozygous for the T allele or heterozygous had higher counts of W.C. 1 cells compared to the animals homozygous for the C allele ($P = 0.023$; $P = 0.079$) respectively. Comparisons among genotypes were made for all the other cell types and have been listed in Appendix A. Only results that had P values less than 0.1 have been discussed.

4.2. *Antibody Titres*

The differences between the rabies antibody titres for the three visits were measured. While there were significant variations in the rabies antibody titres between days 0 and 21 or 42 among genotypes of leptin (Table 4.4. and Figure 4.4), there was no such variation among genotypes of POMC for any of the 3 visits. All results of rabies antibody titre measurements for POMC as well as that for leptin have been indicated in Appendix B and those having P values less than 0.1 have been discussed.

Table 4.4 Rabies antibody titres versus corresponding genotypes of leptin

Day	Genotype	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
Logdiff0to21	CC-TT	0.137	-0.015	0.290	0.078
	CT-TT	0.141	0.002	0.280	**0.047**
	CC-CT	-0.004	-0.098	0.091	0.940
Logdiff0to42	CC-TT	0.188	0.009	0.367	**0.039**
	CT-TT	0.179	0.014	0.345	**0.034**
	CC-CT	0.009	-0.095	0.113	0.866

Logdiff0to21 refers to the logarithm of differences in rabies antibody titres on day 0 and day 21
Logdiff0to42 refers to the logarithm of differences in rabies antibody titres on day 0 and day 42

C.I. refers to Confidence Interval

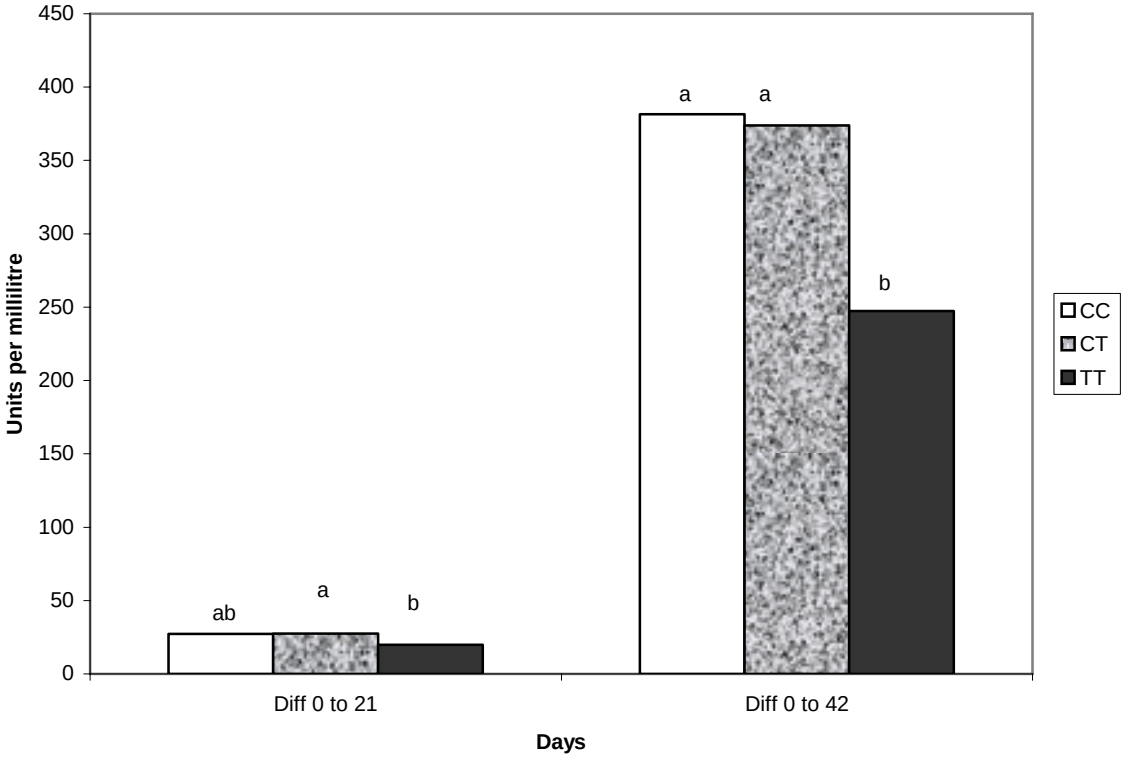

Figure 4.4 Least square means of rabies antibody titres in peripheral beef cattle blood with significant variation with leptin genotype

Within a short period, i.e. within the first twenty-one days, there was a significant difference in the rabies antibody titres of the CT animals in relation to the TT animals. The titres of the CT animals were significantly higher than those of the TT animals while there was a trend with CC animals having higher titres than those of the TT animals.

Over a longer period, i.e. between day 0 and day 42, there was again a significant difference between the titres of the CT animals and those of the TT animals as well as a significant difference between the titres of the CC animals and the TT animals. Both the titres of the CT and CC animals were significantly higher than those of the TT animals. There was no measurable difference in the response to the second vaccination (day 21-day 42) associated with genotype (Appendix B).

4.3 *Pro-opiomelanocortin*

Figure 4.5 is representative of a PCR-RFLP gel of POMC alleles. An uncut product is visible at 390 bp indicating a C allele whilst a cut product represents the T allele with products of 233bp and 157bp. Lanes 3, 5-7 are CCs, lanes 8 and 9 are TTs. Remaining lanes are CTs.

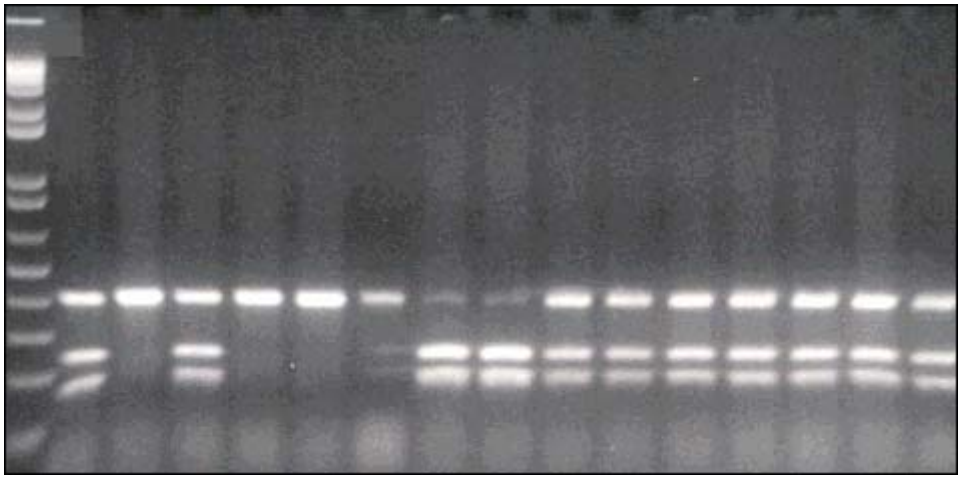

Figure 4.5 PCR-RFLP gel showing genotypes of POMC. Lane 1, 100 bp ladder. Band at 390 bp represents the C allele and bands at 233 bp and 157 bp represents the T allele.

The allele frequencies of POMC for both day 0 and 42 are presented in Table 4.5. Although the relative gene frequency remained very similar, some animals were not available for study on day 42. The association between POMC and immune system outcome are reported for days 0 and 42 in Tables 4.6 and 4.7. The presented outcomes have probabilities of less than 0.1 ($P \leq 0.1$). Least square means per genotypes ($P \leq 0.05$) are graphed for day 0 in Figures 4.6 and 4.7 for day 42. The differences among genotypes of POMC were compared for all the other cell types and have been listed in Appendix C while results with P value less than 0.1 have been discussed.

Table 4.5 Allele frequencies for pro-opiomelanocortin

Allele	C	T	N	Frequency
Day 0	767	271	519	C = 0.74 T = 0.26
Day 42	610	220	415	C = 0.73 T = 0.27

On day 0, the changes in cell count in relation to the different genotypes of POMC occurred in seven cell types (Table 4.6). The cells involved were the activated $CD_{4,}$ regular CD_8, $CD_{8\_25\_45}$ activated $CD_{8,}$ $CD_{8\_26\_45}$ activated $CD_{8,}$ ratio of CD_4 to CD_8 cells, the combination of CD_5CD_{21} and B cells and B cells. Figure 4.6 represents the least square means per genotype of cell types with $P \leq 0.05$ on day 0. The animals responded in a similar pattern with activated $CD_{4,}$ regular CD_8, and $CD_{8\_25\_45}$ activated CD_8 cells. In all of these three cell types, the CT animals had a tendency for higher cell numbers compared to the TT animals while CT animals had significantly higher numbers of these cells compared to CC animals.

Table 4.6 Relationship between POMC genotypes and numbers of immune cell types on day 0

Cell Type	Genotype	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
Log Activated CD_4 ($CD_{4\_26\_45}$)	CC-TT	0.001	-0.063	0.065	0.966
	CT-TT	0.059	-0.004	0.122	0.065
	CC-CT	-0.058	-0.095	-0.021	**0.002**
Log CD_8 (regular CD_8 cells)	CC-TT	0.015	-0.039	0.070	0.580
	CT-TT	0.048	-0.006	0.101	0.083
	CC-CT	-0.032	-0.064	-0.001	**0.046**
Log Activated CD_8 ($CD_{8\_25\_45}$)	CC-TT	0.008	-0.055	0.070	0.812
	CT-TT	0.058	-0.003	0.120	0.064
	CC-CT	-0.051	-0.087	-0.014	**0.006**
Log Activated CD_8 ($CD_{8\_26\_45}$)	CC-TT	0.001	-0.068	0.070	0.983
	CT-TT	0.034	-0.033	0.102	0.318
	CC-CT	-0.034	-0.073	0.006	0.095
Log Ratio of CD_4 to CD_8	CC-TT	-0.020	-0.062	0.023	0.364
	CT-TT	-0.042	-0.084	0.000	**0.051**
	CC-CT	0.022	-0.002	0.047	0.077
Log CD_5CD_{21} & B cell	CC-TT	0.058	-0.006	0.123	0.077
	CT-TT	0.037	-0.027	0.101	0.256
	CC-CT	0.021	-0.016	0.059	0.266
Log B cell	CC-TT	0.017	-0.053	0.073	0.553
	CT-TT	-0.019	-0.074	0.037	0.509
	CC-CT	0.035	0.003	0.068	**0.032**

