INNATE AND ACQUIRED IMMUNE RESPONSES IN CROSSBRED CATTLE

Fiona Jane Young
B.Sc. (Hons.) M.Sc. (Vet. Sci.)

A THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW VETERINARY SCHOOL FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY
of
GLASGOW

DEPARTMENT OF VETERINARY CLINICAL STUDIES
UNIVERSITY OF GLASGOW VETERINARY SCHOOL

MARCH 2002

Abstract

Resistance to infection by pathogenic organisms is critical for survival of livestock under commercial farming conditions. Understanding the complex processes involved in immunity to infection is essential to allow future selection of animals for disease resistance to become an attainable goal in livestock production.

The overall aim of this study was to assess various aspects of immune function in a genetically defined population of male and female Holstein-Friesian cross Charolais cattle from six months of age. One aspect of the study assessed the *in vitro* proliferation of peripheral blood mononuclear cells (PBMC) induced by *Staphylococcus aureus* or Phytohaemagglutinin (PHA) in over 300 second generation animals consisting of F2, Holstein-Friesian backcross and Charolais backcross individuals. Variation in *S. aureus*-induced, PHA-induced and control PBMC proliferation was found among individuals at each of the time points assessed. Statistical analyses by Residual Maximum Likelihood (REML) identified a significant effect of sex on all of the parameters assessed and a significant effect of control PBMC proliferation on *S. aureus*-induced and PHA-induced PBMC proliferation. Overall, males had higher levels of both specific and non-specific immunity measured by *S. aureus*-induced, PHA-induced and control *in vitro* PBMC proliferation. Following REML analysis, the control *in vitro* PBMC proliferation was found to have a regression coefficient greater than zero and less than one, which indicated that in this study, calculation of immunity using stimulation indices (SI) or delta counts per minute (Δ c.p.m.) would have been inaccurate and would have resulted in misleading interpretation of the data. On several occasions, sample age and year of birth were also identified as significant factors. The sample age and year of birth were found to have different effects on specific and non-specific immunity. Specific immune function, measured by *S. aureus*-induced PBMC proliferation, was shown to increase with sample age of the individual, whereas, non-specific immune function, measured by PHA-induced PBMC proliferation, was shown to decrease with sample age. Specific immune function, measured by *S. aureus*-induced PBMC proliferation, was shown to decrease in the animals born in the 1998 to 2000 cohorts, whereas, non-specific immune function, measured by PHA-induced PBMC proliferation was shown to increase in the animals born in the 1998 to 2000 cohorts. Following REML analyses, the cross and sire of the animal were found to be significant at several of the time points. Variation in immune function was identified among crosses with the Holstein-Friesian backcross animals having greater levels of *S. aureus*-induced PBMC proliferation than the Charolais backcross animals. In contrast, the Charolais backcross animals were identified as having greater levels of PHA-induced PBMC proliferation than the Holstein-Friesian backcross animals. In addition, a small subset of animals for which additional MHC RFLP patterns were available, were ranked for *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation. This study identified that animals with high specific immune responses, measured by *S. aureus*-induced PBMC proliferation, may have low non-specific immune responses, measured by PHA-induced PBMC proliferation, and also, conversely, that animals with high non-specific immune responses may have low specific immune responses. The variation identified in this study among individual animals, cross and sire in specific and non-specific immunity indicates that the immune function of these animals may be under genetic control and may differ with type of immune response measured.

Another aspect of the study developed a method to assess the polymorphonuclear (PMN) phagocytic ability of the study animals. Phagocytic ability was determined by assessing the phagocytosis of Fluorescein Isothiocyanate labelled (FITC-labelled, 2.0μM) latex beads by isolated PMN in 140 second generation animals. Following development and optimisation of the assay, variation among 140 individual animals in the levels of phagocytosis of FITC-labelled (2.0μM) latex beads was identified at each of the time points assessed. Following statistical analyses, sex was identified as a significant factor in the complete data set, with males having consistently higher levels of phagocytosis of FITC-labelled (2.0μM) latex beads than females. Variation was identified among sires in their PMN phagocytic ability however, as this variation was not statistically significant, larger numbers of animals may be required to accurately assess this factor in future.

Determining the existence of correlations between specific and non-specific immunity and genes involved in control of immunity, possibly MHC or other complexes, may lead ultimately to the identification of Quantitative Trait Loci (QTL) for immune function. Incorporation of QTL for disease resistance to specific pathogens may be exploited in future breeding programmes for improved livestock production and animal welfare.

Contents

List of Tables

List of Figures

Acknowledgements

I am greatly indebted to Prof. Julie Fitzpatrick for her supervision, enthusiastic encouragement and invaluable guidance during the course of this study. I am also greatly indebted to Dr Lubna Nasir and Arlene Macrae for their help and support throughout this project. I would also like to thank Dr. John Woolliams of the Roslin Institute for all his help and guidance with regards to the data analyses, especially the REML analysis, and for all the time he spent answering queries about Genstat.

I wish to express my gratitude to:

Linda Andrews and Lesley Shields for all their invaluable help with the FACS machine.

Laura Nicoll, Wilson Lee, Reg, and all the other farm staff at Blythbank for their willingness on Monday mornings to sample the animals, whatever the weather.

Andrew Kerr for his assistance with some of the neutrophil assays.

I would also like to thank Stuart Mitchell, Neil Henderson, Marge Robinson and all the other Medicine Research Laboratory staff for their help and support throughout this study.

I must also thank Ronan and my mum and dad for all their support and encouragement throughout this thesis.

Finally, I would like to thank the Department of Veterinary Clinical studies.

Dedication

For Ronan, my mum and dad and Ritchie.

Apart from the help acknowledged I declare that the work described was carried out by me and is not that of any other person and, further has not been submitted, in full or in part, for consideration for any other degree or qualification.

Fiona J. Young, January, 2002

Presentations

British Mastitis Conference, Stoneleigh (1999). Breeding Cattle for Mastitis Resistance. Fitzpatrick, J.L., Logan, K.E., **Young, F.J.**, Stear, M.J., Platt, D.J., and McGuirk, B.J.

AVTRW 54th Annual Conference (2000) *In vitro* immunological responses of cattle to *Staphylococcus aureus*. **Young, F.J.**, Logan, K.L., Stear, M.J., and Fitzpatrick, J.L.

Symposium on Immunology of Ruminant Mammary Gland, Stresa, Italy (2000) The systemic T cell proliferative response of Holstein/Friesian bulls, induced by *Staphylococcus aureus in vitro*, is negatively correlated with their predicted transmitting ability for milk somatic cell counts. Fitzpatrick, J.L., Logan, K.E., **Young, F.J.**, Stear, M.J., Platt, D.J. and McGuirk, B.J.

AVTRW Annual Conference (2001) Assessment of *in vitro* neutrophil phagocytosis in cattle by flow cytometric analysis. **Young, F.J.**, Kerr, A.E., Stear, M.J., and Fitzpatrick, J.L.

6th International Veterinary Immunology Symposium, Sweden (2001) Analysis of immune traits in cattle:correlation with sire and MHC. Glass, E.J., Craigmile, S.C., Douglas, A., Fitzpatrick, J.L., Miltiadou, D., Russell, G.C., Springbett, A., Woolliams, J., **Young, F.J.**, and Williams, J.L.

Chapter 1

Introduction

1.1 Immunity to disease

Resistance to infection is critical for the survival of any human or animal. It is essential that humans and animals can exclude infectious agents that may cause disease, or be able to reduce the ability of pathogens to survive once they have invaded the host (Tizard, 2000). The immune system has evolved in many complex ways to deal with pathogens (Engelhard, 1994), and to be effective and reliable, multiple defence systems must be available. The protection of the body comes from a complex system of interlinked defence mechanisms which can be classified into the 'non-specific' (innate) and 'specific' (acquired) immune systems: together these systems have the combined ability to destroy or control most pathogens (Kaufmann and Kabelitz, 1998). Innate immune responses are triggered rapidly and focus on structural components associated with pathogens such as bacterial cell wall components, whereas the acquired immune system requires a longer period of time to respond than the innate system, but focuses specifically on epitopes expressed by a pathogen (Bachmann and Kopf, 1999).

Variation among individual animals in their ability to mount a successful immune response against a pathogen may be influenced by a variety of factors such as the efficiency of antigen processing, the association between antigenic peptides and

MHC class II and, ultimately, presentation to T helper cells (Buus *et al.*, 1987). Immunity to disease is associated with the hosts' ability to mount an effective immune response. Pathogens have been shown to affect all aspects of immune function. Bacteria such as *Escherichia coli* and *Mycobacterium tuberculosis* have the ability to secrete molecules that depress neutrophil phagocytosis (Prescott *et al.*, 1990). Resistance to parasitic infections such as trypanosomosis in cattle has been shown to be associated with the hosts' ability to resist the development of anaemia and control parasitaemia once infected (Dargie *et al.*, 1979; Murray *et al.*, 1979). Protection from viral infections such as equine infectious anaemia, or AIDS in humans, is dependent on a successful innate immune response in the individual that prevents successful intracellular invasion by virus (Tizard, 2000). In cattle, bovine virus diarrhoea virus (BVDV) can cause a lymphopenia and destruction of both B and T cells in lymph nodes, spleen, thymus and Peyer's patches. Surviving B cells fail to make immunoglobulins and respond poorly to mitogens, BVDV also depresses neutrophil functions such as degranulation and antibody-dependent cytotoxity (Radostits *et al.*, 1994). In addition, malfunction of the immune system resulting in the occurrence of immunodeficiencies or autoimmune diseases, highlight the importance of a balanced immune system, as either under-production or over-production of specific immune factors can result in susceptibility to disease in many species (Klein and Horejsi, 1997; Tizard, 2000).

1.2 Innate immune system

1.2.1 Primary defences

Animals have multiple levels of defence against pathogenic challenge. The first defence against pathogens is the physical barrier of the skin (Tizard, 2000). The surface epithelia of the skin acts initially as a mechanical barrier but also has additional chemical factors such as fatty acids or a low pH, which help to prevent microbial invasion (Kaufmann and Kabelitz 1998).

1.2.2 Secondary defences

Once successful pathogenic invasion across the surface epithelia occurs, cellular effector mechanisms are triggered which focus on the site of invasion or tissue damage. Local changes in tissues such as increased blood flow and accumulation of cells capable of destroying invading bacteria occur, along with the activation of the alternative complement system (Tizard, 2000). These initial cellular responses comprise the professional phagocytes such as neutrophils and macrophages, in addition to non-professional phagocytes such as dendritic cells (Roitt *et al.*, 1993).

1.2.3 Complement

The complement system is part of the innate immune system and consists of many proteins that act as a cascade, where each enzyme acts as a catalyst for the next (Roitt *et al.*, 1993). The complement system is a multi-component, complex biochemical system that covalently binds specific proteins to the surface of invading micro-organisms (Klein and Horejsi, 1997). The complement system can be triggered either by the presence of antibodies on the surface of an invading organism or simply by the presence of specific carbohydrate structures on the surface of the invading organism (Lachmann and Hughes-Jones, 1984). At least 30 components of the complement system, which are synthesised at various sites throughout the body, have been identified (Manderson *et al.*, 2001). The main production of complement components C3, C6, C8 and B occurs in the liver, whereas C2, C3, C4, C5, B, D, P and I are primarily produced by macrophages. Neutrophils have been shown to store large quantities of C6 and C7. As a result, these complement components are readily available at sites of inflammation where macrophages and neutrophils accumulate. Two main branches of the complement system have been defined: the classical pathway and the alternative pathway, which converge into the lytic pathway (Roitt *et al.*, 1993).

The classical pathway is triggered when antibodies bind to an invading micro–organism, triggering an alteration in the shape of the immunoglobulin exposing the F_C region. When several immunoglobulin molecules are bound to an organism and multiple F_C regions are exposed, subsequent immune reactions in the complement cascade are triggered (Cooper, 1985). In contrast, the alternative pathway runs continuously and spontaneously at a low level in the absence of activators such as the C3 component (Pangburn and Muller-Eberhard, 1980). The final outcome of both the classical and alternative pathways is the lytic pathway, where the cleavage of C5 components into C5a and C5b fragments is an essential step in the cascade.

At present, deficiency of bovine complement receptor (CR) 3 is the only reported deficit in the bovine complement system to date. This deficiency is associated with the bovine leucocyte adhesion deficiency (BLAD) (Shuster *et al.*, 1992, Gerardi, 1996; Cox *et al.*, 1997) and is associated with impaired microbicidal activities of phagocytes (Nagahata *et al.*, 1994, 1996).

1.2.4 Cytokines

Cells of the immune system secrete a vast number of proteins that regulate the immune responses between cells: these proteins are defined as cytokines (Godson *et al.*, 1997; Pastoret *et al.*, 1998). Cytokine production is stimulated by a variety of signals, antigens, or antigen-MHC complexes acting through T cell receptors or B cell receptors, antigen-antibody complexes acting through F_c receptors, superantigens acting through T cell receptors, or via the presence of microbial components such as lipopolysaccharides (Slifka and Whitton, 2000). Some cytokines such as Interleukin (IL)-1, IL-6, Interferon alpha (IFN-α) and beta (β), and tumour necrosis factor (TNF)-α are produced by a wide variety of cells, whereas others such as IL-2, IL-3, IL-4 and IL-5 are produced by only a small number of cell types (Carter and Swain, 1997).

The structure, function and predominant production of a cytokine by one specific cell type are several of the ways used to identify and classify the cytokines. Cytokines

act on different cellular targets by binding to cell surface receptors on the cell that produced them, binding to receptors on other cells in close proximity and also by spreading throughout the circulatory system and binding to cells in distant locations (Slifka and Whitton, 2000).

1.2.4.1 Interleukin-1

Interleukin-1 plays a critical role in inflammation and the immune response and is produced by mononuclear phagocytes and other cells in response to bacteria, bacterial products and various soluble signals (Yu *et al.*, 1998). The biological effects of IL-1 are diverse, including the induction of fever, initiation of the acute phase response and activation of endothelial cells and neutrophils. Interleukin-1 has been shown to act as a co-factor for the proliferation and activation of lymphocytes (Dinarello, 1996). Interleukin-1 has been shown to be of critical importance to host defences against infectious agents. Independent studies by Dinnarello (1996) and Lennard (1995) have shown that administration of recombinant IL-1 enhances resistance to a variety of pathogenic microbes and, conversely, treatment with IL-1 receptor antagonist, or monoclonal antibodies against the IL-1 receptor, impairs resistance to infection.

1.2.4.2 Interleukin-4

Interleukin-4 was first described as B-cell stimulating factor-1 (Klein and Horejsi, 1997). It is produced primarily by activated T helper (Th) 2 cells and acts on B cells, T cells, macrophages, endothelial cells, fibroblasts and mast cells. Interleukin-4 is essential in the stimulation and differentiation of B cells (Estes *et al.*, 1995), and has been shown to enhance the development of cytotoxic T cells from resting T cells (Klein and Horejsi, 1997).

1.2.4.3 Interleukin-6

Interleukin-6 is a multi-functional cytokine (Montero-Julian, 2001) produced by several cell types including activated macrophages, T cells, B cells, bone marrow

stromal cells, fibroblasts and vascular endothelial cells (Van Snick, 1990). Interleukin-6 exerts its biological activity through interaction with specific receptors expressed on the surface of target cells by binding initially to a low affinity subunit, 80kDa glycoprotein IL-6 receptor (IL-6Rα) or gp80. The IL-6/IL-6Rα complex recruits the signal-transducing β-subunit, a 130kDa glycoprotein, gp130 (Montero-Julian, 2001). Interleukin-6 is a mediator of the acute phase response, co-stimulator of haemopoiesis and an essential factor in plasma-cell development (Lotz, 1995). High levels of IL-6 are found in autoimmune diseases, osteoporosis, multiple myeloma, cardiac myxoma and mesangial glomerulonephritis (Kishimoto, 1989).

1.2.4.4 Tumour necrosis factor family

There are three members of the tumour necrosis factor family: TNF-α, TNF-β, also known as lymphotoxin-α, and lymphotoxin-β (Gruen and Weissman, 2001). Tumour necrosis factor-β is produced by Th1 cells and activates cluster of differentiation (CD)8^+T cells, and has been shown to cause apoptosis of tumour cells and activation of neutrophils, macrophages, endothelial cells and B cells (Gruen and Weissman, 2001). The TNF family are encoded in three closely related genes within the Major Compatibility Complex (MHC). The TNF superfamily is also involved in immune regulation and inflammation (Eigler *et al.*, 1997). Due to the position of the TNF genes close to the MHC, a 'masking' of the role of TNF genes in immune regulation may have occurred. For example, the relative closeness of the two gene complexes within the same region may have resulted in early disease-association studies attributing effects to the MHC region, whereas, in effect they may have been due to the TNF region or *vice versa* (Ruuls and Sedgwick, 1999; Furuya *et al.*, 2001; Tsukasaki *et al.*, 2001).

1.2.5 Interferons

Fibroblasts, T cells and macrophages are the three main cell types responsible for production of the interferons. There are three types of interferon (IFN), IFN-α, -β and -γ. Interferon-γ is unrelated to the other interferons and is produced by mitogen-

or antigen-stimulated T cells (Hochrein *et al.*, 2001). Interferon-γ activates many cell types and controls many functions, such as the enhancement of T cell expression of MHC class I, induction of Th1 cells to produce IL-2 and IL-2R, inhibition of IL-4 production by Th2 cells, induction of maturation in resting B cells, and the activation of mature neutrophils and monocytes (Billiau, 1996).

1.2.6 Chemokines

Members of this family are small molecules which, when bound to their receptors on vascular endothelial cells, form a concentration gradient down which leucocytes can migrate (Zlotnik and Yoshie, 2000). At present, between forty and fifty chemokines have been identified in humans (Rollins, 1997). Many of the genes encoding chemokines have been mapped, and they cluster at specific loci. Chemokines have been divided into the two major sub-families on the basis of the arrangement of the two N-terminal cysteine residues, CXC and CC, depending on whether the first two cysteine residues have an amino acid between them (CXC) or adjacent to them (CC) (Zlotnik and Yoshie, 2000). Two other classes of chemokines have been described: lymphotactin (C, Kelner *et al.*, 1994), and fractaline (CX3C, Bazan *et al.*, 1997). Members of the CXC sub-family have been shown to be potent chemoattractants and activators of neutrophils but not monocytes, whereas members of the CC sub-family have been shown to be chemoattractants for monocytes and lymphocytes but not neutrophils (Klein and Horejsi, 1997). It has recently been observed that chemokines are responsible for basic functions in the immune system, which are not directly related to the inflammatory processes (Kim and Broxmeyer, 1999). These functions include regulation of trafficking of immature blood cells and naïve lymphocytes (Springer, 1994; Butcher and Picker, 1996). Chemokines have been shown to influence the trafficking of lymphocytes at various stages of maturation from primary to secondary, and finally to tertiary lymphoid tissues.

1.3 Acquired immune system

The acquired immune system consists of two major 'arms'. One of the 'arms' is directed against extracellular or exogenous pathogens and is antibody driven (Roitt *et al.*, 1993). This system is termed the humoral immune system. The other major 'arm' is directed against the intracellular or endogenous pathogens and is termed the cell-mediated immune response (Janeway and Travers, 1994).

1.3.1 Humoral immune system

1.3.1.1 Immunoglobulins

Immunoglobulin is the term used to describe soluble B cell receptor or antibody. Immunoglobulins are glycoproteins and can be divided into five distinct classes or isotypes, each with structural and functional variations. Each immunoglobulin class: IgA, IgD, IgE, IgG and IgM, is optimised for 'action' within a specific environment or against a specific group of pathogens. Research in several species has revealed that different types of mammals have evolved divergent molecular and cellular strategies for generating immunoglobulin (Ig) diversity (Meyer *et al.*, 1997). In cattle, four heavy chain classes have been identified: IgM, IgG, IgA and IgE, with three IgG subclasses (IgG1, IgG2 and IgG3) and two light chain types (λ and κ) also identified (Pastoret *et al.*, 1998). A feature of the mucosal system in ruminants is the prominence of IgG1 relative to IgA, particularly in secretions from the mammary gland, whereas IgA is associated with milk fat globule membranes (Honkanen-Buzalski *et al.*, 1981). This suggests the existence of an additional transport mechanism for IgG1 in ruminants. As no placental transfer of maternal antibodies to the foetus occurs in ruminants, transfer of passive immunity in cattle is ensured by the accumulation of extremely high concentrations of antibodies, particularly IgG1 in colostrum and an efficient uptake of the proteins by the neonatal calf.

1.3.2 Cell mediated immune system

The term 'cell-mediated immunity' was originally used to describe localised reactions to organisms, usually intracellular pathogens, mediated by lymphocytes and phagocytes rather than antibody (Roitt *et al.*, 1993). The term is now considered in a more general sense and is used to describe any response against organisms or tumours in which antibody plays a subordinate role. Cell mediated immunity is dependent on many factors including direct interactions between T cells and cells bearing molecules, such as MHC, that T cells recognise (Janeway and Travers, 1994). It should not be assumed, however, that cell-mediated immunity is solely dependent on T cell function. Studies using severe combined immuno-deficiency (SCID) mice, which have no mature T cells, have shown the presence of rapid T-cell independent mechanisms, involved in the recognition of bacterial pathogens, which resulted in immunity to some pathogens in these mice (Roitt *et al.*, 1993).