C.I refers to Confidence Interval

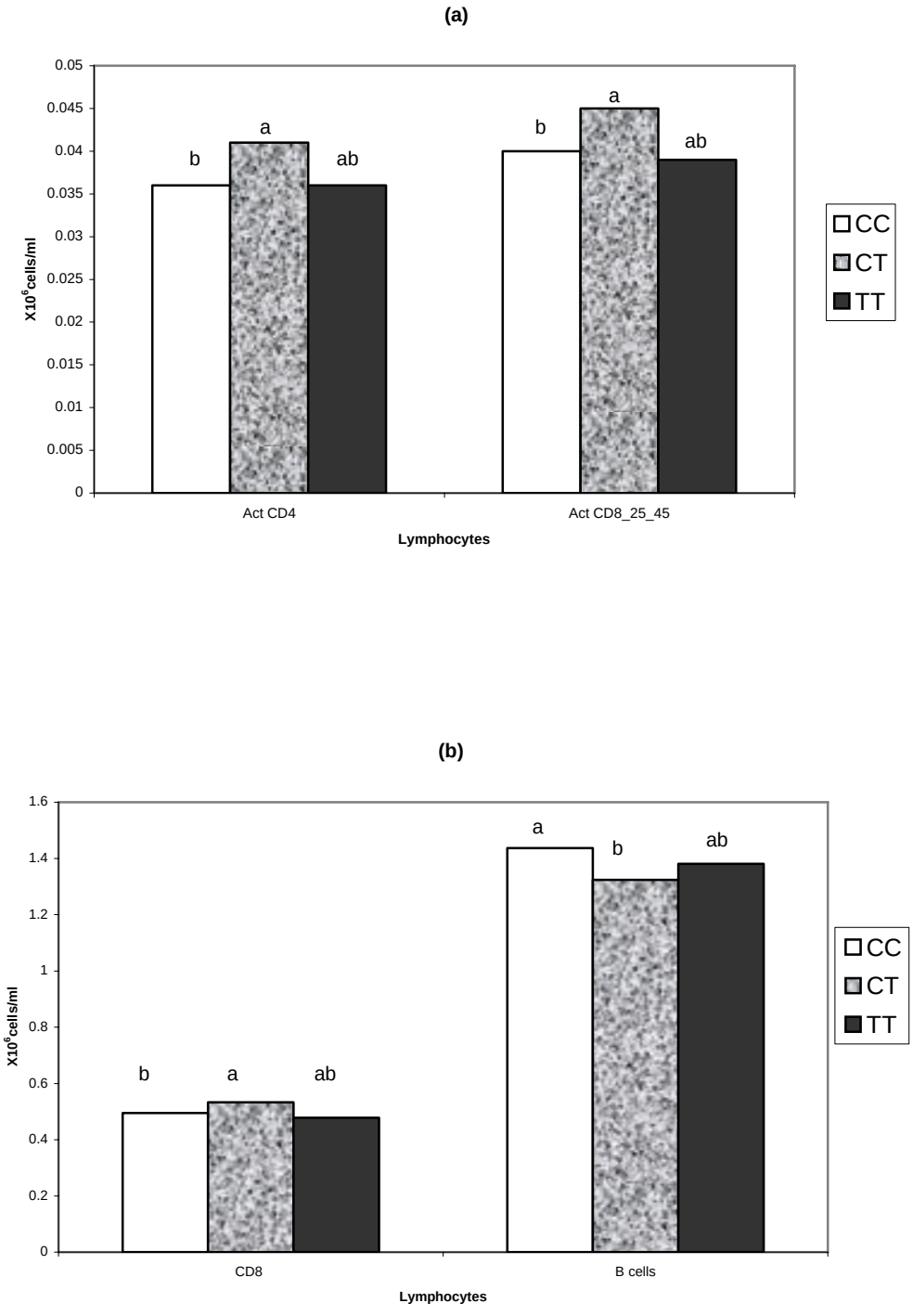

Figure 4.6 Least square means of immune cell types found in peripheral blood in beef cattle having significant variation with POMC genotype on day 0.

The CT animals had a higher number of $CD_{8\_\mathbf{26}\_45}$ activated CD_8 cells compared to the animals with homozygous C genotype. Animals homozygous for the T allele had a higher ratio of CD_4 to CD_8 cells compared to the CT animals. However, the CC animals were higher than the CT animals in that same cell count. With the combination of $CD_5CD_{21}$and B cell count, the homozygous C animals were higher than the homozygous T animals. B cell count was higher in animals with CC genotype than those with CT genotype ($P = 0.032$).

On day 42, five cell types varied ($P \leq 0.1$) with the POMC genotype (Table 4.7); two of these had $P \leq 0.05$. Figure 4.7 indicates the graphed least square means of the two cell types. The CT animals had elevated counts of activated CD_4, $CD_{8\_\mathbf{25}\_45}$ activated CD_8, and $CD_{8\_\mathbf{26}\_45}$ activated CD_8 cells over the CC animals. The TT animals maintained a higher ratio of CD_4 to CD_8 cell count over CT animals ($P = 0.043$). Animals homozygous for the C allele also maintained a higher B cell count relative to the CT animals.

Table 4.7 Relationship between POMC genotypes and numbers of immune cell types on day 42

Cell Type	Genotype	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
Log Activated CD_4 ($CD_{4\_26\_45}$)	CC-TT	-0.020	-0.096	0.055	0.599
	CT-TT	0.019	-0.056	0.093	0.619
	CC-CT	-0.039	-0.081	0.003	0.069
Log Activated CD_8 ($CD_{8\_25\_45}$)	CC-TT	-0.005	-0.075	0.066	0.894
	CT-TT	0.029	-0.040	0.099	0.408
	CC-CT	-0.034	-0.073	0.005	0.090
Log Activated CD_8 ($CD_{8\_26\_45}$)	CC-TT	-0.009	-0.085	0.067	0.812
	CT-TT	0.034	-0.041	0.109	0.376
	CC-CT	-0.043	-0.085	-0.001	**0.047**
Log Ratio of CD_4 to CD_8	CC-TT	-0.030	-0.077	0.017	0.206
	CT-TT	-0.047	-0.093	-0.002	**0.043**
	CC-CT	0.017	-0.008	0.043	0.186
Log B cell	CC-TT	0.008	-0.052	0.067	0.802
	CT-TT	-0.024	-0.083	0.035	0.430
	CC-CT	0.031	-0.002	0.065	0.064

C.I refers to Confidence Interval

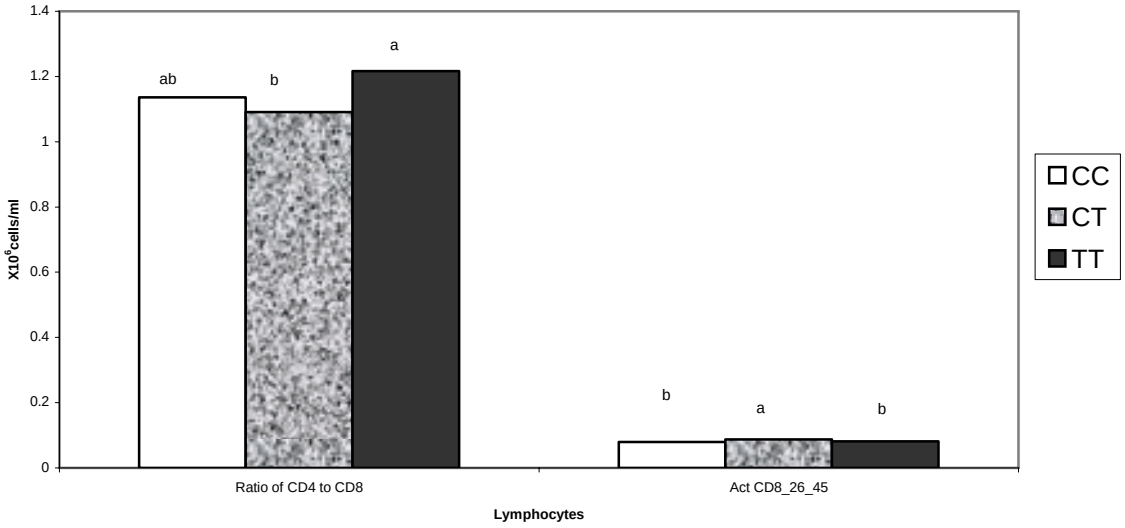

Figure 4.7 Least square means of immune cell types found in peripheral blood in beef cattle having significant variation with POMC genotype on day 42.

Descriptive statistics and least square means were calculated for all the immune cells types that were affected by leptin and POMC. These results have been tabulated in appendices D and E respectively. However, least square mean calculations made for the cell types that had significantly different outcome among the various genotypes have been indicated above in graphs.

5.0. DISCUSSION

5.1. *Data Collection*

The difference in the number of animals sampled was a result of the cattle owners' inability to keep all the animals throughout the period of the research. Hence, some of the animals that were tested on day 0 were not available for testing on day 42. Some of the animals had been sold or put out to pasture with the bull. A number of the herd owners did not want to sample the heifers after the start of the breeding season. The herd owners were concerned about the effect of stress on conception rates. For this reason, the initial twenty-seven herds that were sampled on day 0 were reduced to twenty-one on day 42.

The twenty-seven herds sampled on day 0 were part of a larger study of immune function in beef cattle. For leptin, on day 0, the herd comprised twenty-three (23) bulls and five hundred and thirty three (533) heifers. For POMC, there were twenty-three (23) bulls and four hundred and ninety six (496) heifers. However on day 42, all the animals for which measurements were taken were heifers and this was also true for subjects used for rabies antibody titres. Of relevance to this research was the immune cell counts for day 0 and day 42, and the rabies titres for all three visits i.e. day 0, 21 and 42. It should also be noted that this study is a reflection of occurrences *in vivo* and the results were drawn from several farm herds. The outcome may serve as a guide to the natural environment as opposed to the available literature that are mainly based on *in vitro* studies.

5.2. *Leptin*

The presence of a significantly higher number of activated CD_4 and CD_8 cells in the animals with TT genotypes for leptin on day 0 could have been due to a sub-acute infection in all the animals for which the immune cells had been activated to combat. In the presence of an infection, immune cells are activated to eliminate the microbes. After the elimination of the microbes, a reservoir of immune cells, the memory cells, remain that function as the first cells to fight disease pathogens in subsequent infections (Abbas and Lichtman 2001).

The difference in the association between leptin and lymphocyte numbers from day 0 to day 42 was considered a reflection of the effect of the rabies vaccine on the animals within that period. Other factors that could also be associated with this difference include changes to the nutritional status of herds during this period and pre-breeding vaccination for BVDV and infectious bovine rhinotracheitis (IBR). For leptin, animals with TT genotypes maintained a higher number of activated CD_4 cells than CT animals, which was consistent with day 0. Gainsford et al (1996) and Lord et al (1998) reported that naïve and memory T cells are differentially affected by leptin, which mainly regulates primary T cell responses. In an *in vitro* study where the effect of leptin on CD_4 proliferation was tested, it was observed that the cell population that had been pre-incubated with leptin for 12 hours had a marked increase in proliferation in the subsequent mixed lymphocyte reaction (Lord et al. 1998).

The CC animals had significantly elevated number of activated CD_8 cells. This was not consistent with the occurrence on day 0. Although available literature do not indicate leptin's ability to influence CD_8 cells, Busso et al. (2002) found leptin to stimulate the proliferative response of CD_4 T cells in the context of mixed lymphocyte reaction *in vitro*. Since both CD_4 and CD_8 cells are subsets of T cells, it is assumed that leptin might also stimulate the production of CD_8 cells. The animals with TT genotypes also maintained a higher number of $CD_8CD_{\mathbf{25}}CD_{45}$ activated CD_8 cells. This was again consistent with the concept of leptin's ability to stimulate proliferative response of CD_4 T cells (Busso et al. 2002). The CC animals had an increased number of activated CD_4 cells over the CT animals. A similar situation occurred with the $CD_8CD_{\mathbf{26}}CD_{45}$ activated CD_8 cells of the CC animals, which was again significantly higher than that of the CT animals on day 42. Animals with CT genotypes had a higher ratio of CD_4 to CD_8 cells, which confirms the outcome of the activated CD_4 and CD_8 cells without making reference to the ratio. In leptin, a change from cytosine (C) to thymine (T) changes the amino acid from arginine to cysteine (Buchanan et al. 2002). It was expected that one allele could consistently be associated with increased number of immune cells. Based on a previous study with leptin, where the T allele was associated with increased linear somatic cell count in dairy cattle (Buchanan et al. 2003), the anticipation was that the T allele might be the one to have that connection.