1.4 Cells of the immune system

Cell surface molecules, identified on specific cell types of the immune system and detectable by their reaction with specific monoclonal antibodies have provided essential information about the cells of the immune system and their interactions (Howard and Morrison, 1991). A number of markers have been described for most of the cells of the haemopoietic progression, especially the T and B lymphocytic lineage (Klein and Horejsi, 1997). The markers on many of the other cell types are less well defined. Lymphocytes express a large number of different markers on their surface that can be used to distinguish the different cell subsets (Reinherz and Schlossman, 1981; Thomas *et al.*, 1981). Analysis of individual cell phenotype allows variations in origin and function of lymphocytes and other cell types to be studied. At present, a large number of cell surface markers have been identified in many species, each one is designated a unique cluster of differentiation number (CD). Considerable homology among species such as humans, rodents and

ruminants has been found between the cell surface markers expressed by leucocytes and the function of the cells that they identify (Howard and Morrison, 1991).

Adherence molecules are cell surface molecules that are essential in the interaction and binding of immune cells (Masinovsky *et al.*, 1990; Meerschaert and Furie, 1995). Some of the common adherence molecules are the integrins, selectins, the immunoglobulin superfamily and the MHC molecules. Integrins bind cells to extracellular matrix proteins, such as fibronectin and collagen (Lobb *et al.*, 1991; Hynes, 1992; Whittard and Akiyama, 2001). Resting T cells express integrins at low levels and activation of the T cells results in increased expression of these adherence molecules. Selectins regulate the circulation and binding of leucocytes to the walls of blood vessels (Jutila *et al.*, 1997). Three selectins have been identified: P-selectin (CD62P), L-selectin (CD62L) and E-selectin (CD62E). P- and E-selectins are found on capillary endothelial cells. When these cells are activated as a result of inflammation, the selectins bind neutrophils, activated T cells and monocytes. In contrast, L-selectin is found on lymphocytes and mediates their binding to high endothelial venules in lymphoid organs (McEver, 1994). The adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), are part of the immunoglobulin superfamily. These adhesion molecules are expressed on cells such as endothelial cells following inflammation which allows lymphocytes and monocytes to adhere and move into inflamed tissues (Tizard, 2000).

1.4.1 Polymorphonuclear cells

Polymorphonuclear granulocytes have a lobed, irregular, polymorphic nucleus (Roitt *et al.*, 1993). Three main cell types of polymorphonuclear granulocytes have been identified: basophils, eosinophils and neutrophils. Basophils and eosinophils primarily attack extracellular pathogens, in particular parasites (Kaufmann and Kabelitz, 1998), and can be differentiated by their ability to take up either acidic or basic dyes. The major cell type of the polymorphonuclear granulocytes is the neutrophil (Tizard 2000), which accounts for around 60-70% of human leucocytes

(Roitt *et al.*, 1993) and approximately 20-30% of bovine leucocytes (Carlson and Kaneko, 1973; Roth, 1994). Healthy cattle have approximately half as many neutrophils as lymphocytes in peripheral blood. A distinctive feature of bovine neutrophils is the presence of a unique third granule type in the cytoplasm (Gennaro *et al.*, 1983).

1.4.1.1 Neutrophils

Polymorphonuclear neutrophils are generally 10-20μm in diameter and have a granular cytoplasm with a distinctive irregular nucleus. They have been shown to be of major importance in the ingestion and destruction of pathogens by antibody and complement-dependent mechanisms (Salgar *et al.*, 1994), and have been described as the primary professional killers of invading micro-organisms (Hasui *et al.*, 1989). Human neutrophils and neutrophils from most other species contain two types of granules: primary and secondary (Paape and Wergin, 1977). The primary granules are lysosomes rich in hydrolases, lysozyme, myeloperoxidase and cationic proteins (Bainton *et al.*, 1971). The secondary granules contain, in addition to lysozyme, lactoferrin, collagenase, and vitamin B_{12}–binding protein (Clark and Klebanoff, 1978). The additional tertiary granules identified in bovine neutrophils are larger than the primary and secondary granules and contain cationic antibacterial peptides (Pastoret *et al.*, 1998). These peptides are unique to cattle and have been shown to have bactericidal properties and are believed to play an essential role in the immune defence system. The plasma membrane of neutrophils contains receptors capable of binding immunoglobulin (F_C receptors) and complement (Paape *et al.*, 1991), as well as β-adrenergic and insulin receptors (Paape *et al.*, 1996). Neutrophils express many different CD molecules on their surface, the most important of which are involved in neutrophil attachment and opsonisation (Gbarah *et al.*, 1991). On neutrophils the main cell surface receptors identified are CD32 and CD35, receptors associated with opsonisation and complement, and the integrins CD11a, CD11b or CD11c which are linked to the common β chain CD18. In cattle, evidence for different CD11b/CD18-dependent mechanisms for neutrophil diapedesis across the various cells of the blood-milk barrier have been identified (Smits *et al.*, 1998; Smits *et al.*, 2000).

1.4.1.1.1 Process of phagocytosis

Neutrophils capture and destroy invading pathogens through the continuous process of phagocytosis, however, the process of phagocytosis can be divided into four distinct stages: chemotaxis, adherence, ingestion and digestion (Hasui *et al.*, 1989).

Neutrophils are normally found circulating in the bloodstream. They are triggered to leave the blood as a result of increased adhesiveness of the endothelial cells and the production of chemoattractants such as C5a (Springer, 1990; Butcher, 1991; Foreman *et al.*, 1994). The expression of adhesive molecules by the endothelial cells is triggered by bacterial products such as lipopolysaccharide, or by molecules produced by damaged tissues, such as thrombin, histamine, TNF α and IL-1 (Lawrence and Springer, 1991). These molecules trigger the expression of P-selectin by the endothelial cells which binds transiently with L-selectin on the neutrophil surface, slowing down the rolling of the neutrophils along the endothelial cell surface. During this rolling, the second stage of activation occurs when platelet-activating factor activates the neutrophils to express the integrin CD11a/CD18 and they undergo a conformational change which results in increased affinity for the endothelial cells. Pseudopodia are then inserted between the endothelial cells by the neutrophils as they migrate out of the blood vessel into the tissue spaces by diapedesis (Lawrence and Springer, 1991).

Adherence of the neutrophil to the 'foreign' particle or bacterium does not happen spontaneously, since both cells are found with negative charges in the body fluids. The negative charge of the bacterium is neutralised by coating the surface with opsonins such as complement component, C3b, or antibody to promote phagocytosis (Scribner and Fahrney, 1976; Newman and Johnston, 1979).

As neutrophils move towards a chemotactic source, a pseudopod advances first, followed by the main portion of the neutrophil (Griffin *et al.*, 1975, 1976). The cytoplasm of the neutrophil pseudopod contains a filamentous network of the proteins, actin and myosin. The pseudopod flows over and around the bacterium or particle allowing the binding of the opsonins and neutrophil surface receptors

(Griffin *et al.*, 1975, 1976). This binding allows the formation of a cup-like pseudopod over the particle, which eventually becomes engulfed by the cytoplasm and is enclosed in the phagosome (Paape and Wergin, 1977).

After phagocytosis of invading bacteria, neutrophils use one of two processes to destroy the invading bacteria: either an oxygen-dependent, or oxygen-independent mechanism is employed (Hoeben *et al.*, 1997). Oxygen-dependent killing is known as the respiratory burst and is a sequence of events which results in the production of active oxygen derivatives such as superoxide, hydrogen peroxide, hydroxyl oxygen and singlet oxygen (Hirabayashi *et al.*, 1985). The oxidative burst can be monitored by a variety of techniques including monitoring oxygen consumption, chemiluminescence (Allen *et al.*, 1972), formation of redox reaction products, and the generation of reactive oxygen species. The oxygen-independent mechanism involves the release of lytic enzymes and antimicrobial peptides from intracellular granules (Lukacs *et al.*, 1985). The enzymes contained in these granules digest bacterial cell walls and destroy most micro-organisms, however, variation in susceptibility to the destruction by oxygen-independent mechanisms between pathogens have been observed (Klein and Horejsi, 1997).

1.4.2 Mononuclear cells

1.4.2.1 Antigen presenting cells

Antigen presenting cells (APC) are a heterogeneous population of leucocytes, which possess a very efficient immunostimulatory capacity (Unanue and Allen, 1987; Roitt *et al.*, 1993). Some antigen presenting cells have a pivotal role in the induction of the functional activity of Th cells, whereas, some interact with other leucocytes and their functions in maintaining an effective immune system (Glass and Spooner, 1989).

1.4.2.2 Dendritic cells

Dendritic cells are professional APC derived from the bone marrow and are distributed at sites suitable for antigen uptake throughout the body tissues (Howard *et al.*, 1999). These bone marrow derived leucocytes are considered to be the only cells able to present antigen to naïve T cells and thus initiate primary immune responses (Banchereau and Steinman, 1998). Dendritic cells have been shown to be widely distributed in small numbers throughout the body tissues, with a number of distinguishable phenotypes with different associated functions having been identified (Steinman, 1991, Shortman and Caux, 1997). Studies in cattle have also shown the existence of phenotypically heterogeneous populations of dendritic cells with different biological properties (Howard *et al.*, 1999).

1.4.2.3 Monocytes

Monocytes are the largest of all blood cells, measuring about 15μM in diameter (Roitt *et al.*, 1993). The cell surface of monocytes is abundant in F_C receptors for immunoglobulins, receptors for complement as well as adherence molecules associated with many cells such as platelets, lymphocytes and endothelial cells (Rabinovitch, 1967; Huber and Fudenberg, 1970). Monocytes have been classified as immature tissue macrophages: monocytes leave the systemic circulation by diapedisis and mature into larger, more effective phagocytes, which are found in the tissues (Van Furth, 1970; Volkman, 1970). The migration of monocytes to the tissues is markedly enhanced in response to tissue injury or infection (Van Furth *et al.*, 1973).

1.4.2.4 Macrophages

Macrophages are mononuclear phagocytes, have a single rounded nucleus, are capable of repeated phagocytic activity unlike neutrophils (Klein and Horejsi, 1997), and are found residing in tissues. Cattle, like other ruminants and pigs, have high numbers of pulmonary intravascular macrophages (Winkler, 1988), which are thought to play a major role in the clearance of blood-borne bacteria. Phagocytosis

of invading pathogens is just one of the functions of macrophages and they are also responsible for the secretion of molecules such as cytokines (Springer, 1994; Mosser and Karp, 1999), which are involved in the amplification of the immune response. Macrophages are essential in the control of inflammation by contributing directly to the repair of tissue damage by the removal of dead and damaged tissues. Another function of macrophages is the processing of antigen in preparation for the specific immune response (Harding *et al.*, 1995). Proteolytic antigen processing produces short peptides that bind to MHC molecules to generate the peptide-MHC complexes that are subsequently presented to T cells (Harding *et al.*, 1995). Upon recognition of the peptide-MHC complex, T cells act as mediators or regulators of the cellular immune reaction in addition to playing a role in the regulation of B cell function (Harding *et al.*, 1995).

1.4.2.5 Lymphocytes

Lymphocytes are central to all adaptive immune responses and play an essential role in the specific recognition of pathogens (Roitt, *et al.*, 1993). The two main types of lymphocytes are the T cells and the B cells (Bachmann and Kopf, 1999). T cell precursors arise in the bone marrow and develop into mature antigen–recognising cells in the thymus (Klein and Horejsi, 1997). In the case of B cells, more than one primary lymphoid organ or diversification mechanism has been identified among different species (Meyer *et al.*, 1997). In mammalian species, such as mice and humans, the bone marrow has been documented as the major site of B cell development (Claman *et al.*, 1966), whereas studies in both the bovine and ovine systems have suggested that the ileal Peyer's patch is the site of primary B cell development (Meyer *et al.*, 1997). A third population of lymphocytes which do not express antigen receptors are called natural killer (NK) cells (Glas *et al.*, 2000). Natural killer cells are derived from lymphoid cell progenitors in the bone marrow and can be functionally distinguished from T and B cells by their ability to lyse certain tumour cell lines *in vitro* (Trinchieri, 1989). However, bovine NK cells have been shown to be less efficient at lysing tumour cell targets except when the NK cells undergo cytokine activation (Roth, 1994). The cell surface marker CD45 is a membrane protein tyrosine phosphatase expressed on all nucleated haematopoetic

cells in various isoforms (Kong *et al.*, 1995). Studies have identified an essential role for CD45 in the activation and antigenic stimulation of T and B cells (Chan *et al.*, 1994). The isoforms CD45RA and CD45RO have been shown to be an indicator of $CD4^+$ T cell status, expression of CD45RA is replaced by expression of CD45RO following the differentiation of an activated T cell into an effector or memory T cell in humans (Beverley *et al.*, 1988; Terry *et al.*, 1988). However, expression of these isoforms is not mutually exclusive and has been observed on a small subset of T cells in humans (Mason and Powrie, 1990). In cattle, approximately 10% of bovine T cells have been shown to be double positive for CD45RA/RO (Bembridge *et al.*, 1995).

1.4.2.6 B cells

Only a small proportion of B cells are found within the circulatory system, the majority of the B cell population is found within the tissues, mainly in the cortex of lymph nodes, marginal zone of the spleen, bone marrow and Peyer's Patches of the intestine (Von Boehmer and Kisielov, 1990). One of the main functions of B cells is to respond to external antigen through the production of antigen-specific antibody (Morafo *et al.*, 1999). Dendritic cells have been shown to be essential for the activation of B cells and are responsible for the modulation of all stages of B cell growth and development.

Antigen binds to B cell receptors in the presence of helper T cells, IL–2 or IL–4. This results in a signal being transmitted into the cell, inducing increased expression of B cell receptors, MHC class II, IL–2, IL-4, IL-5, IL-6, TNF-α and TGF-β receptors which leads to the initiation of B cell division (Morafo *et al.*, 1999). Following successful activation, B cells enlarge and divide repeatedly, as the immune response progresses a gradual increase in antibody affinity for antigen is seen. This increase in antibody affinity is in response to somatic mutation and selection within responding B cells. T cell cytokines bind to receptors on the B cells to induce their proliferation, and subsequent immunoglobulin isotype switching and synthesis which results in secretion of IgM specific for the inducing antigen. Most of the mature B cells differentiate into plasma cells, however some of the activated B

cells will enter lymphoid follicles in the cortical area of lymph nodes where they form germinal centres. These germinal centres containing memory B cells are critical to B cell existence (Berek, 1992).

1.4.2.7 T cells

T cells play a central role in the antigen-specific immune response to various pathogens (Saalmuller, 1998). In the majority of cases, T cells recognise antigen only when it is presented to them in the form of small fragments bound to MHC molecules on the surface of another cell (Germain, 1994). In addition to the signal delivered via the T cell receptor (TCR) engaged by the MHC molecule plus antigen, the T cell must also receive co-stimulatory signals from the APC. Only then does it become fully activated and is able to proliferate and differentiate into an effector cell. Each T cell clone has a unique TCR that is non-covalently associated on the surface of the T cell with a complex of transmembrane polypeptides known as CD3 (Tanaka *et al.*, 1995). The CD3 complex consists of three molecules γ, δ and ε and is essential for T cell development, as well as for the assembly and efficient surface expression of the TCR (Exley *et al.*, 1995). T lymphocytes can be divided into subsets according to the markers they express and their function and generally fall into two categories: αβ T cells or γδ T cells. The α, β, γ and δ peptide chains are encoded on separate genes. In humans, in the mature T cell pool, αβ T cells predominate with γδ T cells as the minority population. In ruminants, however, the number of γδ T cells is high compared with the proportions observed in humans and mice (Mackay and Hein, 1990; Hein and Mackay, 1991). Workshop cluster (WC)1 is a cell-surface protein which has been identified in the major domestic mammals, but has not been currently recognised in humans or mice (Tizard, 2000). In cattle, the $WC1^+$, $CD2^+$, $CD8^+$ phenotype predominates in blood but other minor subsets, $WC1^-$, $CD2^+$ and $CD8^+$ or $CD8^-$, are evident and have been shown to predominate in the spleen and gut mucosa (Clevers *et al.*, 1990; Wyatt *et al.*, 1994).

Induction of a proliferative response induced by antigen *in vitro* is representative of cellular immunocompetence (Kristensson *et al.*, 1994). The recognition of antigenic peptide by lymphocytes results in proliferation and differentiation of cells and

ultimately in clonal selection (Roitt *et al.*, 1993). Resting lymphocytes appear as a homogeneous population with a low RNA and DNA content and are referred to as G_0 cells (Kristensen *et al.*, 1982). On stimulation the G_0 cells transform into the G_1 phase which is characterised by increased levels of RNA synthesis (Stadler *et al.*, 1980). The cells progress from the G_1 phase to the S phase, which is detectable by the presence of DNA synthesis in the cells. Following completion of DNA synthesis the lymphocytes enter the G_2 phase before returning to the G_0 phase following mitosis (Stadler *et al.*, 1980). Studies in mice and humans have indicated that IL-2 is required for activation and proliferation to occur (Baker and Knoblock, 1979; Gillis *et al.*, 1980). Lymphocyte proliferation is an essential aspect of cell-mediated immunity, in children suffering from DiGeorge syndrome, the lack of mature T cells due to an athymic condition, results in the failure to produce a proliferative response when stimulated by mitogens (Markert *et al.*, 1998).

1.4.2.7.1 αβ T cell receptor

The αβ TCR distinguishes lymphocytes into two categories, those that recognise peptides presented by MHC class I molecules, and those that recognise peptides presented by MHC class II molecules (Van Neerven *et al.*, 1990). Mature human αβ T cells can be further divided into $CD4^+$ and $CD8^+$ molecules, with the $CD4^-CD8^-$ accounting for approximately 60% of the cells. The $CD4^-CD8^+$ cells account for the majority of the remaining cells along with the double negative $CD4^-CD8^-$ cells, which account for approximately 5% of the population. In cattle, MHC class II restricted $CD4^+$ T cells (Baldwin *et al.*, 1986; Howard *et al.*, 1989) and MHC class I restricted $CD8^+$ T cells (Ellis *et al.*, 1986) have also been described. Traditionally, based on their function, there are two main subsets of T cells: the T_C cells which are $CD4^-CD8^+$ and MHC class I restricted, and the Th cells which are $CD4^+CD8^-$ and MHC class II restricted. Further divisions exist within the Th subgroup based on the functional capabilities of the cells and the cytokines they produce, which divides the Th cells into Th1 and Th2 cells (Mossman *et al.*, 1986). The Th1 cells have been shown to produce cytokines associated with inflammation such as IL-2, IFN-γ and TNF-β which induce cell mediated responses. The Th2 cells produce cytokines associated with B cell proliferation and differentiation such as IL-4, IL-5, IL-6, IL-10

and IL-13 and induce humoral immune responses (Constant and Bottomly, 1997). Another subset of cells, classified as Th0 cells, has also been identified within the Th group. The Th0 cells have been shown to produce IL-2, IFN-γ and IL-4, however, their function and relationship with the Th1 and Th2 cells remains controversial (Klein and Horejsi, 1997). Current evidence suggests the existence of a much more complex system involving more than just the traditional Th1:Th2 paradigm (Kelso, 1995; Brown *et al.*, 1998).

1.4.2.7.2 γδ T cell receptor

In humans and mice, γδ T cells have been shown to emigrate from the thymus to colonise the skin, mammary gland, the reproductive organs and the intestinal wall (Mackay, 1988; Mackay *et al.*, 1988). Human γδ T cells directly recognise proteins such as heat-shock proteins and phospholigands, in the absence of MHC binding (Correa *et al.*, 1992; Bigby *et al.*, 1993). Mouse γδ T cells can destroy cells such as chicken red blood cells, cells infected with mycobacteria and some leukaemic cells (Tizard, 2000), and they lack expression of CD4. Possible roles for γδ T cells may be to regulate macrophage activation, mediate oral tolerance, or regulate the activation of αβ T cells (De Libero, 1997).

In ruminants, γδ T cells can bind a wide variety of antigens, suggesting that they are of major functional significance (Hein *et al.*, 1991). In sheep, γδ T cells express OvWC1 and do not express CD2, CD4 or CD8. In lambs, at birth, 60% of peripheral blood T cells are γδ, and by one year of age this has decreased to 30% and continues to decrease with age (Washington *et al.*, 1992). Studies in cattle have shown that proportions of γδ T cells are consistently higher in young calves (25-30%) than in older animals (3-10%), and that the numbers of γδ T cells also continually decrease with age (Clevers *et al.*, 1990). Several subsets of the bovine γδ TCR^+ have been identified in cattle. WC1 is found on a major subset of γδ T cells. It has been suggested that ruminants possess large numbers of γδ T cells when young (Hein and Mackay, 1991), in order to provide an early cell-mediated response in animals whose immunoglobulins are received after birth through colostrum and not by placental transfer prior to birth (Clevers *et al.*, 1990; Leid *et al.*, 1998).