In addition to the activated CD_4 and CD_8 cells, the CC animals also had a significantly higher number of CD_8 gamma-delta cells, a subset of T lymphocytes with receptors that recognize lipids and non-peptide antigens displayed by molecules other than MHC molecules. They are abundant and function in the epithelial surfaces. The

increased number of regular CD_4 cells in the TT animals as compared to both CC and CT animals was expected based on the outcome of day 0. In previous studies with leptin, the T allele has been associated with increased linear somatic cell count in dairy cattle (Buchanan et al. 2003). This may reflect a role in immune response since it was the high milk yielding cattle that also had increased somatic cell count and part of the components of somatic cells are white blood cells as well as other immune cells. Animals with TT and CT genotypes had an elevated number of the W.C. 1 cells, a subset of T cells on day 42.

5.3. *POMC*

The expression of the POMC gene in the hypothalamus is partially regulated by leptin (Mori 2001). It is therefore possible that the POMC genotype will influence immune cell counts. The SNP in POMC however, does not change the amino acid; the amino acid remains serine whereas in the case of leptin, a change from cytosine (C) to thymine (T) changes the amino acid from arginine to cysteine (Buchanan et al. 2002). Hence, although there was an expected association in the case of POMC, the beneficial allele could not be predicted. The results for POMC on day 0 showed that animals with CT genotypes had a significantly ($P = 0.02$) higher number of activated CD_4 cells than the CC animals. The CT animals again showed a trend of higher activated CD_4 cells over the TT animals. The CT animals also had a higher number of regular CD_8 cells than TT animals ($P = 0.083$) and CC animals ($P = 0.046$).

The situation was not any different with both the $CD_8CD_{25}CD_{45}$ and $CD_8CD_{26}CD_{45}$ activated CD_8 cells. Animals with CT genotypes had higher numbers of these cells compared to both the CC and TT counterparts. TT genotypes had a significantly higher CD_4 to CD_8 cell ratio than their CT counterparts. However, there was a trend with the CC animals having a higher CD_4 to CD_8 ratio cell counts than the animals with CT genotypes. With the $CD_5CD_{21}$and B cell, the animals with CC genotypes had a trend for higher immune cell count than the TT animals. The CC animals however had a higher number ($P = 0.032$) of B cells than the animals with CT genotypes.

Day 42 is of more physiological and immunological significance because of the response of the animals to the rabies vaccine although other factors such as the BVDV and nutrition might also be playing some roles. The effect of the vaccine was manifested in four of the immune cell types, which were: the activated CD_4, $CD_8CD_{25}CD_{45}$ and $CD_8CD_{26}CD_{45}$ activated CD_8 cells and B cells. Adachi et al. (1999) found α-MSH, a POMC derived neuropeptide, to down modulate some aspects of inflammation through direct effects on T-lymphocytes, B-lymphocytes and monocytes. This was true in the outcome of this research. Animals with TT genotypes had a significantly elevated CD_4 to CD_8 cell type ratio ($P = 0.043$). The $CD_8CD_{26}CD_{45}$ activated CD_8 cell counts for animals with a CT genotype were also significantly ($P = 0.047$) high. In all, the effect of POMC in modulating inflammation through direct effects on T and B cells was manifested.

5.4. *Possible Causative Factors of Variations in Outcome*

Generally, the number and type of cell did not follow a clear pattern for the various genotypes of animals. There were inconsistencies in the outcome. In some instances, animals with the T allele produced more of a particular immune cell type whilst animals with the C allele produced an elevated level of other immune cell types. Several factors could have come into play to result in this occurrence including nutrition. Since animals used in this research were from different herds, there is the possibility of nutritional variations.

Deficiencies or excessive use of trace minerals in animal rations could result in discrepancies in the immune functions of these animals. Minatel and Carfagnini (2000) observed that in ruminants copper deficiency results in a change in the production of acute phase proteins. They also noted that in ruminants, copper deficiency affects components of the innate immune response especially the production of neutrophils, even though there is no established finding about the effect of copper on macrophage production (Minatel and Carfagnini 2000). Graham (1991) also found that in cattle selenium deficiency results in immune dysfunction causing changes in the normal immunoresponsiveness or control of inflammation. Selenium deficiency also causes a reduced mixed lymphocyte reaction and a reduced responsiveness to interleukin 1 (IL-1) and interleukin 12 (IL-12). Zinc deficiency affects humoral immunity by altering antibody formation and affects cellular immunity by reducing responsiveness to interleukins (Graham 1991). According to his findings, zinc deficiency also changes macrophage and neutrophil function.

The source of dietary lipids could also be a contributing factor to the animals' ability to resist diseases. Drouillard et al. (2001) observed that feeding flaxseed to cattle could positively influence both growth performance and immunity. The results of their research indicated that cattle fed flaxseed produced more TNF-α and had a higher total white blood cell count compared to those fed either tallow or soybean as a dietary source of lipid.

It should however be noted that the occurrence of a high immune cell output in an animal does not make it better. This is because animals may be responding to other challenges and not necessarily to the challenge in question. Also, up regulating one cell type in an animal may result in the down regulation of other cell types and might have potential effects on cytokines as well. For instance, T_H1 cytokines such as IL-2 and T_H2 cytokines such as IL-4 work differently (Abbas and Lichtman 2001); T_H2 down regulates T_H1 responses. Since cytokines are pleiotropic in nature, the outcome of this project might not be wholly attributable to leptin and/or POMC. These results are representative of cell types appearing only in the blood of the animals. No measurements were taken from the bone marrow, lymph nodes, spleen or any body cells of the animals.

DNA extraction was from frozen blood that had been stored at minus 80 degrees Celsius for nearly a year. DNA had to be purified by the MagNa Pure kit as a result of poor amplification. The use of fresh blood might potentially limit or exclude such poor amplification suspected to be due to inhibitors present in the DNA. Counting of the lymphocyte subtypes would have been more uniform by just using one method of

counting instead of using both manual and mechanical counting methods. It is also suggested that in future research, animals in the same herd be used to ensure that sources of variations such as that of management are minimized.

5.5. *Rabies Antibody Titres*

Rabies titres were measured on days 0, 21 and 42 and the differences between them were evaluated. There was a significant response to vaccination in all animals. For leptin, the significant increase in the rabies titres, which occurred within the first 21 days, was expected. This is because generally, when animals are vaccinated their immune system is activated to produce immune cells. T cells, B cells and macrophages are all required for maximal antibody production against most antigens. It takes about three days to detect anti-rabies immunoglobulin M after vaccination and seven to fourteen days to detect peak antibody titres (Dietzschold et al. 1978). The glycoprotein of the rabies virus is the only varion component that induces the production of, and reacts with neutralizing antibodies and that protects animals from challenge (Wiktor et al. 1973). Dietzshold et al. (1978) supported this finding and added that the antibodies raised against purified rabies virus glycoprotein have been shown to neutralize infectious rabies virus and the purified glycoprotein was more immunoprotective when used alone.

The effect of the booster vaccine administered on day 21 was seen in all the animals however; there was no association between genotype and the increase in antibody concentration in response to the second rabies vaccination. However, the

overall difference (log diff 0 to 42) was significantly higher in both CC and CT animals than in TT animals. The rabies antibody titres for all POMC genotypes increased steadily from day 0 to day 42 although it did not have any significant differences between the measurements on days 0, 21 and 42 for any of the CC, CT and TT animals.

6.0. CONCLUSION

The motive behind this research was to ascertain whether there is a correlation between the genotypes of both leptin and POMC and immune response by measuring the number and types of immune cells as well as antibody titres after introducing a rabies vaccine. The outcome indicates that there is an interrelationship between leptin genotypes and immune response. On day 0 there was significant variation with the leptin genotype in only two cell types but in four cell types on day 42. Rabies antibody titres also showed significant differences among the leptin genotypes.

It should however be noted that some researchers have indicated that leptin's effect is more pronounced in the control of body weight but not in inflammatory or hematopoietic processes (Agnello et al. 1998). They also found that it is only at doses higher than those required to cause body weight loss *in vivo* that leptin functions as a pro-inflammatory cytokine.

The effect of POMC on immune cells was not well pronounced. POMC genotype appeared to have had a negative effect on the number of immune cell output. There were four immune cell types that had variations among the POMC genotypes on day 0 and two cell types on day 42. There was also no significant outcome in the rabies antibody titres with POMC genotype. Adachi et al. (1999) observed that the POMC derived peptide α-MSH down modulates the cytokines IL-1 β, TNF-α and lymphotactin. It is possible that α-MSH could also have an effect on the stimulation of immune cells. Blalock (1999) also supported the finding of Adachi et al. (1999) with his finding that α-MSH functionally acts as an IL-1 antagonist.

Although there were significant differences in the specific immune cell output in relation to SNPs in both hormones, there was no consistent pattern observed with regards to allelic preference. This was also true for the rabies antibody titres for leptin genotype. In previous studies, the T allele in leptin has been associated with carcass quality in beef and with milk yield in dairy cattle (Buchanan et al. 2002; 2003). The T allele in POMC has also been linked with yield in beef cattle (Buchanan et al. 2005). This was however not the situation in terms of immune response as neither the T or C allele seem to be distinctively exhibiting a clear expediency in immune response. Hence, there is no allele advantage at either gene with leucocyte numbers. Consequently, selection based on either the C or T allele should not negatively affect animal immune function.

6.1. Future Research

Based on the fact that animals used in this research were from different herds, there is the possibility of variation in diets fed to these animals. Feed could be a potential confounding factor to the outcome of this research. Dietary source of lipids and deficiencies or excessive use of trace minerals such as copper and zinc in animal rations could result in discrepancies in the immune functions of ruminants (Drouillard et al. 2001; Minatel and Carfagnini 2000). Graham (1991) also found that in cattle, selenium deficiency results in immune dysfunction causing changes in the normal immunoresponsiveness or control of inflammation.

In view of the aforementioned, an experimental model which combines the present immune data and genotypes with nutrition, could be designed for researching into the possible effect nutrition could have on immune response and hence on the outcome of this research.

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APPENDIX A

Effect of leptin genotypes on numbers of immune cell types on day 0

Cell Type	Genotype Comparison	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
$LogCD_8gd$ (CD_8 gamma delta cells)					0.620
	CC-TT	-0.007	-0.061	0.046	0.790
	CT-TT	0.009	-0.039	0.057	0.715
	CC-CT	-0.016	-0.049	0.017	0.339
$LogCD_4CD_8Ratio$ (Ratio of CD_4 to CD_8)					0.874
	CC-TT	-0.010	-0.051	0.030	0.618
	CT-TT	-0.009	-0.045	0.027	0.629
	CC-CT	-0.001	-0.027	0.024	0.917
$LogCD_5CD_{21}$ & B cells					0.552
	CC-TT	-0.006	-0.066	0.054	0.834
	CT-TT	0.013	-0.040	0.067	0.625
	CC-CT	-0.020	-0.057	0.017	0.297
$LogCD_{4\_25}$ (IL-2)					0.300
	CC-TT	-0.014	-0.057	0.029	0.523
	CT-TT	0.007	-0.031	0.046	0.706
	CC-CT	-0.021	-0.048	0.005	0.117
$LogCD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen					0.928
	CC-TT	0.009	-0.039	0.057	0.725
	CT-TT	0.008	-0.035	0.051	0.706
	CC-CT	0.000	-0.030	0.030	0.983
$LogCD_{4\_26\_45}$ (Activated CD_4)					0.111
	CC-TT	-0.063	-0.123	-0.004	0.036
	CT-TT	-0.045	-0.098	0.008	0.095
	CC-CT	-0.018	-0.055	0.019	0.330
$LogCD_8CD_{25}CD_{45}$ - (Activated CD_8)					0.144
	CC-TT	-0.058	-0.117	-0.000	0.050
	CT-TT	-0.043	-0.095	-0.009	0.106
	CC-CT	-0.015	-0.052	0.021	0.401
$LogCD_8CD_{26}CD_{45}$ (Activated CD_8)					0.834
	CC-TT	0.016	-0.048	0.080	0.629
	CT-TT	0.018	-0.039	0.075	0.547

	CC-CT	-0.002	-0.041	0.038	0.929
LogCD_8 (CD_8-regular cells)					0.613
	CC-TT	-0.015	-0.066	0.036	0.574
	CT-TT	0.001	-0.045	0.047	0.957
	CC-CT	-0.016	-0.048	0.016	0.326
LogCD_4 (CD_4-regular cells)					0.471
	CC-TT	-0.024	-0.072	0.024	0.324
	CT-TT	-0.007	-0.050	0.036	0.738
	CC-CT	-0.017	-0.046	0.013	0.269
LogW.C.1 cells					0.661
	CC-TT	-0.030	-0.097	0.037	0.380
	CT-TT	-0.017	-0.077	0.043	0.583
	CC-CT	-0.013	-0.055	0.029	0.535
Log B cells					0.545
	CC-TT	-0.016	-0.068	0.037	0.560
	CT-TT	0.003	-0.044	0.050	0.910
	CC-CT	-0.018	-0.051	0.014	0.272

Effect of leptin genotype on numbers of immune cell types on day 42

Cell Type	Genotype Comparison	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
LogCD_8gd (CD_8 gamma delta cells)					0.019
	CC-TT	0.009	-0.052	0.069	0.780
	CT-TT	-0.039	-0.095	0.017	0.173
	CC-CT	0.048	0.013	0.083	0.007
LogCD_4CD_8Ratio (Ratio of CD_4 to CD_8)					0.183
	CC-TT	-0.026	-0.070	0.018	0.245
	CT-TT	-0.003	-0.043	0.037	0.884
	CC-CT	-0.023	-0.048	0.002	0.075
LogCD_5CD_{21} & B cells					0.963
	CC-TT	0.009	-0.057	0.075	0.788
	CT-TT	0.006	-0.055	0.067	0.846
	CC-CT	0.003	-0.035	0.041	0.878
LogCD_{4_25} (IL-2)					0.201
	CC-TT	-0.018	-0.065	0.028	0.440
	CT-TT	-0.034	-0.078	0.009	0.121
	CC-CT	0.0159	-0.011	0.043	0.249
Log$CD_4CD_{25}CD_{45}$-					0.286

LeucocyteCommonAntigen					
	CC-TT	-0.009	-0.063	0.044	0.730
	CT-TT	-0.029	-0.079	0.020	0.245
	CC-CT	0.020	-0.011	0.051	0.206
$LogCD_{4\_26\_45}$ (Activated CD_4)					0.071
	CC-TT	-0.016	-0.085	0.053	0.651
	CT-TT	-0.054	-0.118	0.010	0.098
	CC-CT	0.038	-0.002	0.078	0.062
$LogCD_8CD_{25}CD_{45}$ - (Activated CD_8)					0.035
	CC-TT	-0.022	-0.087	0.043	0.499
	CT-TT	-0.061	-0.121	-0.000	0.048
	CC-CT	0.038	0.001	0.076	0.046
$LogCD_8CD_{26}CD_{45}$ (Activated CD_8)					0.068
	CC-TT	0.002	-0.069	0.072	0.961
	CT-TT	-0.042	-0.107	0.023	0.202
	CC-CT	0.044	0.004	0.085	0.033
$LogCD_8$ (CD_8-regular cells)					0.148
	CC-TT	-0.017	-0.072	0.038	0.549
	CT-TT	-0.040	-0.091	0.011	0.121
	CC-CT	0.024	-0.008	0.055	0.147
$LogCD_4$ (CD_4-regular cells)					0.180
	CC-TT	-0.043	-0.094	0.008	0.095
	CT-TT	-0.044	-0.091	0.003	0.067
	CC-CT	0.001	-0.029	0.030	0.967
Log W.C. 1 cells					0.050
	CC-TT	-0.096	-0.179	-0.014	0.023
	CT-TT	-0.054	-0.130	0.023	0.169
	CC-CT	-0.043	-0.090	0.005	0.079
Log B cells					0.561
	CC-TT	-0.027	-0.083	0.029	0.342
	CT-TT	-0.028	-0.080	0.024	0.286
	CC-CT	0.001	-0.031	0.033	0.949

APPENDIX B

Rabies antibody titres for leptin

Day	Genotype Comparison	Coefficient of Regression (β)	Lower 95%C.I	Upper 95%C.I	P-value
Logdiff0to21					0.133
	CC-TT	0.137	-0.015	0.290	0.078
	CT-TT	0.141	0.002	0.280	0.047
	CC-CT	-0.004	-0.098	0.091	0.940
Logdiff0to42					0.090
	CC-TT	0.188	0.009	0.367	0.039
	CT-TT	0.179	0.014	0.345	0.034
	CC-CT	0.009	-0.095	0.113	0.866
Logdiff21to42					0.942
	CC-TT	0.028	-0.158	0.214	0.770
	CT-TT	0.030	-0.142	0.203	0.730
	CC-CT	-0.003	-0.110	0.105	0.961

Rabies antibody titres for POMC

Day	Genotype Comparison	Coefficient of Regression (β)	Lower 95%C.I	Upper 95%C.I	P-value
Logdiff0to21					0.386
	CC-TT	-0.092	-0.264	0.081	0.297
	CT-TT	-0.121	-0.294	0.053	0.173
	CC-CT	0.029	-0.067	0.125	0.555
Logdiff0to42					0.470
	CC-TT	0.038	-0.157	0.233	0.703
	CT-TT	-0.030	-0.224	0.165	0.765
	CC-CT	0.067	-0.040	0.175	0.219
Logdiff21to42					0.624
	CC-TT	0.093	-0.106	0.292	0.360
	CT-TT	0.061	-0.137	0.260	0.545
	CC-CT	0.032	-0.077	0.141	0.570

APPENDIX C

Effect of POMC genotypes on numbers of immune cell types on day 0

Cell Type	Genotype Comparison	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
$LogCD_8gd$ (CD_8 gamma delta cells)					0.291
	CC-TT	0.000	-0.056	0.057	0.987
	CT-TT	0.025	-0.030	0.081	0.372
	CC-CT	-0.025	-0.058	0.008	0.137
Ratio of CD_4 to CD_8					0.065
	CC-TT	-0.020	-0.062	0.023	0.364
	CT-TT	-0.042	-0.084	0.000	0.051
	CC-CT	0.022	-0.002	0.047	0.077
$LogCD_5CD_{21}$ & B cells					0.176
	CC-TT	0.058	-0.006	0.123	0.077
	CT-TT	0.037	-0.027	0.101	0.256
	CC-CT	0.021	-0.016	0.059	0.266
$LogCD_{4\_25}$ (IL-2)					0.592
	CC-TT	0.016	-0.030	0.062	0.490
	CT-TT	0.023	-0.022	0.069	0.320
	CC-CT	-0.007	-0.034	0.020	0.616
$LogCD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen					0.236
	CC-TT	0.038	-0.014	0.090	0.152
	CT-TT	0.018	-0.034	0.069	0.498
	CC-CT	0.020	-0.010	0.051	0.187
$LogCD_{4\_26\_45}$ (Activated CD_4)					0.006
	CC-TT	0.001	-0.063	0.065	0.966
	CT-TT	0.059	-0.004	0.122	0.065
	CC-CT	-0.058	-0.095	-0.021	0.002
$LogCD_8CD_{25}CD_{45}$ (Activated CD_8)					0.012
	CC-TT	0.008	-0.055	0.070	0.812
	CT-TT	0.058	-0.003	0.120	0.064
	CC-CT	-0.051	-0.087	-0.014	0.006
$LogCD_8CD_{26}CD_{45}$ (Activated CD_8)					0.210
	CC-TT	0.001	-0.068	0.070	0.983
	CT-TT	0.034	-0.033	0.102	0.318
	CC-CT	-0.034	-0.073	0.006	0.095

LogCD_8 (CD_8-regular cells)					0.064
	CC-TT	0.015	-0.039	0.070	0.580
	CT-TT	0.048	-0.006	0.101	0.083
	CC-CT	-0.032	-0.064	-0.001	0.046
LogCD_4 (CD_4-regular cells)					0.848
	CC-TT	-0.005	-0.056	0.046	0.854
	CT-TT	0.004	-0.047	0.054	0.880
	CC-CT	-0.009	-0.039	0.021	0.566
LogW.C.1 cells					0.709
	CC-TT	-0.030	-0.102	0.043	0.418
	CT-TT	-0.021	-0.092	0.051	0.566
	CC-CT	-0.090	-0.051	0.033	0.677
Log B cells					0.101
	CC-TT	0.017	-0.053	0.073	0.553
	CT-TT	-0.019	-0.074	0.037	0.509
	CC-CT	0.035	0.003	0.068	0.032

Effect of POMC genotypes on numbers of immune cell types on day 42

Cell Type	Genotype Comparison	Coefficient of Regression (β)	Lower 95% C.I	Upper 95% C.I	P-Value
LogCD_8gd (CD_8 gamma delta cells)					0.746
	CC-TT	-0.010	-0.076	0.055	0.759
	CT-TT	0.004	-0.061	0.068	0.907
	CC-CT	-0.014	-0.050	0.022	0.447
LogCD_4CD_8Ratio (Ratio of CD_4 to CD_8)					0.091
	CC-TT	-0.030	-0.077	0.017	0.206
	CT-TT	-0.047	-0.093	-0.002	0.043
	CC-CT	0.017	-0.008	0.043	0.186
LogCD_5CD_{21} & B cells					0.606
	CC-TT	0.031	-0.040	0.102	0.396
	CT-TT	0.036	-0.034	0.106	0.317
	CC-CT	-0.005	-0.045	0.035	0.804
LogCD_{4_25} (IL-2)					0.860
	CC-TT	-0.013	-0.063	0.038	0.625
	CT-TT	-0.007	-0.057	0.043	0.783
	CC-CT	-0.006	-0.034	0.023	0.696