1.5 Major Histocompatibility Complex

The MHC has been identified in at least 11 species of higher vertebrates (Gotze *et al.*, 1977). The genes in the MHC determine which antigens and processed and presented and were originally identified by investigators working on tissue transplantation (Engelhard, 1994). The MHC has been shown to be a major genetic component of infectious or autoimmune disease resistance or susceptibility in a variety of species (Klein, 1986; Tizard, 2000). Loci of the MHC fall into three classes: class I loci, which regulate antigen recognition by cytotoxic T cells; class II loci, which govern antigen recognition by other lymphocytes; and class III loci which encode a variety of proteins including complement components (Klein *et al.*, 1982). Class I loci can be divided into those that are highly polymorphic, the class Ia loci, and those that show very little polymorphism, the class Ib, Ic and Id loci, with the class Id loci located outside the MHC region. Class II loci code for polymorphic molecules and class III loci code for a wide variety of proteins with a range of functions (Tizard, 2000). In different species, each MHC contains all three classes of loci however their number, location and arrangement may differ. The nomenclature of the MHC also differs between species and is known as H-2 in the mouse (Snell, 1958), B complex in chickens (Briles *et al.*, 1950), human leucocyte antigens (HLA) in humans (Klein, 1986) and BoLA in cattle (Spooner *et al.*, 1979). Recently, in humans, several genes have been described that are encoded in the telomeric end of the Class III region, that appear to be involved in both general and specific inflammatory responses. This gene-rich region has been named the Class IV region and includes the TNF gene family (Gruen and Weissman, 2001). This Class IV region may play a role in resistance or susceptibility to autoimmune diseases (Gruen and Weissman, 2001).

1.5.1 Class I

The MHC class I molecules each consist of one heavy protein chain and a much smaller light chain, β2m (Engelhard, 1994). Peptides that bind to MHC class I are usually 8 or 9 amino acid residues long, which allow the amino and carboxy terminal ends of the peptide to fit into pockets at opposite ends of the MHC binding cleft (Stern and Wiley, 1994). Class Ia molecules are highly polymorphic and expressed on the surface of most nucleated cells. Each mammal studied has two or three class Ia gene loci, in humans they are known as *A*, *B* or *C*, and in mice they are known as *K* and *D*. Within each locus multiple allelic genes exist, with great variation in the number of class I genes among species. The role of class Ib molecules is poorly understood and, in mice, the gene loci are found in three clusters termed *Q*, *T* and *M* where they may code for proteins found on the surface of regulatory and immature lymphocytes and on haemopoietic cells. Class Ic genes are molecules with low levels of polymorphism found within or close to the MHC, whereas the class Id nonpolymorphic molecules are found outwith the MHC (Barnstable *et al.*, 1978; Ploegh *et al.*, 1981; Bjorkman *et al.*, 1987).

1.5.2 Class II

The class II molecule is highly polymorphic and consists of two chains which are similar in size, but smaller than the class I heavy chain (Engelhard, 1994). Peptides that bind to class II vary in length and are, on average, longer than those associated with class I. The binding cleft of the class II molecules is similar in structure to class I molecules, however, the class II molecule lacks pockets for specifically binding the end of the peptide, resulting in peptide binding occurring in the middle of the cleft (Stern and Wiley, 1994). Within the MHC class II region of humans, five sets of loci have been identified: *DP*, *DN*, *DO*, *DQ* and *DR* (Hardy *et al.*, 1986; Klein and Figueroa, 1986) which can consist of either, or both α and β chains and are designated A and B respectively. If multiple genes are present in a given family,

they are distinguished by Arabic numerals, for example, *DQA1*, *DRB3* (Klein and Horejsi, 1997). In all gene families, except *DR*, the number of loci in humans is the same. In the case of *DR*, all humans have one *DRA* locus but they differ in the number of *DRB* loci per chromosome.

1.6 Bovine lymphocyte antigen system

The bovine MHC (*BoLA*) and at least 32 structural genes have been mapped to bovine chromosome 23 (Fries *et al.*, 1993). More than 50 *BoLA* antigens have been recognised, nearly all of which behaved as alleles of a single locus (*BoLA-A*). Major histocompatibility complex class I typing techniques such as cell-mediated lympholysis (Spooner and Morgan, 1981), immunoblotting (Viuff *et al.*, 1991), Southern blotting (Lindberg and Andersson, 1988) and polymerase chain reaction (Sawhney *et al.*, 2001) have been used to demonstrate class I polymorphism. At least 12 class I genes have been identified, however, the function of some of these genes is still unclear (Gellin *et al.*, 2000).

The BoLA class II region is unique, in that it is divided into two distinct regions, a and b. A large inversion has moved several class II genes to the centromere of bovine chromosome 23, which results in the class IIb genes being separated from the classI/IIa genes by a gap of 17 centimorgans. Eleven class II loci have been identified, but only products encoded at the *DR* and *DQ* loci in the class IIa region (*DRA*, *DRB3*, *DQA*, *DQB1* and *DQB2*) have been identified on bovine leucocytes. These two MHC class II regions have been identified and defined on the basis of genetic mapping (Andersson *et al.*, 1988; van Eijk *et al.*, 1992 (a); Russell *et al.*, 2000). Expression of MHC class II has been identified on many bovine cell types including B cells (Lewin *et al.*, 1985), activated T cells (Taylor *et al.*, 1993), alveolar macrophages, monocytes, and mammary epithelial cells (Fitzpatrick, 1992). In addition to the class I and class II genes, class III genes have also been mapped to the bovine MHC (Tizard, 2000).

1.7 Major histocompatibility complex and disease

The function of presenting antigen to the immune system by MHC molecules is critical in the production of an effective immune response, and ultimately, the control of disease. The association between the MHC and resistance or susceptibility to disease has been defined in many species (McDevitt, 2000). Analysis of haplotypes in disease association studies has been shown to be helpful in mapping disease resistance and susceptibility to localised regions within the MHC (van Eijk *et al.*, 1992; Xu *et al.*, 1993).

Associations have been found between both class I and class II genes and the incidence of disease including persistent lymphocytosis (Stear *et al.*, 1988), ketosis (Mejdell *et al.*, 1994) and mastitis (Oddgeirsson *et al.*, 1988, Schukken *et al.*, 1994 and Sharif *et al.*, 1998). The *BoLA-DR* genes and their products are among the best characterised of the MHC genes in cattle (Ledwidge *et al.*, 2001). In particular, the *BoLA-DRB3* has been widely studied and has been shown to be highly polymorphic with at least 170 *BoLA-DRB3* haplotypes identified in major dairy and beef breeds (Lewin, 1996).

Resistance or susceptibility to Marek's disease in chickens (Briles *et al.*, 1977) has been associated with the haplotypes B^{21} and B^{19} respectively. In mice, high and low antibody responder lines of mice were created by selective breeding to antigens of different specificity (Biozzi *et al.*, 1979). In these mice, immune responsiveness was shown to be under polygenic control, with MHC-linked genes playing a partial and irregular role. Following seven generations of selective breeding, high and low antibody titre lines were associated with the effect of alleles at four independent loci (Cabrera *et al.*, 1982). In humans, the MHC haplotype has been associated with susceptibility to many diseases, in particular the autoimmune diseases such as rheumatoid arthritis (Nepom and Erlich, 1991) or type I insulin-dependent diabetes mellitus (Todd *et al.*, 1987). In pigs, the Swine Lymphocyte Antigen (porcine MHC)

has been shown to have a major influence on major reproduction traits and growth rates (Vaiman *et al.*, 1998). Associations between allelic variants of the bovine MHC (*BoLA*) genes and occurrence of disease, as well as immune responsiveness in cattle, has been well documented (Lewin *et al.*, 1999). Associations between production traits, health traits and MHC class I alleles in cattle have been reported. Certain *BoLA* alleles have been associated with percentage milk fat (Hines *et al.*, 1986), protein yield (Batra *et al.*, 1989), increased neutrophil function and numbers (Weigel *et al.*, 1991), tick infestation (Stear *et al.*, 1989) and with resistance to leukosis (Xu *et al.*, 1993). Different *BoLA* haplotypes have been associated with resistance or susceptibility to mastitis in different breeds of cattle (Mejdell *et al.*, 1994; Mallard *et al.*, 1995). The A11 and A16 haplotypes are associated with susceptibility to mastitis in Norwegian Red and Swedish Red and White cattle (Lunden *et al.*, 1990), while A11 is associated with resistance to mastitis in Holstein cattle (Weigel *et al.*, 1990).

Association between *BoLA* MHC class II alleles and bull breeding have been shown with respect to many factors including clinical mastitis, ketosis, retention of the placenta and milk fever (Lunden *et al.*, 1991). In one study in cattle, it was reported that allele *DRB3.2*16* increased the susceptibility to mastitis as estimated by an increase in somatic cell count, with alleles *DRB3.2*11*, *12* and *23* associated with resistance to clinical mastitis (Dietz *et al.*, 1997 (b)). More recent studies have identified the MHC II haplotypes *DRB3.2*23* with an increased susceptibility and *DRB3.2*16* with a decreased risk of mastitis in Canadian Holsteins (Sharif *et al.*, 1998). The study by Sharif *et al.* (1998) studied Holstein-Friesian cows in at least their second lactation and reported that *DRB3.2*16* was associated with a significant reduction in somatic cell counts. The study by Sharif *et al.* (1998) graded the level of mastitis which occurred in these animals and identified the pathogens responsible for the cases within the grades. The identification of the causative agent of diseases such as mastitis is an essential aspect of the identification of resistant or susceptible *BoLA* alleles as the influence of the *BoLA* alleles on immune function may differ between the major disease-causing pathogens (Dietz *et al.*, 1997 (a)).

1.8 Cattle breeding

Clearly defined breeding objectives are vital for effective genetic improvement of all livestock species (Amer *et al.*, 2001). Breeding objectives were first proposed for animals by Hazel (1943), and since then, multiple-trait selection indexes have become the method of choice for maximising genetic gain in a chosen breeding programme. At present, selection of dairy cattle for breeding worldwide is based on profitability. Until recently, the selection of dairy cattle in the United Kingdom utilised indices such as the profit index number (PIN), index of total economic merit (ITEM) and profitable life index (PLI). These indices combined many measurements including production and type traits to calculate an economic estimate of specific sires through analyses of their daughters. From February 2001, the multiple trait across country evaluation (MACE) index has been introduced. The MACE index has been introduced due to the globalisation of dairy cattle breeding and the requirement for accurate information regarding the performance and genetics of animals worldwide, irrespective of initial variations in ranking systems between countries (Holstein Friesian Society, www.hfs-gbi.org). Dairy cattle have typically been selected largely on production and conformation-associated traits such as udder depth, teat length, angularity and hoof angle (Brotherstone and Hill, 1991). Traits for selection such as clearance of the udder from the ground and tighter fore udder attachment have been correlated with a reduction in log somatic cell count, somatic cell score and subsequently in a reduction in the incidence of clinical mastitis (Rogers *et al.*, 1991; Lund and Jenson, 1996; Boettcher *et al.*, 1998). Selection of dairy cattle based on production traits has been extremely successful, resulting on average in an improved annual yield of approximately 2-3% (Honhenboken, 1987). The emphasis of beef selection traits have focussed on factors affecting traits such as growth rates, feed intake and conversion rates and carcass composition (Simm, 1998).

Traditionally, maintenance of animal health has relied upon livestock that have not been selected specifically to optimise their genetic potential for health (Gavora and Spencer, 1983). In dairy cattle, production traits such as yield of milk and milk solids

are examples of traits of primary economic concern to the dairy breeder, in addition to traits such as longevity, disease resistance and reproductive efficiency. Selection for a combination of production related and disease resistance traits is likely to be more beneficial and cost effective. In beef cattle, economic traits such as postweaning growth and feed efficiency are important components of the overall efficiency of beef production systems (Smith *et al.*, 1976), with traits related to carcass characteristics, and the rate and efficiency of lean tissue gain becoming increasingly important because of the consumer trend for leaner meat (Kempster *et al.*, 1988). In addition, reproductive traits are also of importance to the beef production industry with estimated breeding values (EBV) of beef sires reflecting the two main economic requirements for the beef industry: the beef value and the calving value (Simm, 1998; Avoncroft Sire Information 2001).

Understanding how immune responses are regulated in ruminants is critical for devising future strategies to direct immune responses toward desired effector functions required to either prevent infection or eliminate disease rapidly (Brown *et al.*, 1998). Such strategies may identify possible genetic markers for these immune responses to be incorporated into future breeding indices for dairy and beef cattle.

1.9 Quantitative trait loci

Quantitative trait loci (QTL) have been identified and mapped onto regions of specific chromosomes in many species for a range of effects including obesity in mice (Snustad and Simmons, 2000), immune function in pigs (Edfors-Lilja *et al.*, 1998, 2001) and susceptibility to cardiovascular disease in humans (Vogler *et al.*, 1997). Primary linkage maps covering all chromosomes are now available for all the major livestock species (Clark, 1998). The majority of traits selected in livestock production are quantitative traits; i.e. the individual phenotype reflects the action of several genes, confounded by environmental effects. Mapping the underlying genes or QTL should allow for marker assisted selection, which is expected to increase the

rate of genetic progress (Georges *et al.*, 1993). Complex traits, such as disease resistance and lactation, are under the control of many genes, each with a different phenotypic contribution (Heyen *et al.*, 1999). The current genetic linkage map of cattle consists of more than 1,500 genetic markers. The majority of these markers are polymorphic microsatellites with known map locations (Heyen *et al.*, 1999). Quantitative trait loci have been identified in cattle in association with milk production (Ashwell *et al.*, 1998, Nadesalingam *et al.*, 2001), health traits (Zhang *et al.*, 1998) and body conformation traits (Mrode *et al.*, 2000).

1.10 Mammary gland function

The bovine mammary gland has many forms of defence against invasion by pathogenic organisms, combining non-specific and specific systems, including anatomical features of the gland and humoral and cellular defence mechanisms (Outteridge and Lee, 1988). Humoral defences include the specific defences such as the immunoglobulins (Duncan *et al.*, 1972) and the non-specific defences such as lactoferrin, lysozyme lactoperoxidase and complement (Reiter, 1978). Neutrophils, macrophages, lymphocytes and epithelial cells are the cells normally found in mammary secretions. The numbers and proportions of these cells vary between individuals and the stage of lactation (Lee *et al.*, 1980). In spite of the presence of considerable numbers of immune cells in the local mammary gland environment, it has been shown that the mammary gland is generally immunologically compromised when compared to the rest of the body (Hurley *et al.*, 1990). The functions of all types of white blood cells in milk: neutrophils, lymphocytes and macrophages, have been shown to be reduced compared to cells isolated from blood (Paape *et al.*, 1975). Neutrophil recruitment from blood is therefore, essential to the defence of the mammary gland against bacteria (Smits *et al.*, 2000). Neutrophils migrate from blood across endothelium into the extracellular matrix and then across mammary epithelium into the infected lumen, making effective recruitment of functional neutrophils from the blood an essential requirement. Lymphocytes also play an

essential role in control of pathogenic invasion of the mammary gland (Kehrli and Goff, 1989). Although less well defined, lymphocytes have many roles within the mammary gland, such as antibody secretion, which has been shown to facilitate phagocytosis by neutrophils (Lukacs *et al.*, 1985). Re-circulation of lymphocytes may also play a critical role in the defence of the mammary gland. Approximately 10% of lymphocytes in adult animals are able to migrate from peripheral blood to tissue and then into the lymph nodes (Trnka and Cahill, 1980). Adhesion molecules have been shown to be of critical importance in the re-circulation of lymphocytes to the lymphatic organs or effector sites (Picker, 1994; Springer, 1994). In addition, T cells expressing a memory phenotype have been shown to preferentially re-circulate to the site of initial antigenic stimulus, known as lymphocyte homing (Mackay *et al.*, 1992).

Assessment of milk cells in the laboratory is problematic, requiring large volumes of milk, resulting in relatively poor yields of lymphocytes and neutrophils. Another complication of assessing milk cell function is the fat may interfere with components of the assay. Due to the potential problems of assessing milk cell function and the well-documented re-circulation of the lymphocytes back into the systemic system, assessing the peripheral blood cells may provide an accurate and true reflection of immune function in the mammary gland of individual animals.

1.11 *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive, catalase producing, coccus, a commensal of the skin, and has been shown to account for 25-30% of bovine mastitis cases in the United Kingdom (Sutra and Poutrel, 1993). *Staphylococcus aureus* is the predominant cause of subclinical mastitis (Wilson and Richards, 1980), where an elevated somatic cell count may act as an indicator of infection. Staphylcocci invade deep into the mammary gland (Blowey, 1990) and can survive and multiply within host phagocytic cells (Sandholm and Mattila, 1986). This evasion mechanism results

in the host defence and subsequent antibiotic treatment being incapable of completely eliminating the micro-organism from the udder. Treatment of staphylococcal mastitis is further complicated by the existence of many strains of *S. aureus* which can cause infection, each with variations in sensitivity to antibiotics (Webster, 1993; McKellar, 1996). Alternative methods to prevent staphylococcal bovine mastitis, such as vaccination, have been attempted, but have had limited success (Slanetz *et al.*, 1963). Due to the lack of successful treatment for staphylococcal mastitis, the identification of animals, which were naturally resistant to such a disease, would be highly beneficial to the dairy industry in the future.

1.12 Aims

A previous study in Holstein-Friesian cattle carried out by Logan (2001) which developed an *in vitro* immunological assay to accurately measure the *in vitro* proliferative response of peripheral blood mononuclear cells (PBMC) induced by *Staphylococcus aureus* identified significant variation among individuals, progeny from two different sire groups and among Holstein-Friesian bulls in their *S. aureus*-induced *in vitro* PBMC proliferation. One aspect of this previous study looked at the assessment of the proliferative ability of bulls selected on the basis of their predicted transmitting ability (PTA) for somatic cell count (SCC), where the PTA for SCC is used as an estimate of the effect of the sires' genes on SCC, and thus, a measure of the likely incidence of subclinical mastitis in the subsequent progeny from that bull. Two groups of five Holstein-Friesian bulls were selected on their extreme values of PTA for SCC: bulls with a high PTA for SCC may have progeny with a higher than average SCC, whereas bulls with a low PTA for SCC may have progeny with a lower than average SCC. The *S. aureus*-induced proliferative response of these two groups of bulls, showed a strong negative correlation (-0.07, $p<0.05$) with PTA for SCC, which indicated that bulls with low PTA for SCC were more likely to have high levels of *S. aureus*-induced *in vitro* PBMC proliferation, and conversely, bulls with high PTA for SCC were more likely to have low levels of *S. aureus*-induced *in vitro* PBMC proliferation. Analysis of MHC class II DRB-RFLP allele frequency, identified that bulls with low PTA for SCC and thus high levels of *S. aureus*-induced *in vitro* PBMC proliferation had RFLP patterns associated with *DRB3.2*16*, an allele associated with resistance to bovine mastitis in many studies. The study by Logan (2001) identified a method to accurately assess *S. aureus*-induced *in vitro* PBMC proliferation in male and female Holstein-Friesian cattle, and highlighted a possible association with MHC-RFLP pattern and resistance or susceptibility to *S. aureus* mastitis.

The overall aim of this current study was to expand on the previous work by Logan (2001) by assessing the immune function in a genetically defined population of Holstein-Friesian cross Charolais male and female cattle from six months of age. Over 300 second generation animals consisting of F2, Holstein-Friesian backcross

and Charolais backcross animals were sampled. One aspect of the project was to determine the specific immune response by assessing *S. aureus*-induced *in vitro* PBMC proliferation and a non-specific immune response by assessing Phytohaemagglutinin-induced (PHA) *in vitro* PBMC proliferation in all the animals at approximately six months of age. The aim was to look for variation among individuals and to assess if any correlation existed between PBMC proliferation and cross or sire.

The second main focus of the project was to develop a method to assess the polymorphonuclear *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads. Once a reliable method was developed, assessment of variation in PMN phagocytic ability among individual animals was carried out. Correlation between PMN phagocytic ability and cross and sire was also assessed. The identification of any correlation between non-specific immune functions in PBMC and PMN and specific and non-specific immune function between PBMC and PMN in terms of PHA-induced or *S. aureus*-induced PBMC proliferation and PMN phagocytosis was also explored.

The ultimate goal of this study is to identify QTL in cattle associated with specific and non-specific immune function in relation to *in vitro* PBMC proliferation and *in vitro* PMN phagocytosis that may be exploited in future to improve animal health and production on commercial farms.

Chapter 2

Materials and Methods

2.1 Introduction

This project was designed to measure the peripheral blood mononuclear and polymorphonuclear cellular responses of cattle from approximately six months of age onwards using a range of *in vitro* assays that assess various aspects of cellular function induced by both non-specific and specific immunological stimuli.