$LogCD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen					0.809
	CC-TT	0.011	-0.048	0.070	0.711
	CT-TT	0.001	-0.057	0.059	0.981
	CC-CT	0.010	-0.022	0.043	0.534
$LogCD_{4\_26\_45}$ (Activated CD_4)					0.190
	CC-TT	-0.020	-0.096	0.055	0.599
	CT-TT	0.019	-0.056	0.093	0.619
	CC-CT	-0.039	-0.081	0.003	0.0686
$LogCD_8CD_{25}CD_{45}$ - (Activated CD_8)					0.217
	CC-TT	-0.005	-0.075	0.066	0.894
	CT-TT	0.029	-0.040	0.099	0.408
	CC-CT	-0.034	-0.073	0.005	0.090
$LogCD_8CD_{26}CD_{45}$-(Activated CD_8)					0.128
	CC-TT	-0.009	-0.085	0.067	0.812
	CT-TT	0.034	-0.041	0.109	0.376
	CC-CT	-0.043	-0.085	-0.001	0.047
$LogCD_8$ (CD_8-regular cells)					0.672
	CC-TT	0.011	-0.049	0.071	0.712
	CT-TT	0.022	-0.037	0.082	0.455
	CC-CT	-0.011	-0.045	0.022	0.508
$LogCD_4$ (CD_4-regular cells)					0.673
	CC-TT	-0.018	-0.073	0.037	0.518
	CT-TT	-0.024	-0.078	0.030	0.382
	CC-CT	0.006	-0.024	0.036	0.699
LogW.C.1 cells					0.727
	CC-TT	-0.017	-0.107	0.072	0.701
	CT-TT	-0.031	-0.119	0.057	0.484
	CC-CT	0.014	-0.036	0.063	0.583
Log B cells					0.171
	CC-TT	0.008	-0.052	0.067	0.802
	CT-TT	-0.024	-0.083	0.035	0.430
	CC-CT	0.031	-0.002	0.065	0.064

APPENDIX D

Descriptive statistics

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_8gd (CD_8 gamma delta cells)						556
Leptin	CC	0.212	0.114	0.189	0.107	149
	CT	0.221	0.115	0.202	0.149	345
	TT	0.205	0.119	0.177	0.131	62
POMC						519
	CC	0.208	0.104	0.189	0.136	288
	CT	0.221	0.113	0.199	0.141	188
	TT	0.199	0.080	0.199	0.100	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_4CD_8Ratio (Ratio of CD_4 to CD_8)						556
Leptin	CC	1.181	0.385	1.128	0.440	149
	CT	1.192	0.392	1.132	0.528	345
	TT	1.252	0.392	1.188	0.522	62
POMC						519
	CC	1.227	0.385	1.176	0.463	288
	CT	1.177	0.393	1.126	0.532	188
	TT	1.214	0.387	1.150	0.504	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_5CD_{21} & B cells						556
Leptin	CC	0.369	0.209	0.313	0.276	149
	CT	0.388	0.209	0.345	0.261	345
	TT	0.410	0.249	0.370	0.362	62
POMC						519
	CC	0.407	0.230	0.354	0.314	288
	CT	0.368	0.187	0.322	0.243	188
	TT	0.322	0.164	0.285	0.196	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_{4_25} (IL-2)						556
Leptin	CC	0.164	0.071	0.155	0.090	149
	CT	0.176	0.081	0.168	0.100	345
	TT	0.176	0.080	0.177	0.108	62
POMC						519
	CC	0.177	0.081	0.167	0.109	288

	CT	0.171	0.077	0.155	0.094	188
	TT	0.155	0.066	0.140	0.082	43
$CD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen						556
Leptin	CC	1.162	0.597	1.011	0.648	149
	CT	1.183	0.805	1.071	0.552	345
	TT	1.149	0.591	1.081	0.627	62
POMC						519
	CC	1.271	0.902	1.113	0.671	288
	CT	1.082	0.482	1.012	0.522	188
	TT	1.036	0.432	1.011	0.442	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
$CD_{4\_26\_45}$ (Activated CD_4)						556
Leptin	CC	0.054	0.037	0.043	0.042	149
	CT	0.061	0.040	0.050	0.054	345
	TT	0.057	0.035	0.050	0.036	62
POMC						519
	CC	0.058	0.041	0.046	0.047	288
	CT	0.060	0.038	0.049	0.047	188
	TT	0.052	0.028	0.048	0.046	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
$CD_8CD_{25}CD_{45}$ (Activated CD_8)						556
Leptin	CC	0.060	0.046	0.047	0.039	149
	CT	0.239	3.238	0.054	0.050	345
	TT	0.059	0.039	0.051	0.041	62
POMC						519
	CC	0.272	3.544	0.049	0.044	288
	CT	0.064	0.040	0.053	0.050	188
	TT	0.055	0.029	0.046	0.038	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
$CD_8CD_{26}CD_{45}$ (Activated CD_8)						556
Leptin	CC	0.068	0.044	0.055	0.054	149
	CT	0.073	0.053	0.060	0.054	345
	TT	0.064	0.053	0.047	0.037	62
POMC						519
	CC	0.066	0.045	0.052	0.053	288
	CT	0.077	0.059	0.060	0.054	188
	TT	0.074	0.051	0.057	0.069	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_8 (CD_8-regular cells)						556
Leptin	CC	0.674	0.325	0.632	0.429	149
	CT	0.702	0.332	0.648	0.454	345
	TT	0.688	0.405	0.616	0.457	62
POMC						519
	CC	0.677	0.324	0.642	0.490	288
	CT	0.698	0.312	0.628	0.423	188
	TT	0.609	0.235	0.547	0.303	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_4 (CD_4-regular cells)						556
Leptin	CC	0.748	0.331	0.690	0.461	149
	CT	0.787	0.354	0.733	0.513	345
	TT	0.789	0.381	0.716	0.510	62
POMC						519
	CC	0.786	0.364	0.738	0.515	288
	CT	0.773	0.340	0.700	0.515	188
	TT	0.706	0.306	0.638	0.400	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
W.C. 1 cells						556
Leptin	CC	0.695	0.404	0.657	0.506	149
	CT	0.765	1.238	0.623	0.462	345
	TT	0.683	0.392	0.591	0.455	62
POMC						519
	CC	0.805	1.360	0.653	0.521	288
	CT	0.663	0.317	0.614	0.435	188
	TT	0.692	0.373	0.623	0.472	43

DAY 0 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
B cells						556
Leptin	CC	1.807	0.919	1.569	1.176	149
	CT	1.793	0.797	1.636	0.949	345
	TT	1.768	0.832	1.740	1.178	62
POMC						519
	CC	1.909	0.918	1.718	1.215	288
	CT	1.664	0.699	1.595	0.916	188
	TT	1.605	0.589	1.493	0.778	43

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_8gd (CD_8 gamma delta cells)						443
Leptin	CC	1.556	0.733	1.416	1.028	129
	CT	1.377	0.656	1.253	0.766	272
	TT	1.497	0.678	1.406	1.057	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	1.426	0.692	1.327	0.899	227
	CT	1.476	0.700	1.334	0.912	156
	TT	1.456	0.565	1.352	0.608	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_4CD_8Ratio (Ratio of CD_4 to CD_8)						443
Leptin	CC	1.144	0.382	1.110	0.427	129
	CT	1.201	0.380	1.160	0.546	272
	TT	1.279	0.367	1.215	0.545	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	1.219	0.396	1.154	0.551	227
	CT	1.169	0.367	1.151	0.527	156
	TT	1.171	0.331	1.161	0.459	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_5CD_{21} & B cells						443
Leptin	CC	0.452	0.242	0.390	0.307	129
	CT	0.459	0.228	0.421	0.314	272
	TT	0.479	0.236	0.451	0.226	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.487	0.243	0.435	0.342	227
	CT	0.451	0.213	0.414	0.257	156
	TT	0.388	0.227	0.348	0.226	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_{4_25} (IL-2)						443
Leptin	CC	0.244	0.096	0.237	0.139	129
	CT	0.240	0.095	0.233	0.126	272
	TT	0.270	0.096	0.271	0.139	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.250	0.097	0.238	0.132	227
	CT	0.240	0.090	0.240	0.119	156
	TT	0.219	0.098	0.223	0.141	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
$CD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen						443
Leptin	CC	1.333	0.473	1.326	0.688	129
	CT	1.315	0.479	1.288	0.621	272
	TT	1.405	0.459	1.342	0.621	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	1.390	0.480	1.380	0.606	227
	CT	1.273	0.435	1.246	0.577	156
	TT	1.229	0.525	1.154	0.680	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
$CD_{4\_26\_45}$ (Activated CD_4)						443
Leptin	CC	0.151	0.082	0.140	0.125	129
	CT	0.143	0.077	0.134	0.110	272
	TT	0.156	0.092	0.136	0.096	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.139	0.080	0.127	0.113	227
	CT	0.154	0.074	0.148	0.098	156
	TT	0.139	0.084	0.129	0.135	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
$CD_8CD_{25}CD_{45}$ (Activated CD_8)						443
Leptin	CC	0.149	0.079	0.138	0.103	129
	CT	0.136	0.072	0.132	0.094	272
	TT	0.152	0.096	0.129	0.098	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.132	0.069	0.125	0.087	227
	CT	0.152	0.077	0.147	0.094	156
	TT	0.134	0.087	0.123	0.149	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
$CD_8CD_{26}CD_{45}$ (Activated CD_8)						443
Leptin	CC	0.134	0.081	0.118	0.106	129
	CT	0.123	0.077	0.112	0.094	272
	TT	0.143	0.095	0.119	0.085	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.128	0.083	0.107	0.096	227
	CT	0.127	0.069	0.120	0.089	156
	TT	0.109	0.081	0.096	0.126	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_8 (CD_8-regular cells)						443
Leptin	CC	0.817	0.352	0.774	0.468	129
	CT	0.795	0.340	0.754	0.417	272
	TT	0.877	0.432	0.809	0.484	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.806	0.361	0.786	0.461	227
	CT	0.802	0.316	0.745	0.378	156
	TT	0.761	0.266	0.721	0.317	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
CD_4 (CD_4-regular cells)						443
Leptin	CC	0.871	0.355	0.819	0.445	129
	CT	0.898	0.370	0.862	0.460	272
	TT	1.043	0.382	1.051	0.409	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.912	0.385	0.858	0.477	227
	CT	0.889	0.346	0.872	0.444	156
	TT	0.884	0.364	0.843	0.503	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
W.C. 1 cells						443
Leptin	CC	0.613	0.395	0.503	0.480	129
	CT	0.675	0.417	0.570	0.510	272
	TT	0.754	0.357	0.736	0.405	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	0.676	0.401	0.579	0.541	227
	CT	0.621	0.367	0.562	0.474	156
	TT	0.679	0.471	0.520	0.518	32

DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
B cells						443
Leptin	CC	1.814	0.756	1.702	1.078	129
	CT	1.818	0.717	1.724	0.947	272
	TT	2.131	0.876	1.892	1.109	42
DAY 42 - OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
POMC						415
	CC	1.946	0.771	1.830	1.039	227
	CT	1.752	0.674	1.695	0.982	156
	TT	1.809	0.823	1.633	1.014	32

RABIES ANTIBODY TITRES

OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
Day 0 Titre						586
Leptin	CC	5.210	1.378	5.000	0.000	163
	CT	5.390	3.034	5.000	0.000	358
	TT	9.230	25.850	5.000	0.000	65
POMC						550
	CC	6.100	11.836	5.000	0.000	316
	CT	5.430	3.855	5.000	0.000	193
	TT	5.200	1.249	5.000	0.000	41

OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
Day 21 Titre						579
Leptin	CC	327.870	532.583	170.000	215.000	163
	CT	325.240	522.065	180.000	264.000	353
	TT	271.860	357.878	210.000	214.000	63
POMC						545
	CC	324.770	481.166	200.000	264.000	315
	CT	296.830	455.584	125.000	214.000	190
	TT	434.180	939.647	255.000	250.000	40