One of the specific aims of this project was to assess the proliferative responses of peripheral blood mononuclear cells (PBMC) *in vitro*. Following previous preliminary studies in commercial Holstein-Friesian cattle (Fitzpatrick *et al*., 1999), that indicated variation in *S. aureus*-induced *in vitro* PBMC proliferation among individual animals, it was considered relevant to look at *S. aureus*-induced proliferation as an indicator of specific immunity in a large population of research cattle from which accurate genetic information was available. T lymphocyte-specific, mitogen-induced proliferation of PBMC, induced by Phytohaemagglutinin (PHA) as an indicator of non-specific immunity was also measured in the research herd. In total, 319 animals were sampled at approximately six months of age over the three years of the study (Table 2.1).

YEAR OF BIRTH	MALES	FEMALES	TOTAL	MALES REPEATED	FEMALES REPEATED	TOTAL REPEATED
1998 Cohort	32	23	55	16	16	32
1999 Cohort	65	57	122	16	16	32
2000 Cohort	69	73	142	16	32	48
TOTAL	166	153	319	48	64	112

Table 2.1: F2 Holstein-Friesian cross Charolais second generation calves sampled for assessment of *in vitro* PBMC proliferation. Columns 2-4 show the number of male and female calves sampled each year at approximately six months of age and the total number of calves sampled each year. Columns 5-7 show the number of male and female calves sampled for assessment of individual animal variation over time within each year and the total number of calves re-sampled each year.

Another aim of this study was to develop various *in vitro* assays to assess PMN cell phagocytosis in cattle. The initial part of this work involved the development of a procedure to isolate PMN effectively from bovine blood, resulting in a highly purified PMN population. This population was then used for assessment of *in vitro* phagocytosis of isolated PMN from individual animals. The development of the assay involved assessment of the *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads and Fluoroscein Isothiocyanate (FITC)-labelled *S. aureus* (Molecular Probes Europe BV, The Netherlands) by an isolated PMN population over a 16 hour incubation period. Assessment of *in vitro* phagocytosis of FITC-labelled latex beads by isolated PMN populations from individual animals was carried out on all calves born in the 2000 cohort. A small subset of animals were used for the preliminary development of an assay which assessed the *in vitro* phagocytosis of FITC-labelled *S. aureus* by isolated PMN populations from individual animals.

Due to the current debate over potential disadvantages of the isolation procedure used to obtain a pure PMN population, such as the alteration of the functional ability of the PMN by the possible modification of adhesion molecules or premature activation of the PMN, development of an *in vitro* assay for assessing phagocytosis of FITC-labelled (2.0μM) latex beads by PMN populations in whole blood in cattle was also carried out. A small subset of animals were employed for this part of the study.

2.1.1 Herd structure

The study involved sampling second generation Holstein-Friesian cross Charolais animals at the Roslin Institute, Edinburgh (Appendix A, Figure 2.1). Previous work at the Roslin Institute had established the initial F1 (50:50 Holstein-Friesian cross Charolais) resource herd. This second generation resource herd included approximately 84 Holstein-Friesian backcross (F0 Holstein-Friesian dams crossed with unrelated F1 sires) animals, and approximately 52 Charolais backcross animals (F1 dams crossed with unrelated F0 Charolais sires). Of the remaining animals, 172 were F2 Holstein-Friesian:Charolais (50:50) crosses and 11 had unconfirmed sire data. There were a total of eight F1 sires, which were coded; RO1, RO2, R05, R11, R12, R15, R19 and R21, and four F0 Charolais sires which were coded; CH108, CH158, CH200 and CH219. This resource herd had previously been established to identify QTL associated with commercially relevant traits in cattle such as meat quality, growth rate, carcass development and included traits relevant to disease resistance or susceptibility. This genetic cross represented extremes of dairy breeds (Holstein–Friesian) and beef breeds (Charolais), and was used to obtain the maximum potential for the detection of relevant QTL within the second generation population when compared to a pure-bred population for both QTL and marker loci. This was designed to achieve as much relevant information as possible from a relatively small population of animals. Selection between breeds can achieve dramatic and rapid genetic change when there are large genetic differences between populations in characteristics of economic importance such as milk production for dairy breeds or growth rate for beef breeds (Simm, 1998).

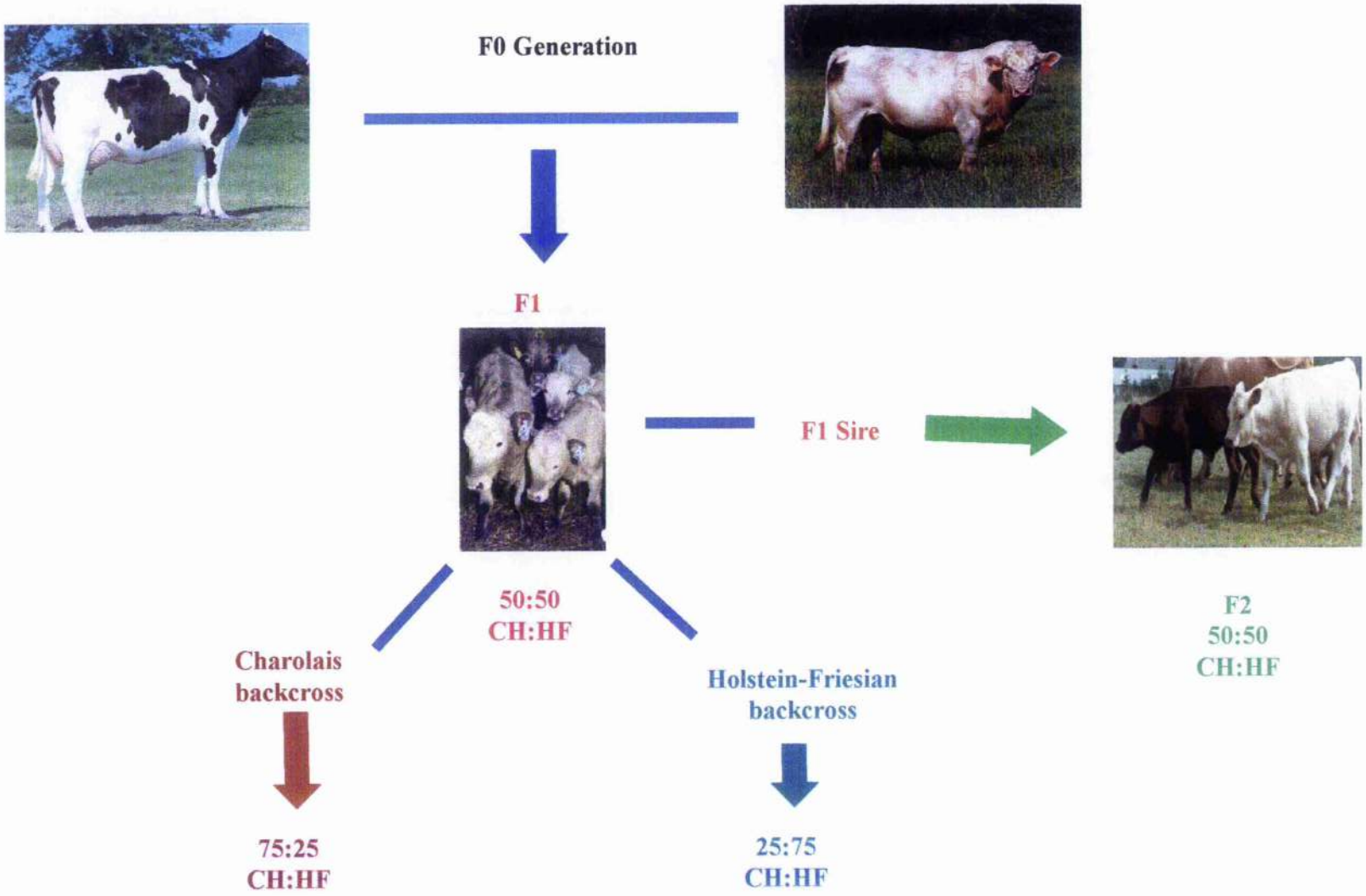


Figure 2.1: Diagrammatic representation of the herd structure. HF = Holstein-Friesian, CH = Charolais

2.2 *In vitro* peripheral blood mononuclear cell proliferation

2.2.1 Experimental animals sampled at approximately six months of age

For this part of the study all male and female second generation calves (Table 2.1), were sampled, at approximately six months of age to determine their *in vitro* PBMC proliferation induced by *S. aureus* NCTC 13047 and PHA (Sigma Aldrich Limited, Poole, Dorset, United Kingdom). This strain of *S. aureus* was originally isolated from a naturally occurring case of subclinical mastitis from a Holstein-Friesian cow (Young, 1997). Approximately three months after the initial sampling, approximately 35% of the total number of second generation animals were re-sampled for measurement of inter-assay repeatability over the three years of the study (Table 2.1)

2.2.2 Experimental animals sampled over 12 months of age

The females were followed throughout the three years of the project at approximately 12 month intervals i.e. the 22 females born in the 1998 cohort of sampling were subsequently sampled at 18 and 30 months of age (Table 2.2). The females from the 1999 cohort were also sampled at 18 months of age (Table 2.2). The male calves were slaughtered at approximately 500kg for the assessment of traits such as meat quality and carcass development.

YEAR OF BIRTH	FEMALES SAMPLED	
	≅18 MONTHS OLD	≅30 MONTHS OLD
1998 Cohort	22	22
1999 Cohort	57	NA

Table 2.2: Females sampled over 12 months of age for assessment of *in vitro* PBMC proliferation. NA=No animals of that age in the study in that year. Column two shows the females sampled at approximately 18 months of age and column three shows the females sampled at approximately 30 months of age.

2.2.3 Antigen

Before the assessment of *in vitro* PBMC proliferation could be carried out in the study, accurate identification of the *S. aureus* NCTC 13047 strain was undertaken. Methods to identify phenotypic traits such as enterotoxin production in conjunction with genotypic identification by restriction enzyme fragmentation pattern (REFP) analysis were used to confirm the Staphylococcus strain type used was NCTC 13047. Optimisation of the concentration of formalin-fixed *S. aureus* used to induce maximal *in vitro* proliferation of PBMC in the assay was also carried out.

2.2.3.1 Confirmation of *Staphylococcus aureus* NCTC 13047 strain type by restriction enzyme fragmentation pattern analysis

Purification of DNA was carried using a method described by Young *et al.* (2001). Briefly, *S. aureus* inoculated brain heart infusion (BHI, Oxoid Limited, Basingstoke, UK) broths were centrifuged (4480g for 10 minutes) and resuspended in 3 x 1ml volumes of Tris ethylene diamine tetra acetic acid (EDTA) sodium chloride buffer (TES). After centrifugation (13,800g for 30 seconds) pellets were resuspended in 200µl TES (50mM sucrose) containing 20µl lysostaphin (1000 units/ml, Sigma Limited, Poole, Dorset, UK) and 100µl lysozyme (40mg/ml, Sigma Limited), vortexed thoroughly and incubated at 37°C. Standard DNA isolation and purification was followed as described by Platt *et al.* (1995), samples were finally microcentrifuged (13,800g for 10 minutes) and resuspended in 60µl TE (10mM Tris base, 1mM disodium EDTA (pH8.0)). Restriction digestion of each sample using restriction enzyme *Hha* I (Gibco Life Technologies Limited, Paisley) was carried out, with bacteriophage λ DNA digested with *Hha* I, *Kpn* I and *Pst* I as controls. Restriction products were electrophoresed in a 0.8% agarose gel at 25mA overnight and stained using ethidium bromide (0.5-1.0µg/ml) before being photographed on a transilluminator at 302nm using Polaroid film type 655.

Confirmation of strain type was carried out using a commercially available programme (Platt and Sullivan, 1992), with λ DNA that had been digested using restriction enzymes *Kpn* I and *Pst* I as calibration controls. Restriction fingerprints were compared using a coefficient of similarity following digestion with the same restriction enzyme (Dice, 1945). The molecular weight of the control fragments was fitted to a robust modified hyperbola, from which the size of restriction fragments in adjacent tracks was estimated. Numerical values were stored for subsequent graphical output which was on a logarithmic scale, the experimental variation in fragment size did not exceed 5% (Plikaytis *et al.*, 1986).

2.2.3.2 Determination of enterotoxin production by *Staphylococcus aureus* NCTC 13047

Enterotoxin production was determined for enterotoxins A, B, C and D using the Staphylococcal enterotoxin rapid passive latex agglutination (SET-RPLA) test kit (Oxoid Limited). Briefly, 10ml of tryptone soya broth (Oxoid Limited) was inoculated aseptically with a single colony of *S. aureus* NCTC 13047 and incubated at 37°C for 24 hours. The sample was then centrifuged at 900g for 20 minutes at 4°C. Following centrifugation, 25 µl of the supernatant was added to the first row of wells in a 96-well plate as indicated by the protocol and doubling dilutions of the sample were carried out across the plate. A duplicate plate containing toxin controls was set up to aid in the interpretation of positive results. Levels of enterotoxin production were determined following 24 hours of incubation without agitation at room temperature by assessment of the levels of agglutination within the wells.

2.2.3.3 Determination of Staphylococcal toxic shock syndrome toxin production by *Staphylococcus aureus* NCTC 13047

Staphylococcal toxic shock syndrome toxin (TSST-1) production was determined using the toxic shock syndrome toxin rapid passive latex agglutination (TST-RPLA) test kit (Oxoid Limited). Briefly, 10ml of brain heart infusion (BHI) broth (Oxoid Limited) was inoculated aseptically with a single colony of *S. aureus* NCTC 13047 and incubated at 37°C for 24 hours. The sample was then centrifuged at 900g for 20

minutes at 4°C. Following centrifugation, 25 µl of the supernatant was added to the first and second row of each well in a 96-well plate as indicated by the protocol and doubling dilutions of the sample were carried out from the second row across the plate. A duplicate plate containing a TSST-1 control was set up to aid in the interpretation of positive results. Levels of TSST-1 production were determined following 24 hours of incubation without agitation at room temperature by assessment of the levels of agglutination within the wells.

2.2.3.4 Preparation of formalin fixed *Staphylococcus aureus* NCTC 13047

Ten millilitres of BHI broth (Oxoid Limited) was inoculated aseptically with a single colony of *S. aureus* NCTC 13047 and incubated at 37°C for 24 hours. Following incubation, sheep blood agar (Oxoid Limited) streak plates were prepared to confirm purity of the culture and to allow single colonies to be used for the inoculation of 4 x 100ml cultures of *S. aureus* NCTC 13047 which were then incubated at 37°C for 24 hours. Following incubation, the bacterial suspension was centrifuged at 1100g for 10 minutes at 20°C. The supernatant was decanted and the cell pellet was resuspended in sterile PBS and the wash step was repeated a further three times. Finally, the cell pellet was resuspended in 2% formalin (vol/vol) in PBS and incubated with continuous stirring at 4°C for 72 hours. After formalin-fixing the sample was washed three times as described above. Finally the cell pellet was resuspended in 50ml sterile PBS and stored at -20°C. Before storing, aliquots were removed: one aliquot was used to determine the concentration by counting the number of bacteria per millilitre of cell suspension of the formalin-fixed suspension in a Neubaeur haemocytometer; the second aliquot was streaked onto sheep blood agar and incubated at 37°C for 96 hours to confirm that formalin fixing had been successful.

In order to assess the *S. aureus*-induced *in vitro* PBMC proliferation in the second generation of cattle over the three years of the project accurately, a single concentrated batch of formalin-fixed *S. aureus* NCTC 13047 was prepared. Several batches of formalin-fixed *S. aureus* NCTC 13047 were prepared before a sufficiently

concentrated batch was obtained. The stock concentration of the batch used in the study was 1.81 x 10^{10} formalin-fixed *S. aureus* per millilitre.

2.2.4 Preparation of autologous serum for *in vitro* culture of peripheral blood mononuclear cells

Twenty millilitres of blood was collected from each animal aseptically by jugular venepuncture into a 'plain' vacutainer (Becton Dickinson, Cowley, Oxford, UK). The samples were incubated at 37°C for 30 minutes and then at 4°C for 30 minutes to aid the clot retraction. Following incubation, the samples were centrifuged at 1300g for 15 minutes at 20°C, the serum fraction was then removed by pasteur pipette and transferred to a sterile 7ml bijoux. The serum samples were then heat inactivated for 30 minutes at 56°C, and subsequently stored at -20°C until required.

2.2.5 Preparation of 'complete' basal medium Eagles

Basal media, Eagles (BME, Gibco, Life Technologies Ltd., Paisley) with Earles salts and without L-glutamine (Gibco) was supplemented with 2mM L-glutamine (Gibco), 20mM Hepes (Gibco), 50µg/50units/ml of streptomycin/penicillin (Gibco) and then stored at 4°C until required.

2.2.6 Isolation of peripheral blood mononuclear cells

Twenty millilitres of anti-coagulated (heparin vacutainer, 143USP units, Becton Dickinson) blood was collected from each animal, aseptically by jugular venepuncture, and transferred to a sterile 50ml tube (Greiner, Stonehouse, Gloucestershire, United Kingdom). The sample was then diluted with 10ml of Hanks Balanced Salt Solution without calcium or magnesium (HBSS, Sigma), and underlayed with 20ml of Histopaque 1077 (Sigma). The sample was centrifuged, without the brake to prevent disruption of the density gradient, at 700g for 40

minutes at 20°C. Following centrifugation, the buffy coat containing the PBMC was removed and transferred to a fresh 50ml tube. The sample was then diluted with HBSS to give a final volume of 50ml and centrifuged at 400g for 10 minutes at 20°C. The supernatant was discarded and the cell pellet was resuspended in HBSS and the wash step was repeated a further three times. Finally, the supernatant was discarded and the cell pellet was resuspended in two millilitres of 'Complete' Basal Medium Eagles (BME, Gibco). Peripheral blood mononuclear cells were counted using a Neubaeur haemocytometer and adjusted to give a final concentration of 2.0 x 10^6 cells/ml/per well. This cell concentration was previously optimised for *in vitro S. aureus*-induced cell proliferation (Logan 2001).

2.2.6.1 *In vitro* peripheral blood mononuclear cell proliferation

One millilitre of PBMC suspension (4.0 x 10^6 cells) and 1ml of either formalin-fixed *S. aureus* NCTC13047 (2.0 x 10^8 cells) or mitogen PHA (5µg per millilitre, Sigma) were added to each well of a 24 well flat bottom plate (Bibby Sterilin, Staffordshire, United Kingdom) that contained 40µl heat inactivated autologous serum. Peripheral blood mononuclear cell cultures were incubated at 37°C with 5% CO_2 over a 10 day period.

2.2.6.2 Assessment of peripheral blood mononuclear cell proliferation by incorporation of ^{3}H - Thymidine

Following thorough mixing of individual PBMC culture wells in the 24 well plates, 100µl aliquots were removed in triplicate into a 96 well round bottomed plate (Bibby Sterilin), and 'pulsed' with 1µC per well of ^{3}H-Thymidine (Amersham Pharmacia Biotech UK Limited, Amersham Place, Buckinghamshire, UK) on days two, three, four, five, nine, and ten of culture. Ninety six well plates were incubated at 37°C with 5% CO_2 for six hours. Following incubation, 96 well plates were removed and stored at -20°C until harvesting.

Ninety-six well plates were 'harvested' using a Filtermat 196™ Cell Harvester (Canberra Packard). Ninety-six well filter plates (Canberra Packard, Pangbourne, Berkshire, United Kingdom) were washed three times with deionised water before the PBMC were harvested onto the filter. The 96 well plate was washed a further five times to ensure the complete removal of the PBMC from the 96 well plate onto the filter and the filter plates were dried at 55°C for approximately 60 minutes. Scintillation fluid was added and the filter plates were counted using a Topcount Microplate Scintillation and Luminescence Counter™ (Canberra Packard). Results were expressed as either counts per minute of ^{3}H-Thymidine incorporation (mean of the triplicate) or as stimulation indices (SI) which were calculated by dividing the test well (PBMC plus antigen or mitogen) mean counts per minute by the control well (PBMC in the absence of antigen or mitogen) mean counts per minute as an indicator of *in vitro* PBMC proliferation. Where stated mean values are expressed as Mean ± Standard Error of the Mean (S.E.M.).

2.2.7 Optimisation of *in vitro* proliferative assay

Optimum concentrations of both *S. aureus* NCTC 13047 and PHA were determined for use in the *in vitro* PBMC proliferation assay. Optimum concentrations were calculated by running preliminary assays over a wide range of concentrations for both *S. aureus* NCTC 13047 and PHA. Optimum concentrations were confirmed by repeating the assay on the same individuals on at least one occasion.

Preliminary assays to determine the optimum dilution of the formalin-fixed *S. aureus* in induction of *in vitro* PBMC proliferation were carried out using a range of concentrations between 0.5×10^{7} - 5.0×10^{8} formalin-fixed *S. aureus* per millilitre. This was assessed in five individual second generation animals. The *S. aureus*–induced response was assessed on days two, four, five, six, seven and nine of *in vitro* culture. The optimisation was repeated using the same animals and conditions with a narrower range of concentrations of between $0.5\text{-}1.5 \times 10^{8}$ *S. aureus* per millilitre.