OUTCOME	GENOTYPE	MEAN	S.D	MEDIAN	IQR	NUMBER
Day 42 Titre						533
Leptin	CC	7349.880	20205.636	1800.000	5600.000	157
	CT	6675.000	17875.214	1600.000	4900.000	325
	TT	6753.240	22322.986	1600.000	5700.000	51
POMC						501
	CC	9262.42	25131.309	1800.000	5200.000	283
	CT	4408.090	6988.967	1600.000	5450.000	181
	TT	4126.080	6594.076	1600.000	4150.000	37

APPENDIX E

Least Square Means

Leptin day 0

Cell Type	GENOTYPE	LSMEAN	L.C.I	U.C.I
CD_8gd (CD_8 gamma delta cells)				
	CC	0.152	0.132	0.176
	CT	0.158	0.138	0.181
	TT	0.155	0.132	0.182
CD_4CD_8Ratio (Ratio of CD_4 to CD_8)				
	CC	1.151	1.051	1.261
	CT	1.155	1.063	1.255
	TT	1.179	1.058	1.313
CD_5CD_{21} & B cells				
	CC	0.275	0.233	0.323
	CT	0.287	0.247	0.335
	TT	0.279	0.232	0.335
CD_{4_25} (IL-2)				
	CC	0.124	0.110	0.140
	CT	0.130	0.117	0.146
	TT	0.128	0.112	0.146
$CD_4CD_{25}CD_{45}$ (LeucocyteCommonAntigen)				
	CC	0.886	0.774	1.014
	CT	0.885	0.779	1.006
	TT	0.868	0.746	1.010
$CD_{4\_26\_45}$ (Activated CD_4)				
	CC	0.037	0.029	0.045
	CT	0.038	0.031	0.047
	TT	0.042	0.034	0.053
$CD_8CD_{25}CD_{45}$ (Activated CD_8)				
	CC	0.040	0.033	0.049
	CT	0.042	0.034	0.051
	TT	0.046	0.037	0.057

$CD_8CD_{26}CD_{45}$ (Activated CD_8)				
	CC	0.046	0.038	0.056
	CT	0.046	0.038	0.055
	TT	0.044	0.036	0.055
CD_8 (CD_8-regular cells)				
	CC	0.498	0.437	0.567
	CT	0.516	0.458	0.582
	TT	0.515	0.444	0.597
CD_4 (CD_4-regular cells)				
	CC	0.573	0.501	0.656
	CT	0.596	0.524	0.677
	TT	0.606	0.521	0.705
W.C. 1 cells				
	CC	0.528	0.453	0.616
	CT	0.544	0.473	0.627
	TT	0.566	0.472	0.678
B cells				
	CC	1.339	1.192	1.505
	CT	1.397	1.258	1.553
	TT	1.388	1.209	1.594
POMC day 0				
Cell Type				
CD_8gd (CD_8 gamma delta cells)				
	CC	0.151	0.132	0.173
	CT	0.160	0.140	0.184
	TT	0.151	0.128	0.180
CD_4CD_8Ratio (Ratio of CD_4 to CD_8)				
	CC	1.179	1.085	1.280
	CT	1.120	1.028	1.220
	TT	1.233	1.099	1.383
CD_5CD_{21} & B cells				
	CC	0.293	0.252	0.341
	CT	0.279	0.239	0.325
	TT	0.256	0.211	0.311

CD_{4_25} (IL-2)				
	CC	0.128	0.114	0.143
	CT	0.130	0.116	0.146
	TT	0.123	0.107	0.142
$CD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen				
	CC	0.907	0.796	1.034
	CT	0.866	0.758	0.990
	TT	0.831	0.706	0.979
$CD_{4\_26\_45}$ (Activated CD_4)				
	CC	0.036	0.029	0.045
	CT	0.041	0.033	0.051
	TT	0.036	0.028	0.046
$CD_8CD_{25}CD_{45}$ (Activated CD_8)				
	CC	0.040	0.033	0.048
	CT	0.045	0.037	0.055
	TT	0.039	0.031	0.049
$CD_8CD_{26}CD_{45}$ (Activated CD_8)				
	CC	0.044	0.037	0.053
	CT	0.048	0.039	0.058
	TT	0.044	0.035	0.055
CD_8 (CD_8-regular cells)				
	CC	0.495	0.440	0.558
	CT	0.533	0.472	0.602
	TT	0.478	0.409	0.559
CD_4 (CD_4-regular cells)				
	CC	0.584	0.513	0.665
	CT	0.596	0.522	0.680
	TT	0.590	0.502	0.694
W.C. 1 cells				
	CC	0.534	0.461	0.619
	CT	0.545	0.468	0.634
	TT	0.572	0.468	0.699

B cells				
	CC	1.436	1.295	1.592
	CT	1.323	1.189	1.473
	TT	1.381	1.192	1.600
Leptin day 42				
Cell Type	GENOTYPE	LSMEAN	L.C.I	U.C.I
CD_8gd (CD_8 gamma delta cells)-				
	CC	0.993	0.837	1.177
	CT	0.889	0.754	1.050
	TT	0.973	0.798	1.187
CD_4CD_8Ratio (Ratio of CD_4 to CD_8)				
	CC	1.130	0.987	1.294
	CT	1.192	1.044	1.360
	TT	1.200	1.029	1.400
CD_5CD_{21} & Bcells				
	CC	0.296	0.244	0.360
	CT	0.294	0.244	0.355
	TT	0.290	0.232	0.363
CD_{4_25} (IL-2)				
	CC	0.162	0.138	0.190
	CT	0.156	0.133	0.183
	TT	0.169	0.141	0.202
$CD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen				
	CC	0.978	0.848	1.128
	CT	0.934	0.814	1.072
	TT	1.000	0.844	1.184
$CD_{4\_26\_45}$ (Activated CD_4)				
	CC	0.098	0.077	0.125
	CT	0.090	0.071	0.114
	TT	0.102	0.078	0.133
$CD_8CD_{25}CD_{45}$ (Activated CD_8)				
	CC	0.092	0.072	0.116
	CT	0.084	0.066	0.106
	TT	0.096	0.074	0.125

$CD_8CD_{26}CD_{45}$ (Activated CD_8)				
	CC	0.085	0.064	0.114
	CT	0.077	0.058	0.102
	TT	0.085	0.062	0.116
CD_8 (CD_8-regular cells)				
	CC	0.513	0.438	0.600
	CT	0.486	0.417	0.566
	TT	0.533	0.444	0.640
CD_4 (CD_4-regular cells)				
	CC	0.581	0.498	0.679
	CT	0.581	0.499	0.675
	TT	0.642	0.538	0.766
W.C. 1 cells				
	CC	0.374	0.288	0.485
	CT	0.412	0.319	0.532
	TT	0.466	0.347	0.626
B cells				
	CC	1.205	1.038	1.398
	CT	1.202	1.041	1.388
	TT	1.282	1.075	1.529
POMC day 42				
Cell Type				
CD_8gd (CD_8 gamma delta cells)				
	CC	0.865	0.725	1.032
	CT	0.894	0.746	1.071
	TT	0.886	0.716	1.096
CD_4CD_8Ratio (Ratio of CD_4 to CD_8)				
	CC	1.136	0.986	1.310
	CT	1.091	0.945	1.261
	TT	1.217	1.033	1.435
CD_5CD_{21} & B cells				
	CC	0.288	0.236	0.351
	CT	0.292	0.238	0.357
	TT	0.269	0.212	0.340

CD_{4_25} (IL-2)				
	CC	0.156	0.132	0.185
	CT	0.158	0.133	0.187
	TT	0.160	0.133	0.194
$CD_4CD_{25}CD_{45}$-LeucocyteCommonAntigen				
	CC	0.960	0.829	1.112
	CT	0.938	0.807	1.090
	TT	0.936	0.780	1.123
$CD_{4\_26\_45}$ (Activated CD_4)				
	CC	0.089	0.069	0.115
	CT	0.098	0.075	0.127
	TT	0.093	0.070	0.125
$CD_8CD_{25}CD_{45}$ (Activated CD_8)				
	CC	0.085	0.066	0.109
	CT	0.092	0.071	0.118
	TT	0.086	0.065	0.113
$CD_8CD_{26}CD_{45}$ (Activated CD_8)				
	CC	0.079	0.058	0.107
	CT	0.087	0.064	0.118
	TT	0.081	0.058	0.112
CD_8 (CD_8-regular cells)				
	CC	0.494	0.420	0.581
	CT	0.507	0.429	0.599
	TT	0.481	0.396	0.585
CD_4 (CD_4-regular cells)				
	CC	0.566	0.482	0.666
	CT	0.559	0.474	0.659
	TT	0.590	0.489	0.713
W.C. 1 cells				
	CC	0.409	0.311	0.539
	CT	0.396	0.300	0.524
	TT	0.426	0.310	0.585

B cells

CC	1.216	1.045	1.414
CT	1.131	0.969	1.320
TT	1.194	0.991	1.440

Least Square Means for Rabies Antibody Titres

Leptin

diff0to21[a]	GENOTYPE	LSMEAN	L.C.I	U.C.I
	CC	27.265	21.372	34.783
	CT	27.498	22.431	33.710
	TT	19.879	14.186	27.857
diff21to42[a]				
	CC	14.041	9.591	20.555
	CT	14.129	9.942	20.079
	TT	13.173	8.066	21.516
diff0to42[a]				
	CC	381.593	272.966	533.446
	CT	373.766	276.198	505.800
	TT	247.286	158.319	386.249

POMC

diff0to21[a]				
	CC	26.724	21.502	33.213
	CT	25.009	19.690	31.766
	TT	33.007	22.025	49.462
diff21to42[a]				
	CC	14.545	10.184	20.771
	CT	13.524	9.334	19.595
	TT	11.749	6.914	19.966
diff0to42[a]				
	CC	383.001	282.283	519.656
	CT	327.944	237.820	452.222
	TT	351.075	215.246	572.617

Legend

L.C.I - Lower Confidence Interval

U.C.I - Upper Confidence Interval

a - diff refers to the difference in rabies antibody titres between the days in question

Unit of LSMEAN = $x10^6$ cells /ml

APPENDIX F

List of Animal Breeds with Genotypes

VIAL No.	SAMPLE/LAB No.	LEPTIN	POMC	BREED	SEX
6001	221	CC	CC	Continental	Female
6002	222	CT	TT	Continental	Female
6003	223	CT	CT	Continental	Female
6004	224	CT	CC	Continental	Female
6005	225	CC	CC	Continental	Female
6006	226	CT	CC	Continental	Female
6007	227	CT	CC	Continental	Female
6008	228	CC	CC	Continental	Female
6009	229	TT	TT	Continental	Female
6081	231	CC	CC	Continental	Female
6082	232	CT	CC	Continental	Female
6083	233	CT	CT	Continental	Female
6084	234	CT	CC	Continental	Female
6085	235	CT	CC	Continental	Female
6086	236	CC	CC	Continental	Female
6087	237	CC	CC	Continental	Female
6088	238	CC	CC	Continental	Female
6089	239	CT	CC	Continental	Female
6090	240	CC	CC	Continental	Female
6091	241	CC	CT	Continental	Female
6092	242	CC	CC	Continental	Female
6093	243	CC	CT	Continental	Female
6094	244	CC	CT	Continental	Female
6095	255	CT	CC	Continental	Female
6072	257	CC	CT	Unknown	Female
6074	259	CT	TT	Unknown	Female
6075	260	CT	CT	Unknown	Female
6077	262	CT	CT	Unknown	Female
6078	263	CC	TT	Unknown	Female
6080	265	CT	CC	Unknown	Female
6213	268	CT	CC	Unknown	Female
6215	270	CC	CT	Unknown	Female
6216	271	CT	CT	Unknown	Female
6218	273	CT	CT	Unknown	Female
6220	275	CT	CC	Unknown	Female
6612	277	CT	CC	Unknown	Female
6621	282	CC	CC	Continental	Female
6622	283	CC	X	Continental	Female
6623	284	CT	CC	Continental	Female