A range of concentrations of PHA of between 1µg-50µg per millilitre were assessed in three individual animals to determine the optimum PHA-induced *in vitro* PBMC proliferation. Phytohaemagglutinin-induced response was assessed on days one, two, three, four and nine of *in vitro* culture.

2.2.8 Major histocompatibility complex *DRB3* allele restriction fragmentation length polymorphism pattern identification in females from 1999 cohort.

Identification of the MHC *DRB3* sequence and RFLP pattern for the females from the 1999 cohort was carried out by Despoina Miltiadou of the Roslin Institute. Despoina Miltiadou kindly gave permission for the data to be used to highlight the existence of any possible association with high or low *S. aureus*-induced or PHA-induced *in vitro* PBMC proliferation and MHC *DRB* allelle pattern identified by RFLP pattern. Briefly, MHC *DRB3* allele patterns generated following PCR amplification and restriction digestion with *Rsa* I, *Hae* III and *Bst* YI were compared with previously published data, which were already attributed to sequenced alleles by prediction of restriction sites (Van Eijk *et al.*, 1992 (a); Gelhaus *et al.*, 1995).

2.3 Polymorphonuclear cell phagocytosis

2.3.1 Experimental animals

The study involved sampling a subset of the second generation Holstein-Friesian cross Charolais animals at the Roslin Institute, Edinburgh as described previously (Section 2.1). All animals in the 2000 cohort were sampled at approximately six months of age for this part of the study

The preliminary optimisation and development of the assay involved using Holstein-Friesian cattle under home office licence at Cochno Farm, University of Glasgow Veterinary School. The use of these Holstein-Friesian cattle was undertaken when sampling of the Robogene herd was prevented temporarily.

2.3.2 Isolation of peripheral blood polymorphonuclear cells

Forty millilitres of anti-coagulated (EDTA, 15%, Becton and Dickinson) blood was collected aseptically from each animal by jugular venepuncture and transferred to a sterile 50ml tube. The samples were centrifuged at 1500g for 10 minutes at 20°C. Following centrifugation, the plasma, buffy coat and upper layer of red blood cells were discarded. The sample was made up to a final volume of 50ml with erythrocyte lysis solution (ACE, 0.15M ammonium chloride, 7 x 10^{-4}M potassium dihydrogen phosphate and 0.003M Trisodium EDTA), inverted several times to ensure thorough mixing, and centrifuged at 500g for five minutes at 20°C. Following centrifugation, the supernatant was discarded and the wash step was repeated with 30ml of the erythrocyte lysis solution. The supernatant was again discarded and the cell pellet was resuspended in 30ml PBS (Sigma). The sample was layered onto 20ml Histopaque 1077 (Sigma) and centrifuged at 1100g for 25 minutes at 20°C without the brake, to prevent the disruption of the density gradient. Following centrifugation the supernatant and the interface were discarded to leave only the cell pellet. The

cell pellet was resuspended in 50ml PBS and centrifuged at 500g for three minutes at 20°C. The PBS wash step was repeated a further three times before the sample was finally resuspended in 1ml RPMI 1640 (Sigma) containing 5% heat inactivated adult bovine serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK). Isolated cell populations were counted using a Neubauer haemocytometer and adjusted to give a final concentration of 0.5 x 10^6 cells per millilitre.

2.3.3 Assessment of *in vitro* phagocytosis in the isolated polymorphonuclear cell population

2.3.3.1 *In vitro* phagocytosis of FITC-labelled latex beads in the isolated polymorphonuclear cell population

Development and optimisation of the *in vitro* phagocytosis of FITC-labelled latex beads involved the assessment of various factors that may affect the assay. The addition of heat inactivated fetal calf serum, heat inactivated adult bovine serum or heat inactivated autologous serum to the media was compared. The phagocytosis of a range of sizes of FITC-labelled latex beads, from 0.1μM to 2.0μM, was also compared. The effect of different PMN:FITC-labelled latex bead ratios was assessed. Another aspect of the development was the assessment of a range of time points, from 0 hours to 20 hours, to determine the most relevant time points to measure phagocytosis. Levels of non-specific adherence of the FITC-labelled latex beads to the PMN cell surface were determined by running a duplicate set of samples for each individual animal at 4°C. Incubating a duplicate sample at 4°C has previously been shown by flow cytometry and scanning electron micrograph to be an indicator of non-specific adherence to the PMN cell surface in humans, as phagocytosis was shown to be inhibited by low temperatures (Santos *et al.*, 1995).

2.3.3.2 Assessment of *in vitro* phagocytosis of FITC-labelled latex beads in the isolated polymorphonuclear cell population

Isolated bovine PMN populations were incubated at 37°C in 5% CO_2 with 2μM FITC-labelled latex beads (Sigma Limited) at a ratio of 1:50 (isolated PMN:FITC-labelled latex beads) with RPMI 1640 containing 5% (vol/vol) heat inactivated adult bovine serum. Duplicate samples were incubated at 4°C. Aliquots from both the 4°C and 37°C samples were removed at 0 minutes, 1 hour, 6 hours and 16 hours after the start of the incubation and the reaction was terminated by the addition of 1ml ice cold 0.4% paraformaldehyde. Following incubation at 20°C for one minute, the samples were centrifuged at 250g for three minutes at 4°C, the samples were then centrifuged and the supernatant was discarded, the cells were washed in ice cold PBS and centrifuged at 250g for three minutes at 4°C. The wash step was repeated a further three times, before the sample was finally resuspended in 0.5ml 1% paraformaldehyde and stored at 4°C and analysed by flow cytometry within one week.

2.3.3.3 Flow cytometry

Flow cytometry was carried using a Coulter EPICS ELITE™ flow cytometer (Becton Dickinson). The cell suspension was passed through the flow cytometer to assess the phagocytosis of FITC-labelled (2.0μM) latex beads or FITC-labelled *S. aureus*. Output was visualised using multiple graphs. Graphs of side scatter versus forward scatter were used to assess the granularity of the cells and the size of the cells respectively. A gate was set on the side scatter versus forward scatter graph to segregate the PMN population. The PMN population was identified as a population containing relatively large, granular cells whereas, the mononuclear cell (MNC) population was a smaller, less granular cell population. A total of 10,000 cells were counted within the PMN gate. Individual gates were set for the *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by PMN in whole blood (section 2.3.5) to

differentiate between the PMN population and the mononuclear cell population. Again, a total of 10,000 cells were counted within the PMN gate.

Linear graphs of fluorescence versus the number of cells were used to assess the levels of fluorescence in the cell populations as an indicator of phagocytosis of FITC-labelled (2.0μM) latex beads. Gates were set on the linear graphs to correspond to the visible peaks of fluorescence which indicated the phagocytosis of one, two, or greater than two FITC-labelled (2.0μM) latex beads (Santos *et al.*, 1995). Logarithmic graphs were used to assess the levels of fluorescence in the cell populations as an indicator of phagocytosis of FITC-labelled *S. aureus*. Gates were set on the logarithmic graphs to correspond to the visible peaks of fluorescence for the phagocytosis of FITC-labelled *S. aureus*. An individual gate was set to monitor the cell population which showed no visible levels of fluorescence and was presumed to not have phagocytosed any FITC-labelled latex beads or FITC-labelled *S. aureus* during the assay.

2.3.4 *In vitro* phagocytosis of FITC-labelled *Staphylococcus aureus* in the isolated polymorphonuclear cell population

2.3.4.1 Experimental animals

A small subset of approximately 20 second generation animals were sampled for this part of the study.

2.3.4.2 Assessment of *in vitro* phagocytosis of FITC-labelled *Staphylococcus aureus* in the isolated polymorphonuclear cell population

Isolation of the PMN population was carried out as described above (Section 2.3.2). The isolated PMN population was incubated at a PMN:FITC-labelled *S. aureus* at a

ratio of 1:50 and incubated under identical conditions as described above for the FITC labelled latex beads (Section 2.3.3.2).

2.3.5 *In vitro* phagocytosis of FITC labelled latex beads by polymorphonuclear cells in whole blood

2.3.5.1 Experimental animals

A small subset of approximately 20 second generation animals were sampled for this part of the study.

2.3.5.2 Assessment of *in vitro* phagocytosis of FITC-labelled latex beads by polymorphonuclear cells in whole blood

The original method used for this part of the work was obtained by personal communication with Dr E. Glass at the Roslin Institute, Roslin, Midlothian. Further development and optimisation was carried out before use. Briefly, 10ml of anti-coagulated (heparin vacutainer, 143 USP units, Becton Dickinson) blood was collected from each animal aseptically by jugular venepuncture. The total number of white cells were calculated per 100µl of whole blood with white cell counting fluid used as a diluent (<0.1µg crystal violet in 2% (vol/vol) acetic acid). The approximate number of PMN per 100 µl was estimated using reference ranges (Radostits *et al.*, 1994) and 100µl of whole blood was added to each Falcon 2054 tube (Becton Dickinson). FITC-labelled latex beads (2.0µM) were added to give a PMN:FITC-labelled latex bead ratio of 1:50, the samples were vortexed for a few seconds and then incubated as described above (Section 2.3.3.2) at 37°C in 5% CO_2 with duplicate samples at 4°C. Following incubation, the samples were placed immediately on ice at 4°C, 1ml of a 1:10 (v/v) FACS Lyse solution (Becton Dickinson) was added to lyse the red blood cells present, the sample was then mixed thoroughly and incubated on ice for a further 10 minutes. Samples were then

centrifuged at 200g for five minutes at 4°C, the supernatant was removed and the lysis step was repeated. Following the second lysis the cell pellet was resuspended in 3ml of cell wash solution (Becton Dickinson) and centrifuged at 200g for 5 minutes at 4°C. This wash step was repeated a further 3 times. Finally, the cell pellet was resuspended with 1% paraformaldehyde in cell wash solution and stored at 4°C and analysed by flow cytometry within one week (Section 2.3.3.3).

2.4 Data exploration and analyses

2.4.1 Distribution of the data

Preliminary analyses of all raw data involved the use of a univariate analysis procedure using the statistical package SAS (version 6.12, USA). The univariate analysis procedure allowed the assessment of the distribution of the raw data by various graphical techniques, normal probability plots, box and whisker plots and histograms. The statistical calculations obtained using the univariate analysis procedure included the number of observations (n), the mean, standard deviation, the skewness and the kurtosis of the data sets. Quantile ranges were obtained for the box and whisker plots and extreme data values were also identified with the univariate analysis procedure.

2.4.2 Box and Cox transformation

Further preliminary data analysis was carried out to determine the optimal methods for transformation of the data to obtain a normal distribution. The Box and Cox analysis was carried out using Minitab (Release 13). Box and Cox analysis estimates an optimal transformation of the data by estimation of a lambda value (λ) which corresponds to a recognised transformation of the data (Box and Cox, 1964) (Table 2.3).

LAMBDA VALUE (λ)	TRANSFORMATION
2.0	Y^2
1.0	No transformation required
0.5	$\sqrt{Y}$
0	$\log_e Y$
-0.5	$1/\sqrt{(Y)}$
-1.0	$\frac{1}{Y}$

Table 2.3: Examples of Box and Cox transformation λ values and the optimal transformations to obtain a data set which would fit a normal distribution. Y=value.

2.4.3 Correlations

2.4.3.1 Correlation of individual animal responses between days of culture (peripheral blood mononuclear cell proliferation) or time points (polymorphonuclear cell phagocytosis)

Data which fitted a normal distribution (either without tranformation or following the relevant transformation suggested following preliminary analyses) was assessed for correlation of individual animal responses between days of culture (PBMC proliferation) or time points (PMN phagocytosis) using Pearson's Product Moment Correlation Coefficient. The Pearson Product Moment Correlation Coefficient is used to give an estimation of the degree of linear relationship that exists between two variables. The significance level (p value) of the correlation between the two variables was also determined.

2.4.3.2 Correlation of individual animal responses at re-sampling (approximately three months after initial sampling)

The correlation of individual animal responses at re-sampling (approximately three months after initial sampling) was assessed using the Pearson Product Moment Correlation Coefficient as described above (Section 2.4.3) when only two variables were analysed.

2.4.4 Analysis of variance

Analysis of variance (ANOVA) was used to assess any correlation between the *in vitro* PBMC proliferative responses in the females from the 1998 cohort when sampled at approximately six, 18 and 30 months of age. Analysis of variance is used when more than two variables are analysed, and was performed in Minitab (Release 13). Post-hoc analyses was used to indicate which variables, if any, were significantly correlated ($p<0.05$).

2.4.5 Paired *t*-test

Variation between two related data sets i.e. the *S. aureus*-induced *in vitro* PBMC proliferation in the 1999 females in the sampling years 1999 and 2000, was assessed using a two-way paired *t*-test (Minitab, Release 13). A significant *p* value, and 95% confidence intervals which did not pass through zero, indicated that the two population means assessed were significantly different.

2.4.6 Data analysis

2.4.6.1 Linear Mixed Models - Residual Maximum Likelihood

Transformed data was further analysed using linear mixed model analysis with residual maximum likelihood (REML) estimation using the statistical software package Genstat (5th Edition, Numerical Algorithms Group Limited, Wilkinson House, Oxford, UK). The Y variate, fixed effect and random effect model was defined for each parameter i.e. *S. aureus*-induced, PHA-induced and control *in vitro* PBMC proliferation and *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads. The significance of fixed effects in the model were determined by examining the significance of the factors using the Wald Test to identify the significance of the factors when the individual terms were consecutively dropped from the full fixed model. Significance was determined by assessment of the 3-way interactions, followed by 2-way interactions and finally, the fixed factors in the model. All non-significant interactions were removed sequentially from the fixed model until only the significant interactions (if any) and the single factors remained. The significance of random effects in the model were determined by calculating the significance of the −2*log likelihood ratio. Significance of the random factors was determined by assessing the difference between the deviance and degrees of freedom in the model, with and without the random factor included, and determining any significance using

a χ^2 statistical reference table. Both the table of effects and the table of predicted means could be used to identify variation between levels within significant factors, for example, variation between males and females, where a significant effect of sex may have been identified. Analysis was carried out on the complete data set, the males and females together, and on the males and females as single sex subsets.

2.4.6.2 Model used in assessment of *in vitro* peripheral blood mononuclear cell proliferation

2.4.6.2.1 Complete data set model

For the complete data set, the *S. aureus*-induced, PHA-induced and control proliferation was assessed as the Y variate in the model. The REML model used in the analyses of the complete data set, except in the analyses of the control as the Y variate, where control was removed as a fixed factor was:

Fixed sex + cross + year of birth + sample date + sample age + control
Random sire

2.4.6.2.2 Subset model

The REML model used for the analyses of the subsets of males and females, except in the analyses of the control as the Y variate, where control was removed as a fixed factor was:

Fixed cross + year of birth + sample date + sample age + control
Random sire

2.4.6.3 Model used in assessment of *in vitro* phagocytosis of FITC-labelled (2.0 μM) latex beads in the isolated polymorphonuclear cell population

2.4.6.3.1 Complete data set model

For the complete data set, the percentage total phagocytosis of FITC-labelled (2.0μM) latex beads at 0, 1, 6 or 16 hours incubation **OR** gated populations (i.e. 1 bead, 2 beads or > 2 beads) was examined. The REML model used was:

Fixed	sex + cross + sample date + sample age
Random	sire

2.4.6.3.2 Subset model

The REML model used for the analyses of the subsets of males and females was:

Fixed	cross + sample date + sample age
Random	sire

Chapter 3

Results

3.1 *In vitro* peripheral blood mononuclear cell proliferation

3.1.1 Confirmation of *Staphylococcus aureus* NCTC 13047 strain type by restriction enzyme fragmentation pattern analysis

Single colonies of *S. aureus* were analysed by REFP analysis before preparation of the formalin-fixed *S. aureus* NCTC 13047 and optimisation of cell proliferation *in vitro*. *Staphylococcus aureus* NCTC 13047 was identified by comparison with a standard stock strain of *S. aureus* NCTC 13047 which had been identified previously and stored as a graphical computer generated image (Young, 1997). Following digestion of the *S. aureus* with the restriction enzyme *Hha* I and subsequent computer analysis, the *S. aureus* NCTC 13047 strain was confirmed as matching the profile of the standard stock strain with 25 fragments identified, ranging from 2.92 kilobases to 9.59 kilobases in size. A graphical computer generated example of *S. aureus* NCTC 13047 is shown in Figure 3.1, lanes four and six.

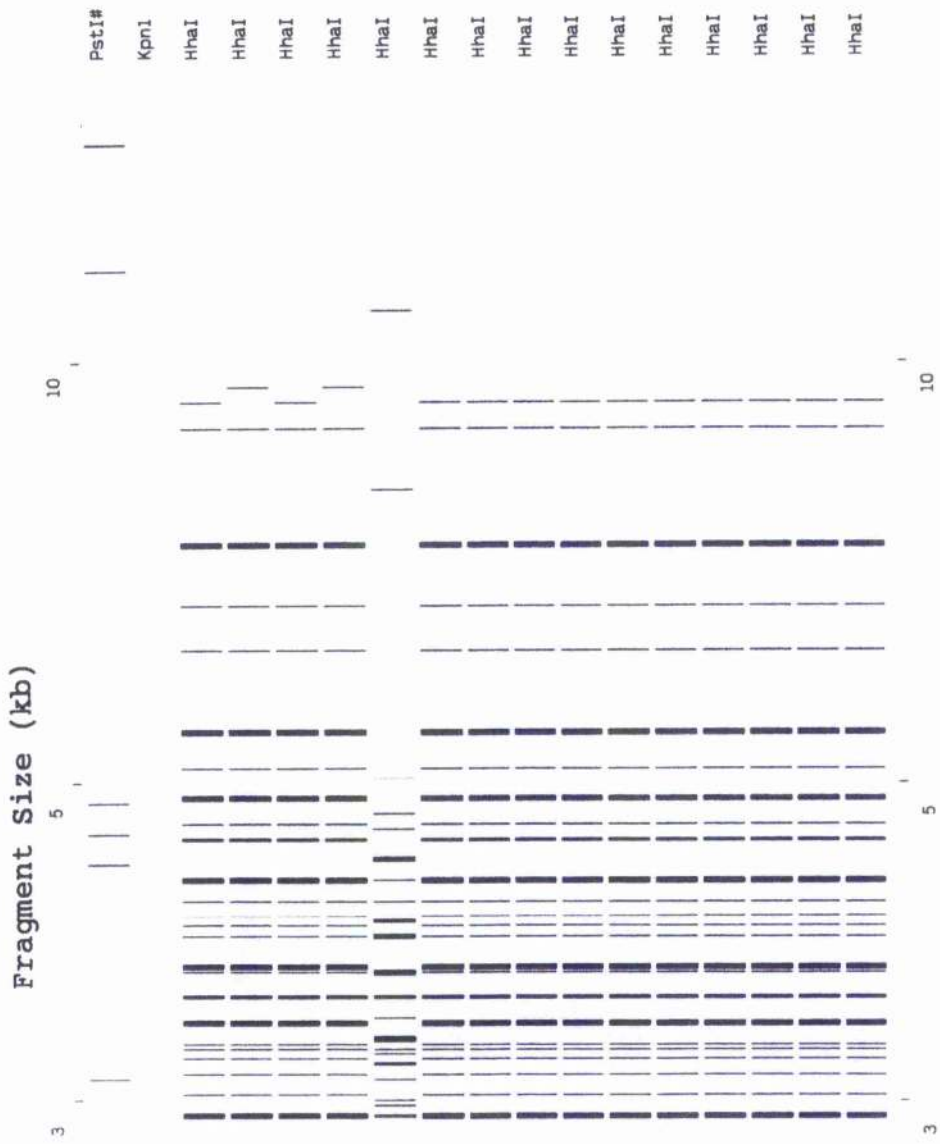


Figure 3.1: Graphical computer generated output showing an example of *S. aureus* NCTC 13047. Lanes one and two show *Pst* I and *Kpn* I digested λ DNA respectively. Lanes four and six show *S. aureus* NCTC 13047. The remaining lanes show two other strains of *S. aureus*, lanes three, five, eight to seventeen show individual colonies from one strain and lane seven shows a different strain.

3.1.2 Determination of enterotoxin production by *Staphylococcus aureus* NCTC 13047

Enterotoxin production by *S. aureus* NCTC 13047 was determined to add to the phenotypic information previously obtained about this strain, including colony morphology and coagulase production. No agglutination was visible in the wells containing *S. aureus* NCTC 13047 supernatant and anti-enterotoxin A sensitised latex particles, anti-enterotoxin B sensitised latex particles or anti-enterotoxin C sensitised latex particles, suggesting that strain NCTC 13047 did not produce enterotoxins A, B or C. Agglutination was seen in the wells containing *S. aureus* NCTC 13047 supernatant and anti-enterotoxin D sensitised latex particles, indicating that this strain produced enterotoxin D.

3.1.3 Determination of Staphylococcal toxic shock syndrome toxin production by *Staphylococcus aureus* NCTC 13047

No agglutination was visible in the wells containing *S. aureus* NCTC 13047 supernatant and anti-TSST-1 sensitised latex particles, indicating that this strain did not produce TSST–1.

3.1.4 Optimisation of *Staphylococcus aureus* NCTC 13047–induced *in vitro* peripheral blood mononuclear cell proliferation

A range of concentrations of *S. aureus* were assessed to determine the *S. aureus*–induced *in vitro* PBMC proliferation in five individual second generation animals. The mean optimal peripheral blood mononuclear cell proliferation was shown to occur at a concentration of 1.5×10^8 *S. aureus* per millilitre (Figure 3.2). At the

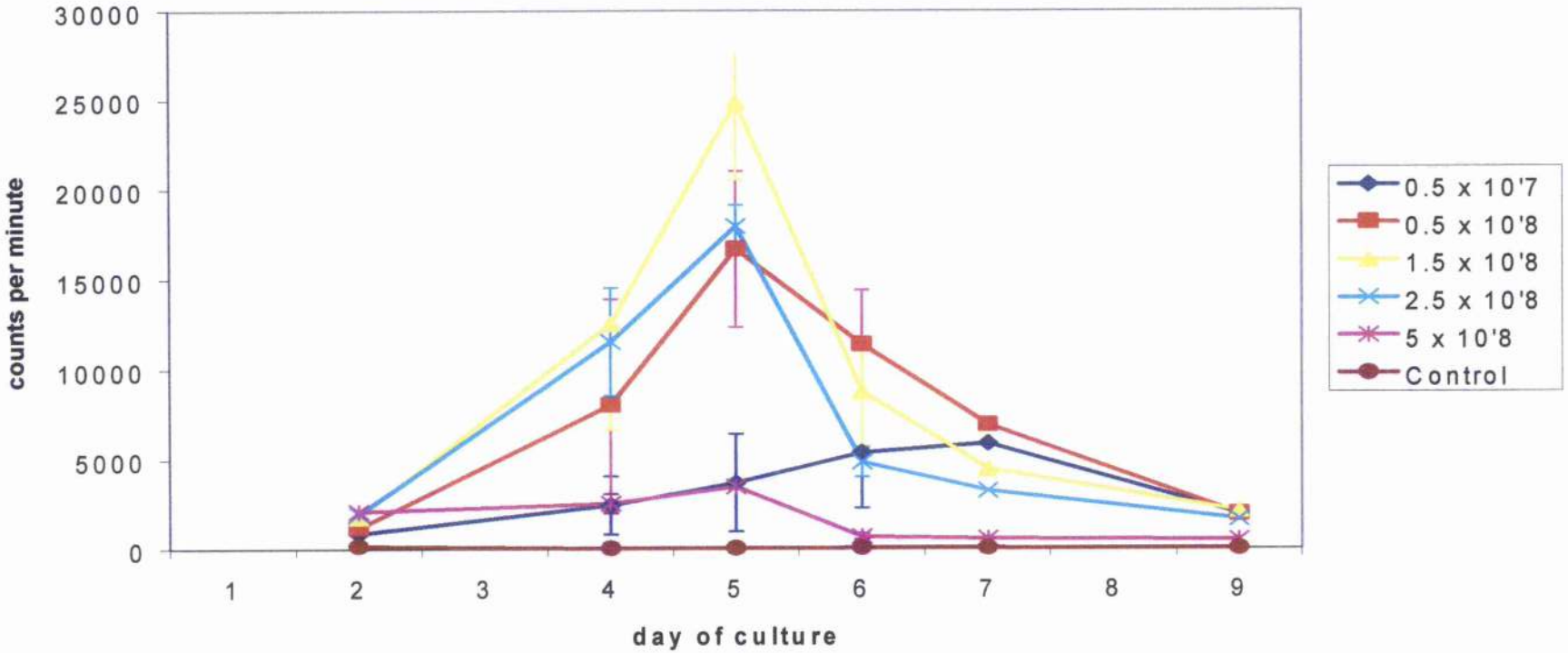


Figure 3.2: Preliminary optimisation of the concentration of formalin–fixed *S. aureus* NCTC 13047 that induced maximal cell proliferation. Each line represents the mean (± S.E.M.) *S. aureus* – induced *in vitro* PBMC proliferation for five individual second generation animals at a range of antigen concentrations from 0.5 x 10^7–5.0 x 10^8 formalin–fixed *S. aureus* per millilitre. Control wells contained PBMC only. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

optimal antigen concentration on day five of culture the mean (± S.E.M.) *S. aureus*–induced proliferation was 24873.1 ± 5799.9 counts per minute. Three of the five individual cattle had an optimal antigen response at a concentration of 1.5 x 10^8 *S. aureus* per millilitre, with the peak response occurring on either day four or day five of culture. The other two individuals had an optimal antigen response at a concentration of 0.5 x 10^8 *S. aureus* per millilitre (data not shown). Control cultures of PBMC, had low levels of proliferation throughout the assay e.g. mean (± S.E.M.) proliferation measured on days two and five of culture were 158.9 ± 76.4 and 42.7 ± 5.9 counts per minute respectively (Figure 3.2).

The optimisation of proliferation was repeated using the same individual animals with a narrower range of concentrations of between 0.5-1.5 x 10^8 *S. aureus* per millilitre. The proliferative response to the antigen at the concentrations 1.0 x 10^8 *S. aureus* per millilitre and 1.5 x 10^8 *S. aureus* per millilitre was similar (Figure 3.3), with means (± S.E.M.) on day four of culture of 68238.9 ± 11342.3 and 72820.6 ± 6252.6 counts per minute, respectively. Three of the five individuals tested had an optimal antigen response at a concentration of 1.0 x 10^8 *S. aureus* per millilitre, with the two remaining animals showing an optimal antigen response at a concentration of 1.5 x 10^8 *S. aureus* per millilitre (data not shown). Control cultures of PBMC, had low levels of proliferation throughout the assay e.g. mean proliferation (± S.E.M.) on days two and four of culture was 644.0 ± 119.6 and 128.1 ± 12.3 counts per minute, respectively. From these preliminary results, 1.0 x 10^8 *S. aureus* per millilitre was chosen as the optimal concentration of antigen and was employed subsequently in the assays.

3.1.5 Optimisation of Phytohaemagglutinin–induced *in vitro* peripheral blood mononuclear cell proliferation

A range of concentrations of PHA were assessed to determine the optimum PHA-induced *in vitro* PBMC proliferation in three individual second generation animals. All three individuals had an optimum response at a PHA concentration of 5µg per

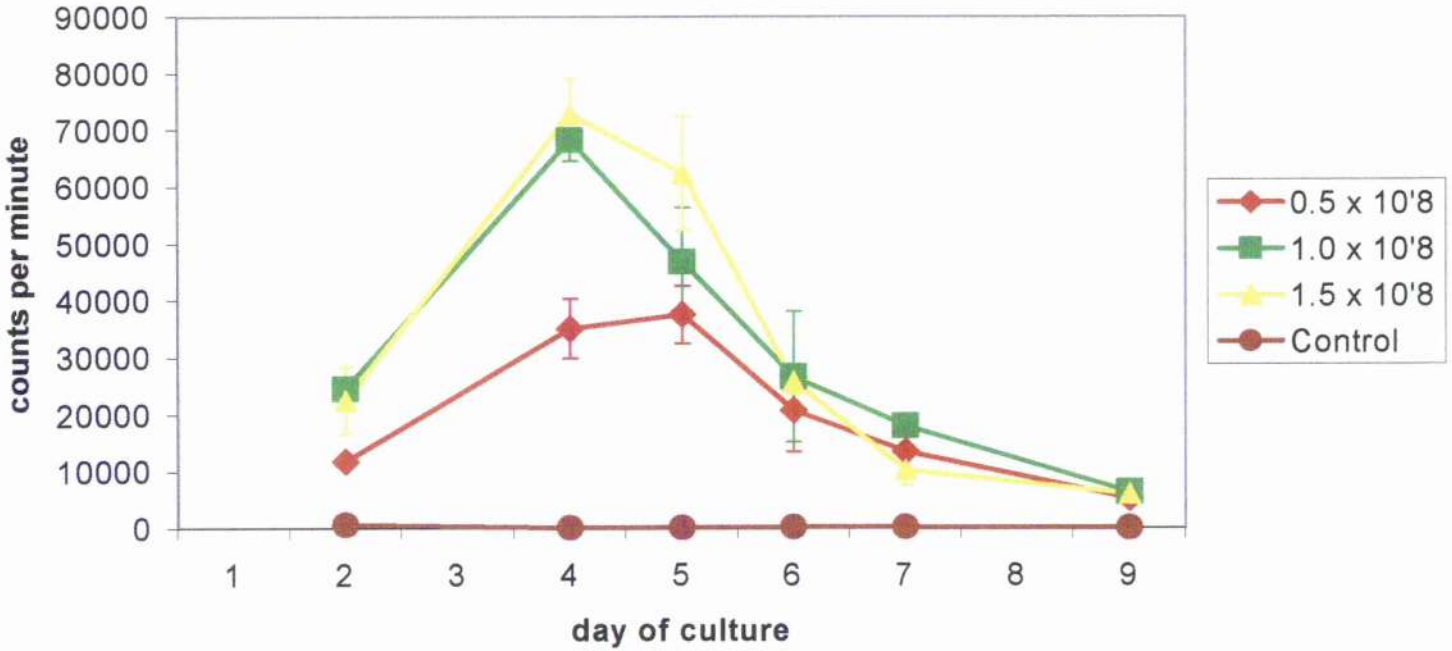


Figure 3.3: Repeat of preliminary optimisation of the concentration of formalin–fixed *S. aureus* NCTC13047 that induced maximal cell proliferation. Each line represents the mean (± S.E.M.) *S. aureus*–induced *in vitro* PBMC proliferation for five individual second generation animals at a range of antigen concentrations from 0.5–1.5 x 10^8 formalin–fixed *S. aureus* per millilitre. Control wells contained PBMC only. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

millilitre. A peak PHA–induced response was shown on day three of culture (Figure 3.4), with a mean (± S.E.M.) of 46476.2 ± 32669.6 counts per minute. A concentration of 5µg per millilitre was used subsequently in all assays. Control culture of PBMC, had low levels of proliferation throughout the assay e.g. mean (± S.E.M.) proliferation on day three of culture was 188.5 ± 35.6 counts per minute (Figure 3.4).

3.2 Assessment of *in vitro* peripheral blood mononuclear cell proliferation in second generation animals.

3.2.1 Experimental animals sampled at approximately six months of age.

The *in vitro S. aureus* and PHA–induced PBMC proliferation was assessed in all animals on one occasion at approximately six months of age. Animals were sampled in batches of approximately 16 per week. Due to the different management and housing regimes that were required for the different sexes under other aspects of the Robogene project, males and females were housed separately. The female calves were housed indoors almost immediately from birth, whereas, the male calves were allowed to remain with their mothers on pasture until they were weaned before winter housing. For this reason, the batches of 16 animals sampled were either all male or all female. Variation in both *S. aureus* and PHA-induced *in vitro* PBMC proliferation was seen among individual animals (Figures 3.5 and 3.6). Variation in *S. aureus* and PHA-induced *in vitro* PBMC proliferation was seen among animals on all days of culture (Figures 3.7 and 3.8, respectively), however, the largest variation among individual animals was seen on the peak days of culture for *S. aureus* and PHA-induced proliferation, i.e. day five and day three, respectively. Mean (± S.E.M.) *S aureus*–induced proliferation was 25835 5 ± 1890.3 on day five of culture

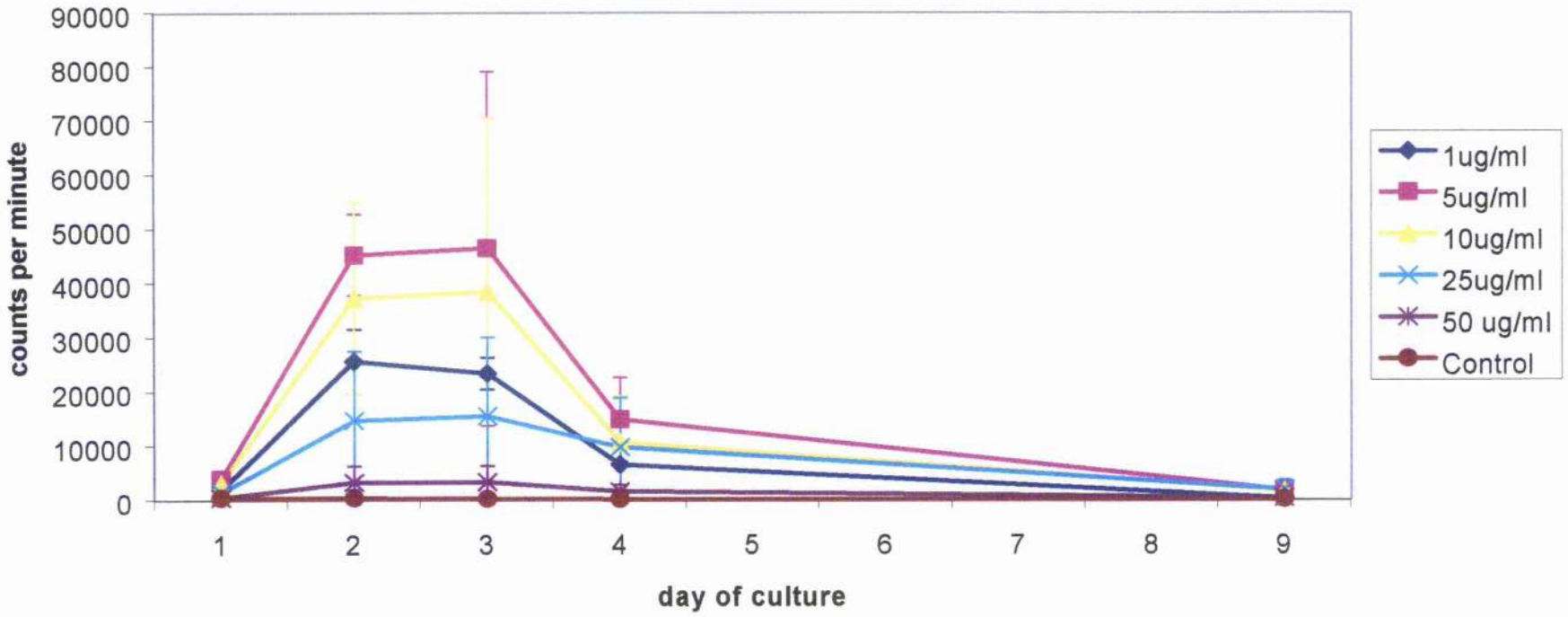


Figure 3.4: Optimisation of the concentration of Phytohaemagglutinin that induced maximal cell proliferation. Each line represents the mean (± S.E.M.) PHA–induced *in vitro* PBMC for three individual second generation animals at a range of mitogen concentrations from 1-50µg/ml. Control wells contained PBMC only. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

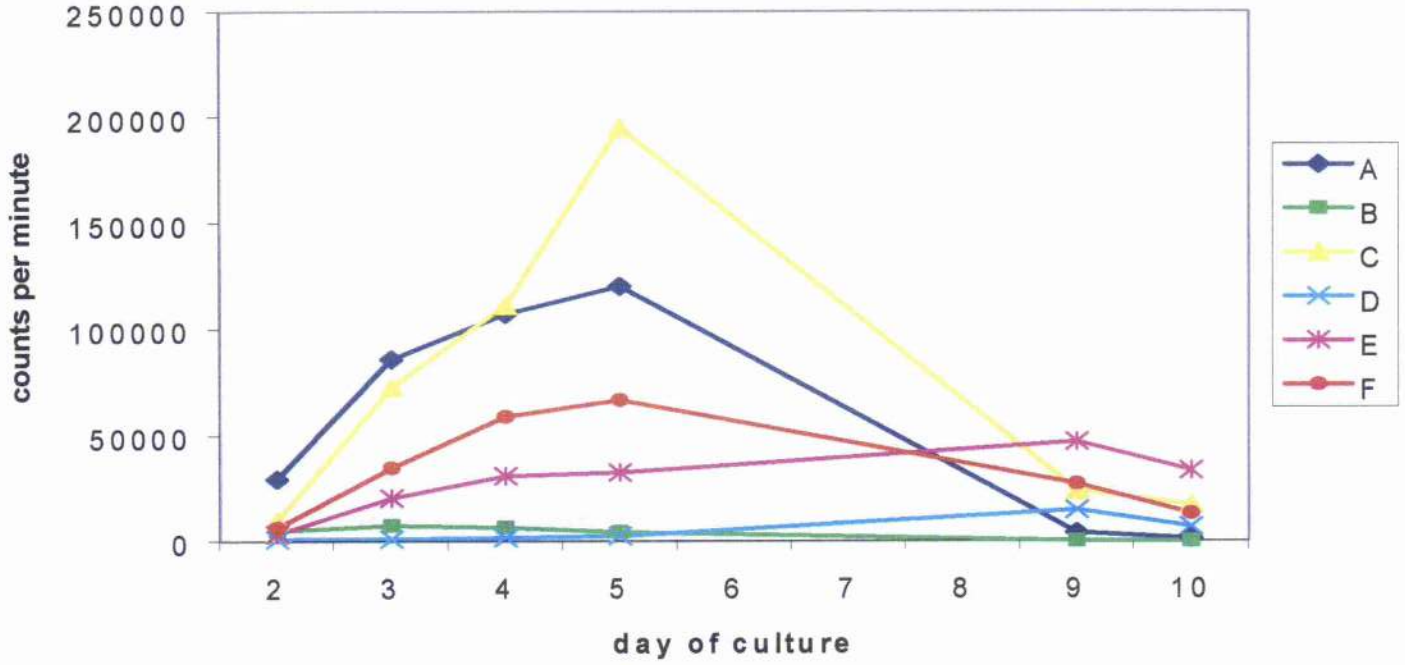


Figure 3.5: Representative example of individual animal variation in *S. aureus*–induced *in vitro* PBMC proliferation in six second generation animals (A-F). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

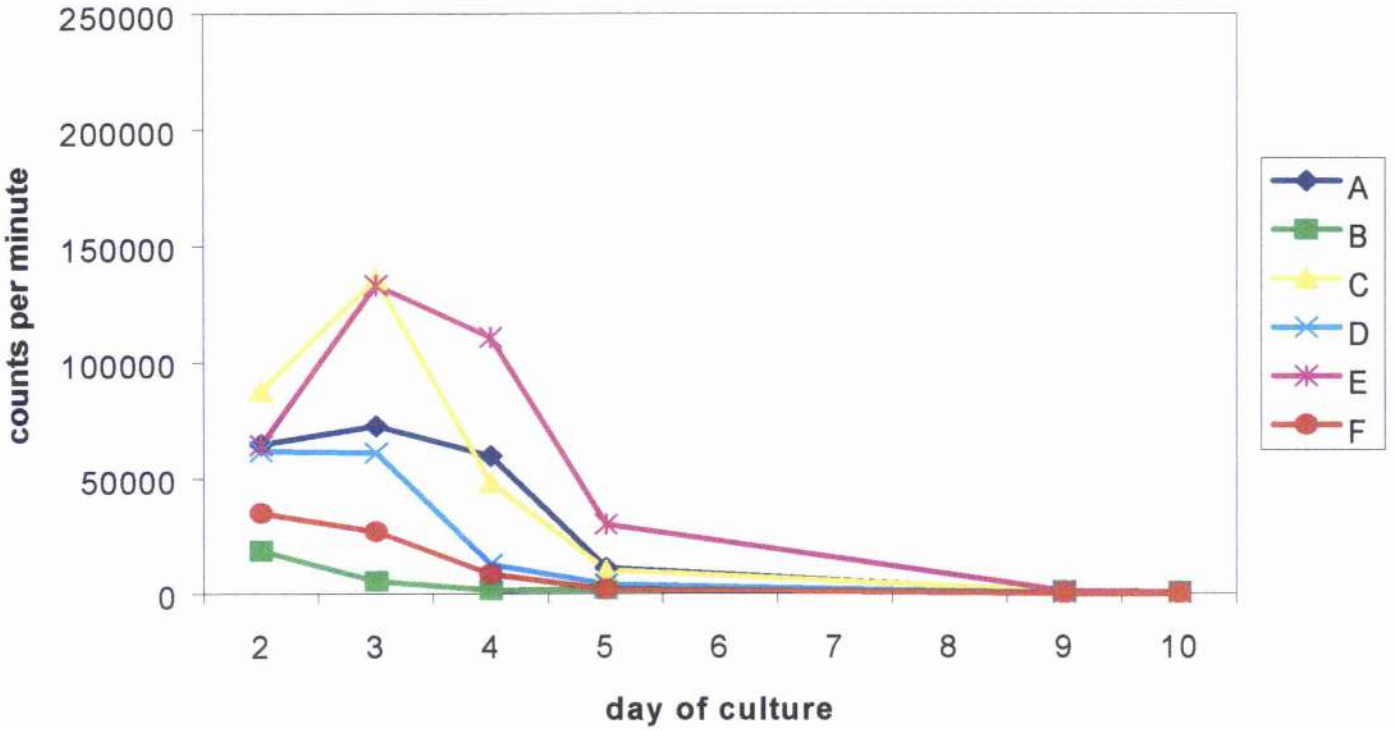


Figure 3.6: Representative example of individual animal variation in Phytohaemagglutinin–induced *in vitro* PBMC proliferation in six second generation animals (A-F). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