6625	286	CT	X	Continental	Female
6626	287	CT	X	Continental	Female
6627	288	CC	CT	Continental	Female
6628	289	CT	X	Continental	Female
6630	291	CC	X	Continental	Female
6631	292	CC	CC	Continental	Female
6632	293	CT	X	Continental	Female
6633	294	CT	X	Continental	Female
6634	295	CT	CC	Continental	Female
6636	297	CT	X	Continental	Female
6637	298	TT	CC	Continental	Female
6638	299	CT	CC	Continental	Female
6639	300	CT	X	Continental	Female
6640	301	TT	X	Continental	Female
6641	302	CC	CC	Continental	Female
6643	304	CC	CT	Continental	Female
6191	305	CT	CT	Continental	Female
6192	306	CC	CC	Continental	Female
6193	307	CT	X	Continental	Female
6194	308	CT	CT	Continental	Female
6195	309	CT	CT	Continental	Female
6196	310	CC	CT	Continental	Female
6197	311	CT	TT	Continental	Female
6198	312	CT	X	Continental	Female
6199	313	CC	CC	Continental	Female
6200	314	CT	X	Continental	Female
6491	315	CT	CC	Continental	Female
6492	316	CT	X	Continental	Female
6493	317	CC	CC	Continental	Female
6494	318	CT	CT	Continental	Female
6495	319	CT	X	Continental	Female
6496	320	CC	X	Continental	Female
6497	321	CT	CC	Continental	Female
6498	322	CT	CC	Continental	Female
6500	324	CC	CT	Continental	Female
6501	325	CT	CT	Continental	Female
6502	326	CT	CT	Continental	Female
6503	327	CC	CC	Continental	Female
6614	328	CT	X	Unknown	Female
6616	330	CT	CT	Unknown	Female
6618	332	CT	TT	Unknown	Female
6619	333	CT	TT	Unknown	Female
6620	334	CT	CC	Unknown	Female
6644	335	CT	CC	Continental	Female

6646	337	CC	CT	Continental	Female
6647	338	CT	CT	Continental	Female
6648	339	CT	CC	Continental	Female
6649	340	TT	CC	Continental	Female
6951	342	CT	CT	Continental	Female
6953	344	TT	CT	Continental	Female
6954	345	TT	CC	Continental	Female
6955	346	CT	TT	Unknown	Female
6957	348	CC	TT	Unknown	Female
6504	352	CC	CT	Continental	Female
6505	353	CC	CC	Continental	Female
6506	354	CC	CC	Continental	Female
6507	355	CT	CC	Continental	Female
6508	356	CT	CC	Continental	Female
6509	357	CT	CC	Continental	Female
6510	358	CT	CC	Continental	Female
6869	359	CC	TT	Continental	Female
6861	360	CT	CC	Continental	Female
6862	361	CT	CC	Continental	Female
6863	362	CT	X	Continental	Female
6864	363	TT	CC	Continental	Female
6865	364	CC	CT	Continental	Female
6866	365	CT	CT	Continental	Female
6867	366	CT	CC	Continental	Female
6868	367	CT	CT	Continental	Female
6870	368	CC	CC	Continental	Female
6871	369	CT	CT	Continental	Female
6872	370	CC	CT	Continental	Female
6873	371	CC	CC	Continental	Female
6874	372	CT	CC	Continental	Female
6875	373	CT	CC	Continental	Female
6877	375	CT	CC	Continental	Female
6878	376	CT	CC	Continental	Female
6879	377	CT	CT	Continental	Female
6880	378	CC	CC	Continental	Female
6881	379	CT	CC	Continental	Female
6882	380	CC	CC	Continental	Female
6883	381	CC	CC	Continental	Female
6884	382	TT	CC	Continental	Female
6885	383	CC	TT	Continental	Female
6891	384	CT	CC	Continental	Female
6892	385	CT	CC	Continental	Female
6893	386	CT	X	Continental	Female
6894	387	CT	TT	Continental	Female

6895	388	CT	TT	Continental	Female
6896	389	TT	CC	Continental	Female
6897	390	CT	CC	Continental	Female
6898	391	TT	CT	Continental	Female
6899	392	CC	CT	Continental	Female
6900	393	CT	CT	Continental	Female
6901	394	CT	CT	Continental	Female
6902	395	CC	CC	Continental	Female
6903	396	CT	CC	Continental	Female
6904	397	TT	CC	Continental	Female
6905	398	CC	CT	Continental	Female
6906	399	TT	CC	Continental	Female
6907	400	CT	TT	Continental	Female
6908	401	TT	CT	Continental	Female
6909	402	TT	CC	Continental	Female
6910	403	CT	X	Continental	Female
6301	404	CT	CT	British	Female
6302	405	TT	CC	British	Female
6304	407	TT	CC	British	Female
6305	408	CT	CC	British	Female
6306	409	CC	CC	British	Female
6307	410	CT	CC	British	Female
6330	411	CT	CT	British	Female
6309	412	CT	CT	British	Female
6310	413	TT	CT	British	Female
6311	414	CT	CC	British	Female
6312	415	CC	CC	British	Female
6313	416	TT	CC	British	Female
6314	417	CC	CC	British	Female
6316	419	CT	CC	British	Female
6317	420	TT	CC	British	Female
6318	421	TT	X	British	Female
6319	422	CC	CC	British	Female
6321	424	CC	CC	British	Female
6322	425	CT	CT	British	Female
6323	426	CT	CT	British	Female
6324	427	TT	TT	British	Female
6326	429	TT	CT	British	Female
6328	431	CT	CC	British	Female
6329	432	TT	CC	British	Female
6721	433	CT	CT	Continental	Female
6722	434	TT	TT	Continental	Female
6723	435	CC	CT	Continental	Female
6724	436	CT	CT	Continental	Female

6725	437	CC	CT	Continental	Female
6727	439	CT	CC	Continental	Female
6728	440	CC	CT	Continental	Female
6729	441	CC	CT	Continental	Female
6732	444	CT	CT	Continental	Female
6733	445	CC	CT	Continental	Female
6734	446	TT	TT	Continental	Female
6735	447	CT	CT	Continental	Female
6736	448	CT	X	Continental	Female
6737	449	CT	CT	Continental	Female
6739	451	CT	CT	Continental	Female
6740	452	CC	TT	Continental	Female
6771	453	TT	CT	Continental	Female
6772	454	CC	CC	Continental	Female
6773	455	CC	CT	Continental	Female
6775	457	CT	CT	Continental	Female
6776	458	CT	CT	Continental	Female
6777	459	CT	X	Continental	Female
6778	460	CT	CC	Continental	Female
6780	462	CT	CC	Continental	Female
6781	463	CT	CC	Continental	Female
6782	464	CT	CC	Continental	Female
6783	465	CT	CC	Continental	Female
6784	466	CT	CC	Continental	Female
6801	468	TT	CC	Continental	Female
6802	469	CT	CT	Continental	Female
6803	470	CT	CT	Continental	Female
6804	471	CT	CT	Continental	Female
6805	472	CT	CC	Continental	Female
6806	473	CC	CT	Continental	Female
6807	474	CT	CT	Continental	Female
6808	475	CT	CC	Continental	Female
6809	476	CT	CC	Continental	Female
6810	477	CC	CC	Continental	Female
6811	478	CT	CT	Continental	Female
6812	479	CC	TT	Continental	Female
6813	480	CT	CT	Continental	Female
6814	481	CT	CC	Continental	Female
6815	482	CT	CT	Continental	Female
6816	483	CT	CC	Continental	Female
6817	484	CC	CT	Continental	Female
6818	485	CT	CC	Continental	Female
6819	486	CT	CC	Continental	Female
6820	487	CT	CC	Continental	Female

6821	488	CC	TT	Continental	Female
6822	489	CT	TT	Continental	Female
6391	490	CT	CT	Continental	Female
6394	493	CT	CT	Continental	Female
6395	494	CT	CT	Continental	Female
6396	495	CT	CT	Continental	Female
6397	496	CT	CT	Continental	Female
6398	497	CT	CT	Continental	Female
6399	498	CT	CT	Continental	Female
6400	499	CT	X	Continental	Female
6411	500	CC	CT	British	Female
6416	501	CT	CT	British	Female
6417	502	CT	CT	British	Female
6419	504	CT	CC	British	Female
6786	506	CC	TT	Continental	Female
6787	507	CT	CT	Continental	Female
6788	508	CC	TT	Continental	Female
6790	509	CT	CT	Continental	Female
6791	510	CC	CC	Continental	Female
6792	511	CC	CC	Continental	Female
6795	514	CC	CT	Continental	Female
6796	515	CT	X	Continental	Female
6921	517	CT	CC	Continental	Female
6922	518	TT	CC	Continental	Female
6923	519	TT	CT	Continental	Female
6924	520	CC	CT	Continental	Female
6925	521	CC	CC	Continental	Female
6401	522	CT	CC	British	Female
6403	523	CT	CC	British	Female
6404	524	CT	CC	British	Female
6405	525	TT	CC	British	Female
6407	527	CT	CT	British	Female
6408	528	CC	X	British	Female
6412	531	CT	X	British	Female
6413	532	CT	X	British	Female
6414	533	CT	X	British	Female
6415	534	CT	X	British	Female
6421	535	CT	X	British	Female
6424	538	CT	CC	British	Female
6831	539	CC	CC	Cross	Female
6832	540	CT	CC	Cross	Female
6833	541	CT	TT	Cross	Female
6834	542	CT	TT	Cross	Female
6835	543	TT	CT	Cross	Female

6836	544	CT	CC	Cross	Female
6837	545	CC	CT	Cross	Female
6838	546	CC	CC	Cross	Female
6839	547	CT	CC	Cross	Female
6841	549	CT	CT	Cross	Female
6842	550	CT	CT	Cross	Female
6843	551	CT	CC	Cross	Female
6844	552	CT	CC	Cross	Female
6845	553	CT	CC	Cross	Female
6846	554	CT	CC	Cross	Female
6847	555	CT	CC	Cross	Female
6848	556	CT	CT	Cross	Female
6850	557	CT	CT	Cross	Female
6851	558	TT	CT	Cross	Female
6852	559	CT	CT	Cross	Female
6926	561	CT	CT	Continental	Female
6927	562	CT	CT	Continental	Female
6928	563	CT	CC	Continental	Female
6929	564	CC	CC	Continental	Female
6930	565	CC	CT	Continental	Female
6931	566	CT	CC	Continental	Female
6932	567	CT	TT	Continental	Female
6933	568	CT	CC	Continental	Female
6934	569	CT	CC	Continental	Female
6935	570	CT	CT	Continental	Female
6936	571	CT	CC	Continental	Female
6937	572	CC	CC	Continental	Female
6938	573	CC	CC	Continental	Female
6939	574	CT	CT	Continental	Female
6941	575	CC	CT	Continental	Female
6131	576	CC	CT	Continental	Female
6133	577	CT	CT	Continental	Female
6134	578	CC	CT	Continental	Female
6135	579	CT	CC	Continental	Female
6136	580	CC	TT	Continental	Female
6137	581	CT	X	Continental	Female
6138	582	CT	CT	Continental	Female
6139	583	CT	TT	Continental	Female
6141	584	CC	CT	Continental	Female
6142	585	CT	CT	Continental	Female
6143	586	CT	TT	Continental	Female
6144	587	CT	CT	Continental	Female
6145	588	CT	TT	Continental	Female
6146	589	CT	CT	Continental	Female