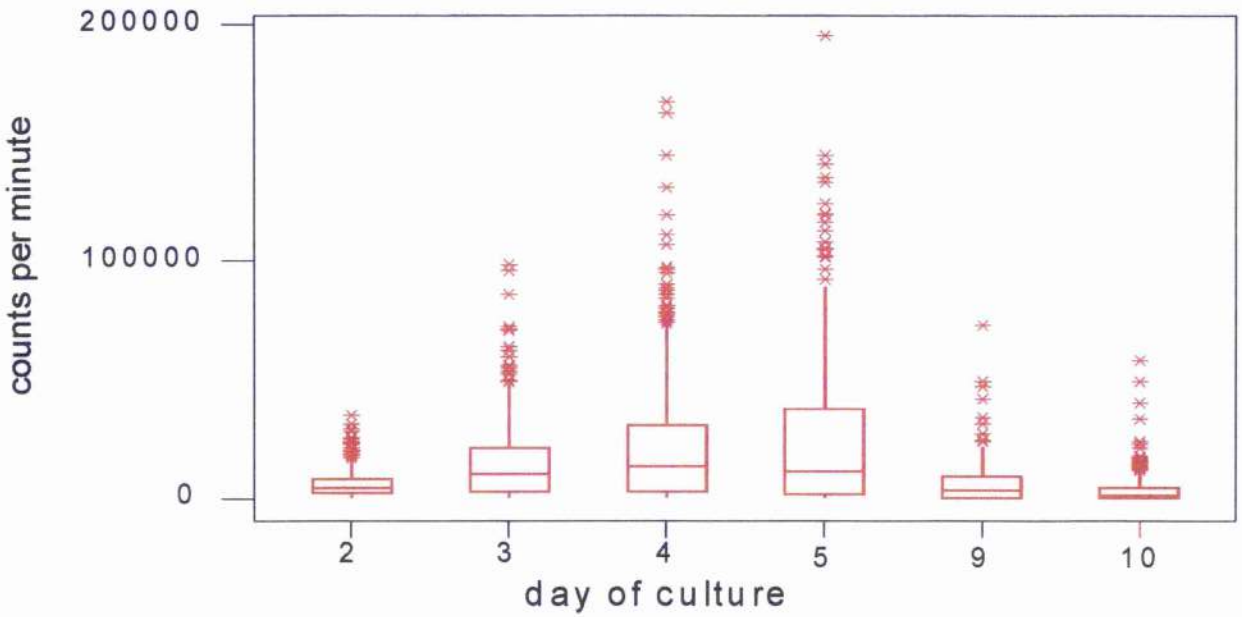


Figure 3.7: *Staphylococcus aureus*–induced *in vitro* PBMC proliferation on each day of culture, showing all second generation animals sampled at approximately six months of age (n=319). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

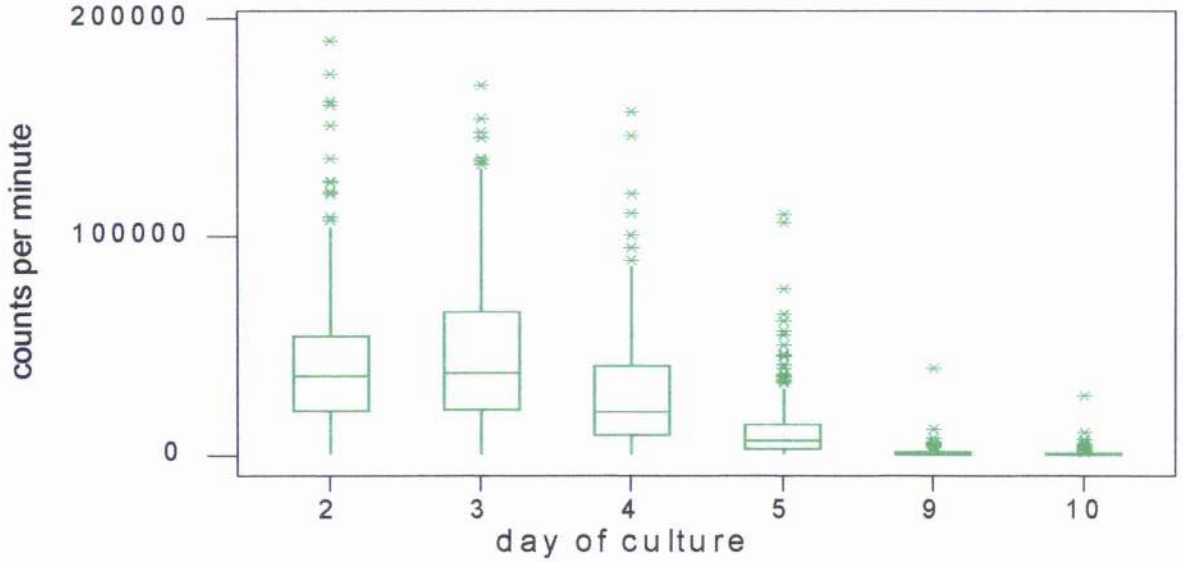


Figure 3.8: Phytohaemagglutinin–induced *in vitro* PBMC proliferation on each day of culture, showing all second generation animals sampled at approximately six months of age (n=319). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

and the mean PHA–induced proliferation was 46247.8 ± 1886.1 on day three of culture. *In vitro* control proliferation of PBMC, was relatively low throughout the assay e.g. mean (± S.E.M.) proliferation on days two and five of culture were 1003.2 ± 2.8 and 730.1 ± 4.8 counts per minute, respectively (Figure 3.9).

Comparison of *S. aureus* and PHA-induced *in vitro* proliferation on individual days of culture allowed observation of the variation in the antigen and mitogen–induced responses and comparison of the kinetics of both responses (Figure 3.10). For example, on day three of culture there was a relatively low level of *S. aureus*-induced proliferation, whereas the PHA-induced proliferation was maximal in most individuals on day three. As the duration of culture increases, a reduction in the PHA-induced proliferation was observed, whereas the *S. aureus*-induced proliferation reached maximal levels of proliferation by day five of culture in most individuals. By day ten of culture, very little cell proliferation was measurable in the presence of either antigen or mitogen (Figure 3.10), due to the large proportion of dead and dying cells in culture at this time point.

3.2.2 Females over 12 months of age

Approximately 22 females born in the 1998 cohort and 57 females in the 1999 cohort were assessed in the subsequent sampling years. Both *S. aureus* and PHA-induced *in vitro* PBMC proliferation was measured.

3.2.2.1 1998 cohort

The mean (± S.E.M.) *S. aureus*–induced proliferation for the 1998 cohort decreased considerably from 1998 to 1999, with only slight variation between 1999 and 2000 (Figure 3.11). On day five of culture there was a significant decrease ($p<0.001$) in the mean (± S.E.M.) *S. aureus*–induced proliferation between 1998 and 1999 from 62550.8 ± 8241.7 counts per minute in 1998 to 18823.1 ± 3214.4 counts per minute in 1999. No significant difference in the mean (± S.E.M.) *S. aureus*–induced proliferation between 1999 and 2000 was identified (Figure 3.11). Variation was,

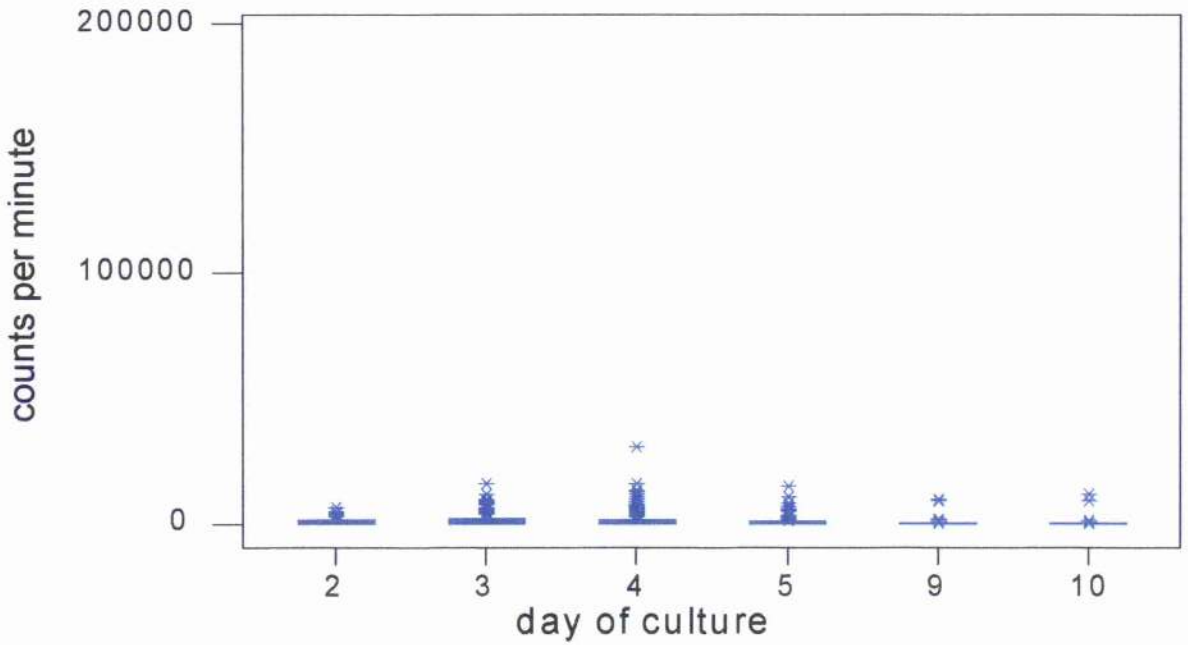


Figure 3.9 *In vitro* control PBMC proliferation on each day of culture, showing all second generation animals sampled at approximately six months of age (n=319). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

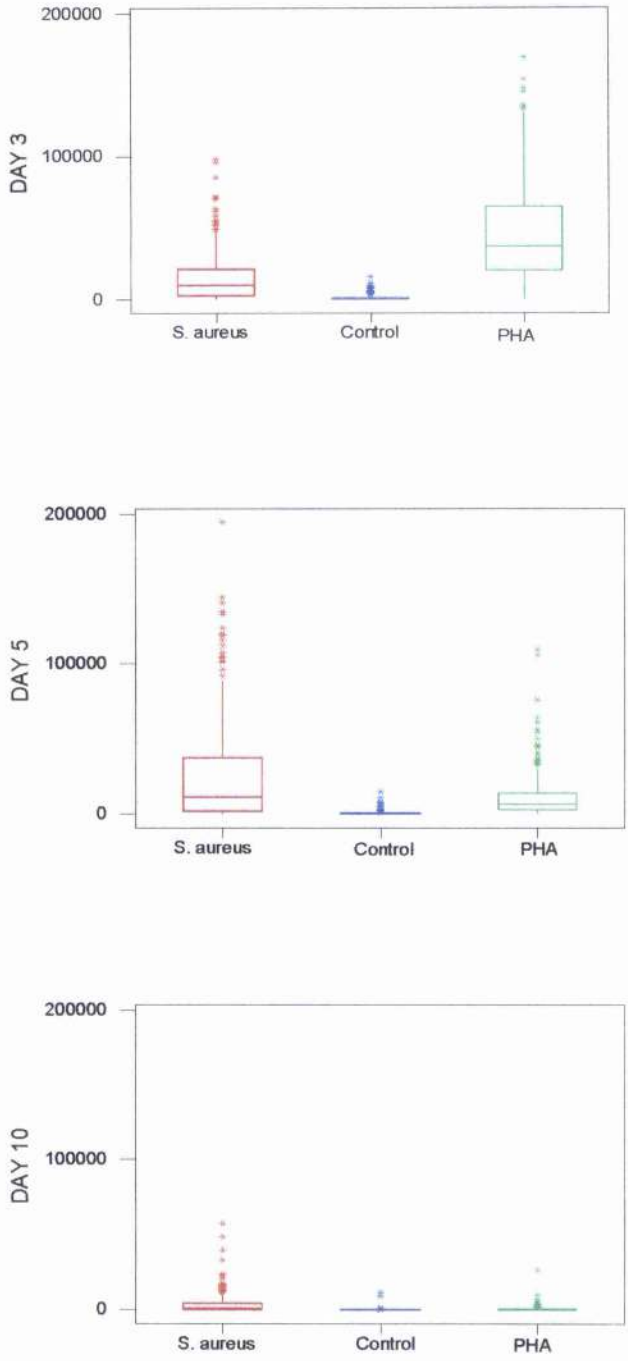


Figure 3.10: A comparison of *in vitro* PBMC proliferation on days three (a), five (b) and ten (c) of culture, showing all second generation animals sampled at approximately six months of age (n=319). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

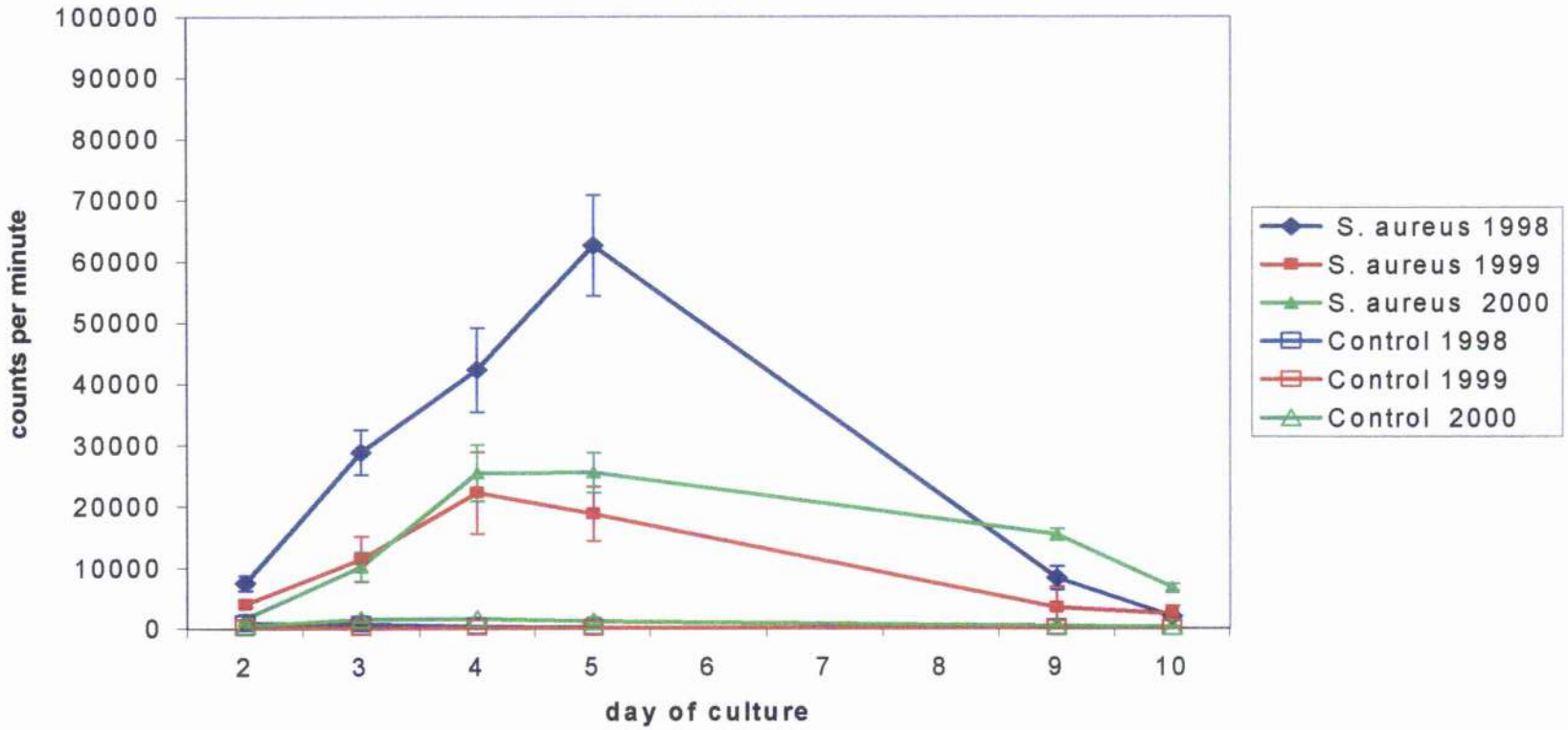


Figure 3.11: *Staphylococcus aureus*-induced *in vitro* PBMC proliferation. Each line represents the mean (± S.E.M.) proliferation of the second generation females from the 1998 (n=22) cohort in the sampling years 1998, 1999 and 2000. Control wells contained PBMC only. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

however, seen in the mean (± S.E.M.) control PBMC proliferation between the sampling years. No significant variation was seen between 1998 and 1999, however, there was a significant increase ($p<0.001$) in the control PBMC proliferation between 1999 and 2000: for example, the mean (± S.E.M.) control PBMC proliferation on day five of culture was 89.4 ±11.0 and 1179.5 ± 656.8 counts per minute, respectively (Figure 3.11).

Mean (± S.E.M.) PHA-induced proliferation increased considerably from 1998 to 2000 (Figure 3.12). Significant variation in the mean (± S.E.M.) PHA-induced proliferation was seen between 1998 and 1999 ($p<0.05$) and 1999 and 2000 ($p<0.05$), e.g. mean (± S.E.M.) PHA-induced proliferation was 58050.9 ± 4369.8 and 89740.1 ± 8076.0 counts per minute in 1999 and 2000, respectively. Significant variation was seen in the mean (± S.E.M.) control PBMC proliferation between 1998 and 1999 ($p<0.001$) and 1999 and 2000 ($p<0.001$): for example, the mean (S.E.M.) control proliferation was 759.5 ± 156.0, 196.9 ± 48.0 and 1592 ± 547.0 counts per minute in 1998, 1999 and 2000, respectively (Figure 3.12).

3.2.2.2 1999 Cohort

The mean (± S.E.M.) *S. aureus*–induced proliferation for the 1999 cohort was very similar over the time course of the assay overall between 1999 and 2000, however there was a marginal decrease on day five of culture from 1999 to 2000 (Figure 3.13). Overall, the mean (± S.E.M.) control proliferation showed very little variation between 1999 and 2000 over the time course of the assay e.g. mean (± S.E.M.) control PBMC proliferation on day five of culture was 268.3 ± 42.1 and 290.8 ± 42.5 counts per minute, respectively (Figure 3.13).