6147	590	CT	CT	Continental	Female
6148	591	CC	CT	Continental	Female
6149	592	CT	TT	Continental	Female
6150	593	CC	CC	Continental	Female
6461	594	CC	CC	Continental	Female
6462	595	CT	CC	Continental	Female
6463	596	CT	CC	Continental	Female
6464	597	CT	CC	Continental	Female
6465	598	CT	CC	Continental	Female
6466	599	CC	CC	Continental	Female
6467	600	CC	CC	Continental	Female
6468	601	CT	CT	Continental	Female
6469	602	CT	CT	Continental	Female
6470	603	CC	CT	Continental	Female
6471	604	CT	CT	Continental	Female
6472	605	CC	CT	Continental	Female
6473	606	CT	X	Continental	Female
6474	607	CC	CT	Continental	Female
6475	608	CT	CT	Continental	Female
6476	609	CC	CC	Continental	Female
6477	610	CC	CC	Continental	Female
6478	611	CT	TT	Continental	Female
6479	612	CC	CC	Continental	Female
6480	613	CT	CC	Continental	Female
6263	614	CC	CT	Cross	Female
6264	615	CT	CT	Cross	Female
6265	616	CT	CT	Cross	Female
6266	617	CT	CC	Cross	Female
6267	618	CT	TT	Cross	Female
6268	619	CC	CC	Cross	Female
6269	620	CC	CC	Cross	Female
6270	621	CT	CT	Cross	Female
6271	622	CT	CT	Cross	Female
6272	623	CC	CT	Cross	Female
6274	625	TT	CC	Cross	Female
6260	626	CT	CC	Cross	Female
6261	627	CT	CT	Cross	Female
6262	628	CT	CT	Cross	Female
6251	629	CT	CT	Cross	Female
6252	630	CC	CC	Cross	Female
6253	631	TT	CT	Cross	Female
6254	632	CT	CC	Cross	Female
6255	633	CC	CC	Cross	Female
6256	634	CT	CC	Cross	Female

6257	635	CT	CC	Cross	Female
6258	636	CT	CC	Cross	Female
6259	637	TT	CT	Cross	Female
9451	638	CT	CC	British	Female
9452	639	TT	CC	British	Female
9453	640	TT	CC	British	Female
9454	641	CT	CC	British	Female
9455	642	CT	CC	British	Female
9456	643	CT	CT	British	Female
9457	644	CT	CC	British	Female
9458	645	CT	CC	British	Female
9459	646	CT	CC	British	Female
9460	647	CT	CC	British	Female
9461	648	TT	CT	British	Female
9462	649	CT	CC	British	Female
9463	650	CT	CC	British	Female
9464	651	CT	CC	British	Female
9465	652	TT	CC	British	Female
9466	653	CT	CC	British	Female
9467	654	CT	CT	British	Female
9468	655	CT	CC	British	Female
9471	658	TT	CT	British	Female
6521	659	CT	CC	Continental	Female
6522	660	CT	CC	Continental	Female
6523	661	CT	CT	British	Female
6524	662	CC	CT	Continental	Female
6525	663	CT	CT	Continental	Female
6526	664	CT	CT	Continental	Female
6527	665	CC	CT	British	Female
6528	666	CC	CC	Continental	Female
6529	667	CT	CC	British	Female
6530	668	CT	CT	Continental	Female
6531	669	CT	X	British	Female
6532	670	CT	CC	British	Female
6533	671	CT	CT	British	Female
6534	672	CT	CC	British	Female
6535	673	CT	CT	British	Female
6536	674	CC	CC	British	Female
6537	675	CC	CC	British	Female
6538	676	CC	TT	British	Female
6539	677	CC	CC	Continental	Female
6540	678	CT	CT	British	Female
6541	679	CT	CT	Continental	Female
6011	680	TT	CC	Cross	Female

6012	681	TT	CC	Cross	Female
6013	682	CT	CC	Cross	Female
6015	684	CT	CC	Cross	Female
6016	685	CT	CC	Cross	Female
6017	686	TT	CC	Cross	Female
6019	688	CT	CC	Cross	Female
6020	689	CT	CC	Cross	Female
6024	693	TT	CC	Cross	Female
6025	694	CT	CT	Cross	Female
6027	696	CT	CC	Cross	Female
6028	697	TT	CC	Cross	Female
6029	698	CC	CC	Cross	Female
6031	700	CT	CC	Cross	Female
6032	701	CC	CC	Cross	Female
6033	702	CT	CC	Cross	Female
6035	704	CT	CT	Cross	Female
6036	705	CT	CC	Cross	Female
6221	706	TT	CC	British	Male
6222	707	CT	CC	British	Male
6223	708	TT	CC	British	Male
6224	709	CT	CC	British	Male
6225	710	TT	CC	British	Male
6226	711	CT	CC	British	Male
6227	712	CT	CC	British	Male
6228	713	TT	CC	British	Male
6229	714	CT	CT	British	Male
6230	715	CT	CC	British	Male
6231	716	TT	CC	British	Male
6232	717	TT	CC	British	Male
6233	718	CT	CT	British	Male
6234	719	TT	CC	British	Male
6235	720	CT	CC	British	Male
6236	721	CT	CC	British	Male
6331	722	CC	CC	Continental	Female
6334	725	CT	CT	Continental	Female
6335	726	CC	CC	Continental	Female
6336	727	CT	CT	Continental	Female
6337	728	CT	CT	Continental	Female
6338	729	CT	TT	Continental	Female
6339	730	CT	TT	Continental	Female
6341	731	CT	CC	Continental	Female
6342	732	CC	TT	Continental	Female
6344	734	CC	CT	Continental	Female
6345	735	CT	TT	Continental	Female

6346	736	CC	CT	Continental	Female
6347	737	CT	CT	Continental	Female
6103	738	CT	CC	Continental	Female
6105	739	CC	CT	Continental	Female
6106	740	TT	CT	Continental	Female
6113	741	CC	CT	Continental	Female
6117	742	CC	CT	Continental	Female
6161	743	CC	CT	British	Female
6167	744	CT	CC	British	Female
6168	745	CC	CC	British	Female
6169	746	CT	CC	British	Female
6170	747	CC	CT	British	Female
6172	748	CC	CC	British	Female
6174	749	CC	CC	British	Female
6176	750	CC	CC	British	Female
6184	751	CC	CC	British	Female
6186	752	CT	CC	British	Female
6190	753	CC	CC	British	Female
6433	754	CT	CC	British	Female
6437	755	CT	CT	British	Female
6443	757	TT	CT	British	Female
6447	758	CT	CC	British	Female
6564	762	CC	CT	British	Female
6566	764	CT	CT	British	Female
6567	765	CT	TT	British	Female
6569	767	CC	CT	British	Female
6570	768	CT	CC	British	Female
6571	769	CT	CC	British	Female
6572	770	TT	CT	British	Female
6573	771	TT	CT	British	Female
6575	773	CC	CT	British	Female
6348	774	CC	TT	Continental	Male
6349	775	CT	CC	Continental	Male
6350	776	CT	CT	Continental	Male
6352	778	CT	TT	Continental	Male
6354	780	CT	CT	Continental	Male
6355	781	CC	CC	Continental	Male
6356	782	CT	CT	Continental	Male
6551	783	CT	CC	British	Female
6552	784	CT	CC	British	Female
6554	786	CT	CC	British	Female
6557	789	CT	CT	British	Female
6558	790	CT	CT	British	Female
6560	792	CT	TT	British	Female

6561	793	CT	CC	British	Female
6562	794	CC	CC	British	Female
6563	795	CT	CT	British	Female
6101	796	CC	CC	Continental	Female
6102	797	CT	CC	Continental	Female
6104	798	CT	CT	Continental	Female
6107	799	CT	CC	Continental	Female
6108	800	CT	CC	Continental	Female
6109	801	CT	CC	Continental	Female
6110	802	CT	TT	Continental	Female
6111	803	CT	X	Continental	Female
6112	804	CT	TT	Continental	Female
6115	805	CT	X	Continental	Female
6116	806	CT	CT	Continental	Female
6118	807	CC	CT	Continental	Female
6119	808	CT	CT	Continental	Female
6120	809	CT	CT	Continental	Female
6121	810	CT	CC	Continental	Female
6162	811	CT	CT	British	Female
6163	812	CC	CC	British	Female
6164	813	CT	CC	British	Female
6165	814	CT	CC	British	Female
6166	815	CT	CC	British	Female
6171	816	CT	CC	British	Female
6173	817	CT	CC	British	Female
6175	818	TT	CC	British	Female
6177	819	CC	CC	British	Female
6178	820	CT	CC	British	Female
6179	821	CT	CC	British	Female
6180	822	TT	CC	British	Female
6181	823	CT	CT	British	Female
6182	824	CT	CC	British	Female
6183	825	CC	CT	British	Female
6185	826	CT	CT	British	Female
6187	827	CT	CC	British	Female
6188	828	CC	CT	British	Female
6189	829	TT	CT	British	Female
6432	831	TT	CT	British	Female
6434	832	CT	CC	British	Female
6435	833	TT	CC	British	Female
6438	835	TT	CT	British	Female
6440	836	CT	CT	British	Female
6441	837	CC	CC	British	Female
6445	840	CT	CC	British	Female

6448	842	TT	CC	British	Female
6450	844	CT	CC	British	Female
6455	846	CT	CC	British	Female
9481	847	CC	CC	British	Female
9482	848	CT	CC	British	Female
9485	851	CT	CT	British	Female
9486	852	CT	CC	British	Female
9487	853	CC	CC	British	Female
9489	855	CT	CC	British	Female
9490	856	CT	CC	British	Female
9492	858	CC	CC	British	Female
9494	860	CC	CC	British	Female
9496	862	CT	CC	British	Female
9497	863	CT	CT	British	Female
9499	865	CT	CC	British	Female
9501	867	CC	CT	British	Female
9503	869	CT	X	British	Female
9505	871	CT	CC	British	Female
9506	872	CC	CC	British	Female
9508	874	CT	CC	British	Female
9509	875	CT	CC	British	Female
9511	877	TT	CC	British	Female
9513	879	CT	CC	British	Female
9514	880	CC	CC	British	Female
9518	884	CT	CC	British	Female
9520	886	CC	CC	British	Female
9522	888	CT	CC	British	Female
9524	890	CT	CC	British	Female
9526	892	CT	CC	British	Female
9527	893	CT	CC	British	Female
9529	895	CT	CC	British	Female
9530	896	CT	CC	British	Female
9531	897	CC	CC	British	Female
9532	898	CT	CC	British	Female
9533	899	CT	CC	British	Female
9534	900	CT	CC	British	Female
9535	901	CT	CC	British	Female
9538	904	CC	CC	British	Female
9540	906	CT	CC	British	Female

Summary of Genotypes of Animals

GENOTYPE	CC	CT	TT	X	Total
LEPTIN	150	348	64	None	562
POMC	292	189	44	37	562

X = DNA samples that failed to amplify on the PCR