Mean (± S.E.M.) PHA-induced proliferation was very similar on days two, nine and ten of culture in 1999 and 2000, however, on days three ($p<0.05$), four ($p<0.001$) and five ($p<0.005$) there was a significant decrease in the PHA-induced proliferation from 1999 to 2000 (Figure 3.14). As mentioned above, the mean (± S.E.M.) control proliferation showed very little variation between 1999 and 2000 over the time

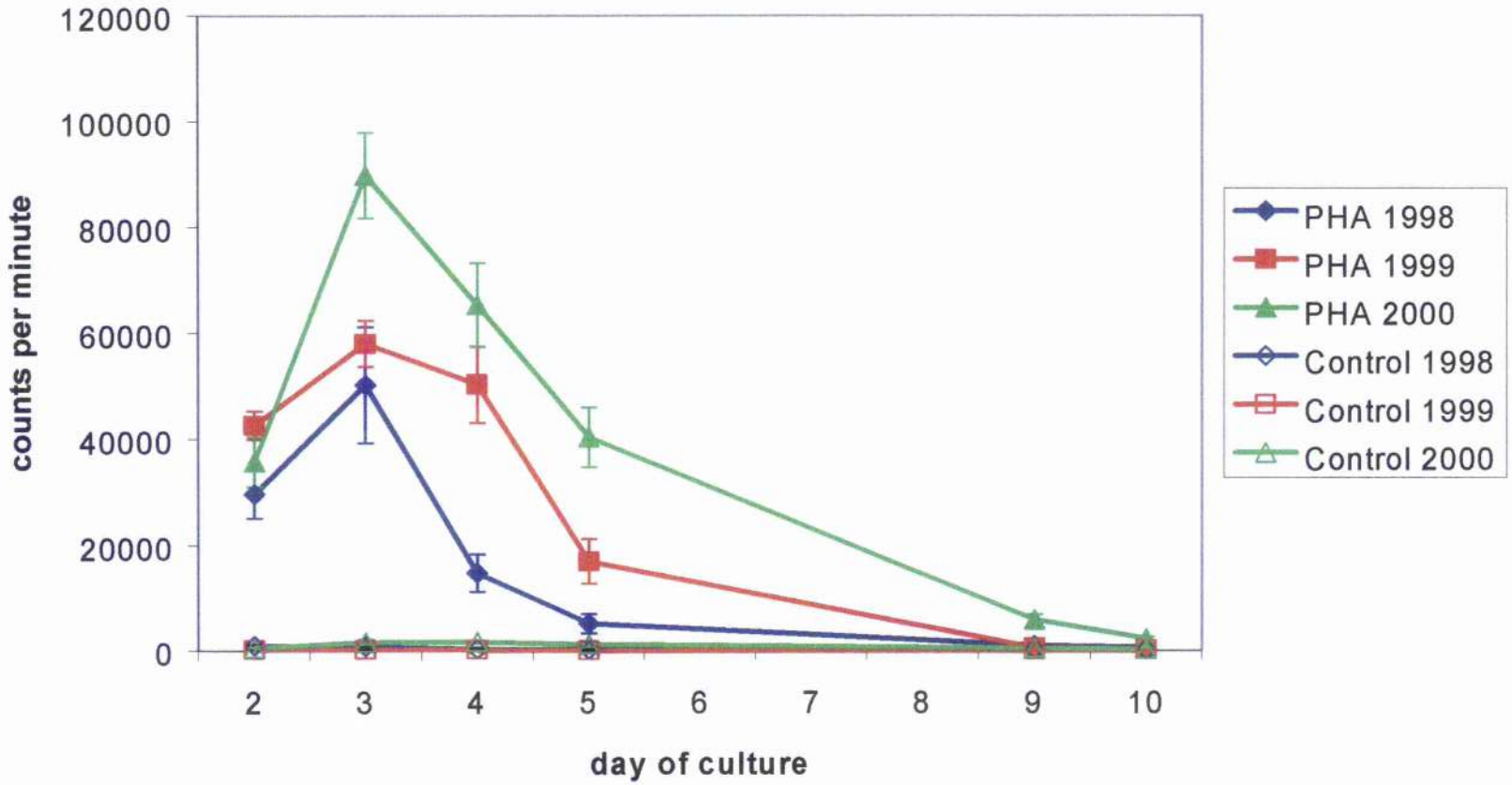


Figure 3.12: Phytohaemagglutinin-induced *in vitro* PBMC proliferation. Each line represents the mean (± S.E.M.) proliferation of the second generation females from the 1998 (n=22) cohort in the sampling years 1998, 1999 and 2000. Control wells contained PBMC only. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

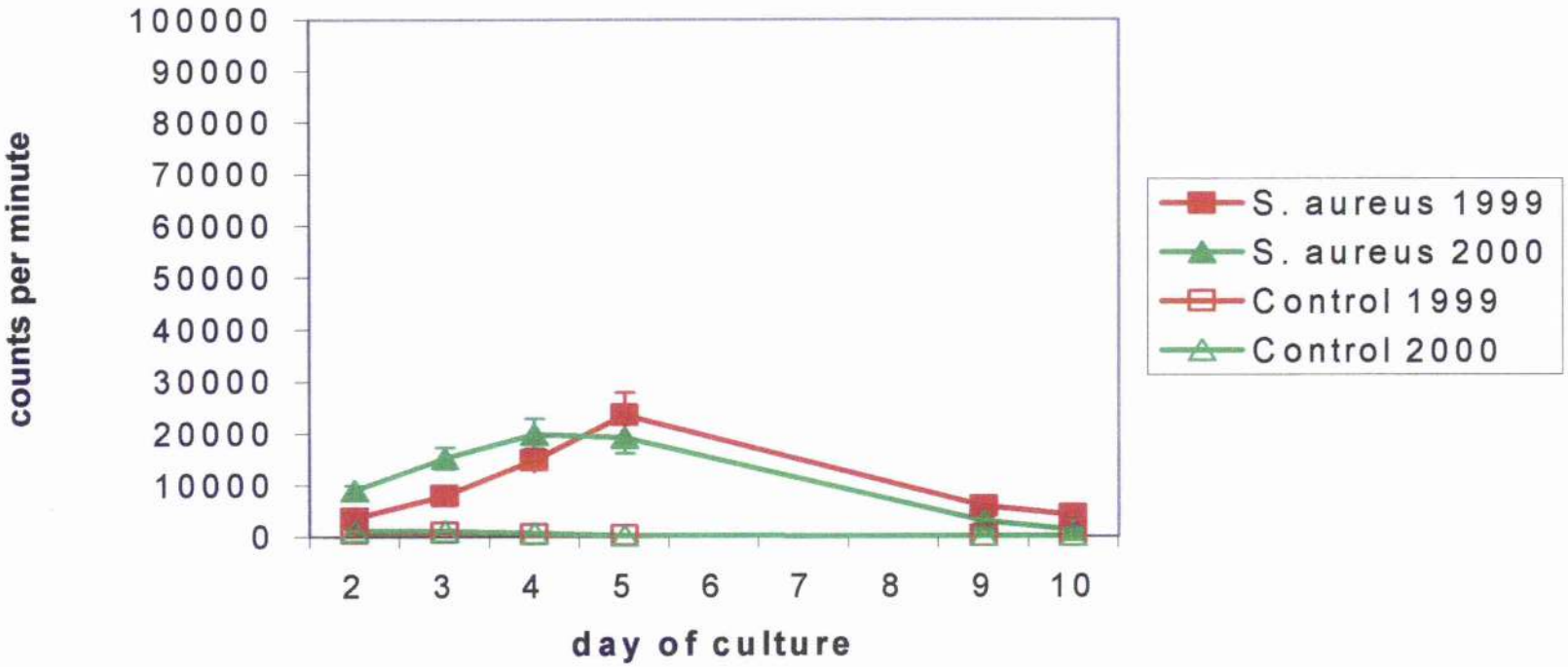


Figure 3.13: *Staphylococcus aureus*-induced *in vitro* PBMC proliferation. Each line represents the mean (± S.E.M.) proliferation of the second generation females from the 1999 (n=57) cohort in the sampling years 1999 and 2000. Control wells contained PBMC only. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

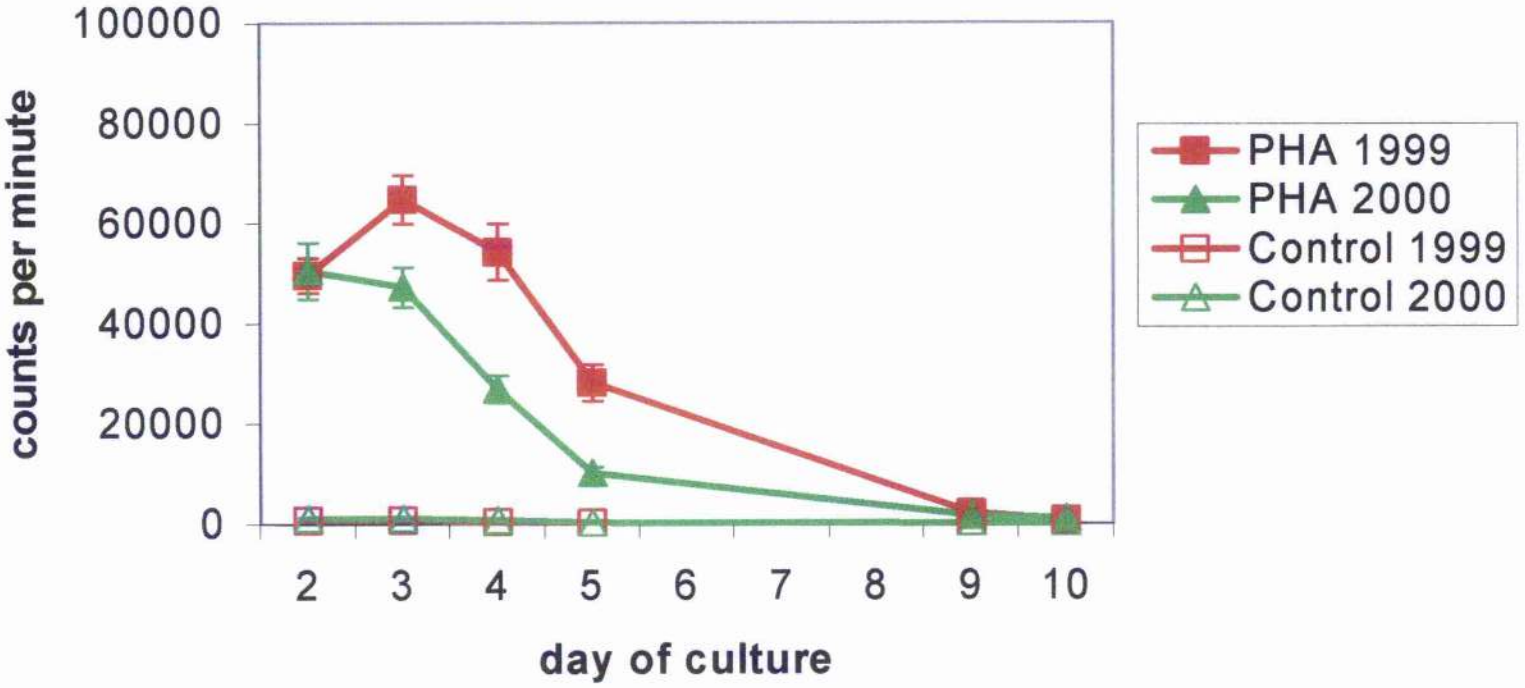


Figure 3.14: Phytohaemagglutinin-induced *in vitro* PBMC proliferation. Each line represents the mean (± S.E.M.) proliferation of the second generation females from the 1999 (n=57) cohort in the sampling years 1999 and 2000. Control wells contained PBMC only. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

course of the assay e.g. mean (± S.E.M.) control PBMC proliferation on day three of culture was 922.8 ± 152.4 and 1236.9 ± 228.1 counts per minute respectively, (Figure 3.14).

3.3 MHC *DRB 3* allele restriction fragmentation length polymorphism pattern identification in females from the 1999 Cohort

Identification of the MHC *DRB3* sequence and the RFLP patterns for the females from the 1999 cohort was carried out by Despoina Miltiadou of the Roslin Institute. These data were used to investigate the possible existence of an association with high or low *S. aureus*-induced, or PHA-induced *in vitro* PBMC proliferation, and MHC *DRB 3* allele patterns identified following RFLP analysis.

3.3.1 Ranking of *Staphylococcus aureus*-induced or Phyatohaemagglutinin-induced *in vitro* PBMC proliferation

The females from the 1999 cohort were ranked according to their level of *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation. The females were ranked by individual animal from those with the highest level of proliferation to those with the lowest level of proliferation on day four of culture (Appendix B), day four was chosen because of the significant correlation of individual animal responses at re-sampling in all the parameters assessed. Table 3.1 shows the animals ranked for *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation and their corresponding RFLP *DRB* allele patterns. The ranked data show that animals with high *S. aureus*-induced proliferation do not necessarily have high PHA-induced proliferation, indeed, only two animals are found in the highest ranked 15 for both *S. aureus*-induced and PHA-induced proliferation. Animal 14804 is ranked number one for *S. aureus*-induced proliferation and number 11 for PHA-induced proliferation, whereas animal 14910 is ranked number 12 for *S. aureus*-

RANK	*S. aureus*-induced PBMC proliferation		PHA-induced PBMC proliferation	
	Cow Number	RFLP Pattern	Cow Number	RFLP Pattern
1	14804	3\23	14882	1\10
2	14898	24\22	14859	23/8
3	14846	15/23	14869	24/22/7/28
4	14815	16\26	14921	23\10
5	14852	7\10	14820	22\24
6	14835	23\10	14876	22/23
7	14879	24\22	14900	18\23
8	14897	24\1	14922	11\3
9	14826	22/24/16	14910	28\23
10	14878	23\13	14811	23\24
11	14829	23\10	14804	3\23
12	14910	28\23	14890	10\22
13	14915	16/23	14838	10\8
14	14820	22\24	14933	24\11
15	14819	11\22	14856	24\11
42	14816	13\23	14829	23\10
43	14882	1\10	14826	22/24/16
44	14901	11\23	14927	10\2
45	14884	12\22	14915	16/23
46	14921	23\10	14835	23\10
47	14861	22\24	14860	10\11
48	14906	3\22	14816	13\23
49	14859	23/8	14909	23
50	14877	23\13	14878	23\13
51	14843	23	14877	23\13
52	14808	1\26	14883	16/3
53	14917	7\23	14861	22\24
54	14876	22/23	14846	15/23
55	14905	24\23	14834	16\7
56	14899	24\22	14863	23

Table 3.1: MHC alleles identified by RFLP pattern for females in the 1999 Cohort (n=57). Column two shows the highest and lowest ranked 15 animals for *S. aureus*-induced PBMC proliferation and column four shows the highest and lowest ranked 15 animals for PHA-induced PBMC proliferation on day four of culture. Animals in red are in the highest ranked 15 for both *S. aureus*-induced and PHA-induced proliferation. Animals in blue are in the lowest ranked 15 for both *S. aureus*-induced and PHA-induced proliferation. Animals in aqua boxes are in the highest ranked 15 for *S. aureus*-induced proliferation and the lowest ranked 15 for PHA-induced proliferation. Animals in the pink boxes are in the highest ranked 15 for PHA-induced proliferation and the lowest ranked 15 for *S. aureus*-induced proliferation. Columns three and five show the RFLP patterns identified for the animals in columns two and four respectively. Allele *DRB 3.2*16* is highlighted in green.

induced proliferation and number nine for PHA-induced proliferation. Conversely, only two animals were identified in the lowest ranked 15 animals for both *S. aureus*-induced and PHA-induced proliferation. Animal 14861 was ranked number 47 for *S. aureus*-induced proliferation and number 53 for PHA-induced proliferation, whereas animal 14877 was ranked number 50 for *S. aureus*-induced proliferation and number 51 for PHA-induced proliferation.

Results show that some animals with high *S. aureus*-induced proliferation have low PHA-induced proliferation and, conversely, that some animals with high PHA-induced proliferation have low *S. aureus*-induced proliferation. Six animals (14846, 14835, 14826, 14878, 14829 and 14915) were identified in the highest ranked 15 animals for *S. aureus*-induced proliferation and in the lowest ranked 15 animals for PHA-induced proliferation, and four animals (14882, 14921, 14859 and 14876) were identified in the highest ranked 15 animals for PHA-induced proliferation and in the lowest ranked 15 animals for *S. aureus*-induced proliferation (Table 3.1).

3.3.2 Restriction fragment length polymorphism allele pattern

The females from the 1999 cohort showed considerable individual variation in the RFLP allele patterns identified. Variation in allele pattern was seen in the animals with both high and low *S. aureus*-induced and PHA-induced proliferation *in vitro* PBMC. Of the five (14815, 14826, 14834, 14883 and 14915) females from the 1999 cohort which possess *DRB 3.2*16*, three of the five (14815, 14826 and 14915) are in the highest ranked 15 animals for *S. aureus*-induced proliferation and four of the five (14826, 14915, 14883 and 14834) are in the lowest ranked 15 animals for PHA-induced proliferation.

Chapter 4

Results

4.1 Polymorphonuclear cell phagocytosis

4.1.1 Assessment of *in vitro* phagocytosis by the isolated polymorphonuclear cell population

4.1.1.1 Development

The yield and viability of the isolated polymorphonuclear cell (PMN) population was assessed as part of the preliminary assay development. Assessment of the lysis buffers, ACE and Bushmans, on four individual second generation animals was carried out. Preliminary isolation of PMN using the lysis buffers, ACE and Bushmans, resulted in very poor yields, which were found to be as low as 10% of the total PMN isolated from whole blood. Preliminary mean (± S.E.M.) PMN yields from four second generation animals following lysis with ACE and Bushmans were 11.1% ± 2.4% and 7.0% ± 1.2% respectively. Use of the lysis buffer, ACE, resulted in a greater yield of polymorphonuclear cells and a greater level of viability of the isolated cell populations compared to use of the Bushmans lysis buffer. The isolation with ACE lysis buffer was repeated until the laboratory technique was developed effectively to obtain an adequate yield of at least 60% of the total PMN available in the blood samples. The mean (± S.E.M.) percentage viability from four

second generation animals, assessed using trypan blue was 90.5% ± 1.5% and 75.0% ± 5.29% for the lysis buffers ACE and Bushmans, respectively.

4.1.1.2 Assessment of non-specific extracellular adherence

Some authors describe the potential problem of non-specific adherence of the FITC-labelled particles to the outside of the PMN cell surface, resulting in an overestimation of total phagocytosis. Many methods of quenching the non-specific fluorescence prior to flow cytometry have been described, however, one author (Santos *et al.*, 1995), showed use of a duplicate sample at 4°C as an indicator of non-specific adherence where, at this temperature, beads adhered to cells but were not phagocytosed. In the current study, assessment of the non-specific adherence was carried out for phagocytosis of FITC-labelled latex beads and FITC-labelled *S. aureus* at each time point (Figure 4.1). Phagocytosis of FITC-labelled latex beads had very low levels of non-specific adherence of approximately 1% throughout the assay, whereas, phagocytosis of FITC-labelled *S. aureus* showed high levels of non-specific adherence at 4°C, ranging from approximately 45.0% ± 8.0% at zero hours, to 48.1% ± 8.3% at one hour, and 74.6% ± 1.4% at eight hours of incubation (Figure 4.1). When the non-specific adherence levels measured at 4°C were subtracted from the phagocytosis at 37°C, a more accurate indicator of the actual phagocytosis was seen (Figure 4.2), especially for the phagocytosis of FITC-labelled *S. aureus*. Due to the low levels of non-specific adherence following phagocytosis of the FITC-labelled latex beads at all the time points, only the non-specific adherence at one hour incubation was used for comparison with phagocytosis at 37°C at all time points. Due to the high levels of non-specific adherence following phagocytosis of the FITC-labelled *S. aureus*, it was considered necessary to continue with a duplicate sample at 4°C for each time point during the incubation.

4.1.1.3 Serum supplementation

The effect of adding heat inactivated fetal calf serum, heat inactivated adult bovine serum or heat inactivated autologous serum to the media at 5% (vol/vol) on *in vitro* PMN phagocytosis was assessed on three Holstein-Friesian lactating cows from

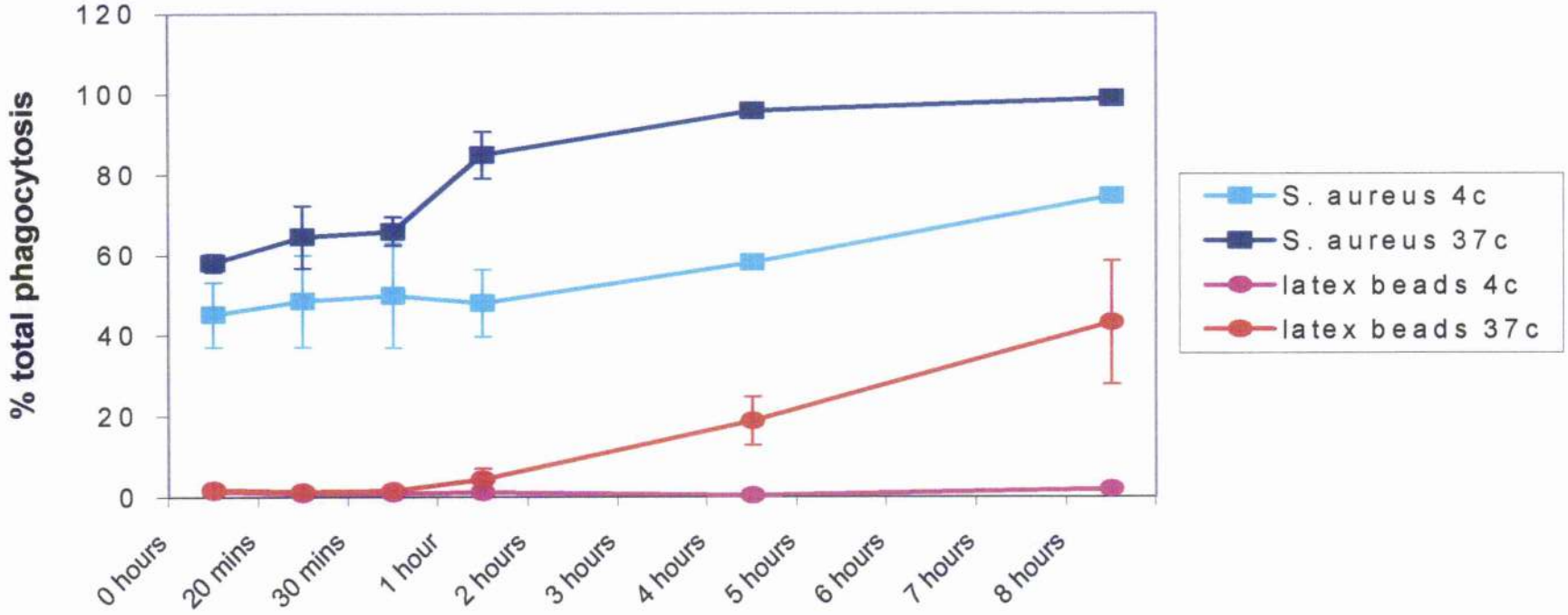


Figure 4.1: Preliminary assessment of *in vitro* PMN phagocytosis. Mean (± S.E.M.) percentage total phagocytosis of FITC-labelled (2.0 μM) latex beads or FITC-labelled *S. aureus* by isolated PMN populations at 37°C and at 4°C (n=3).

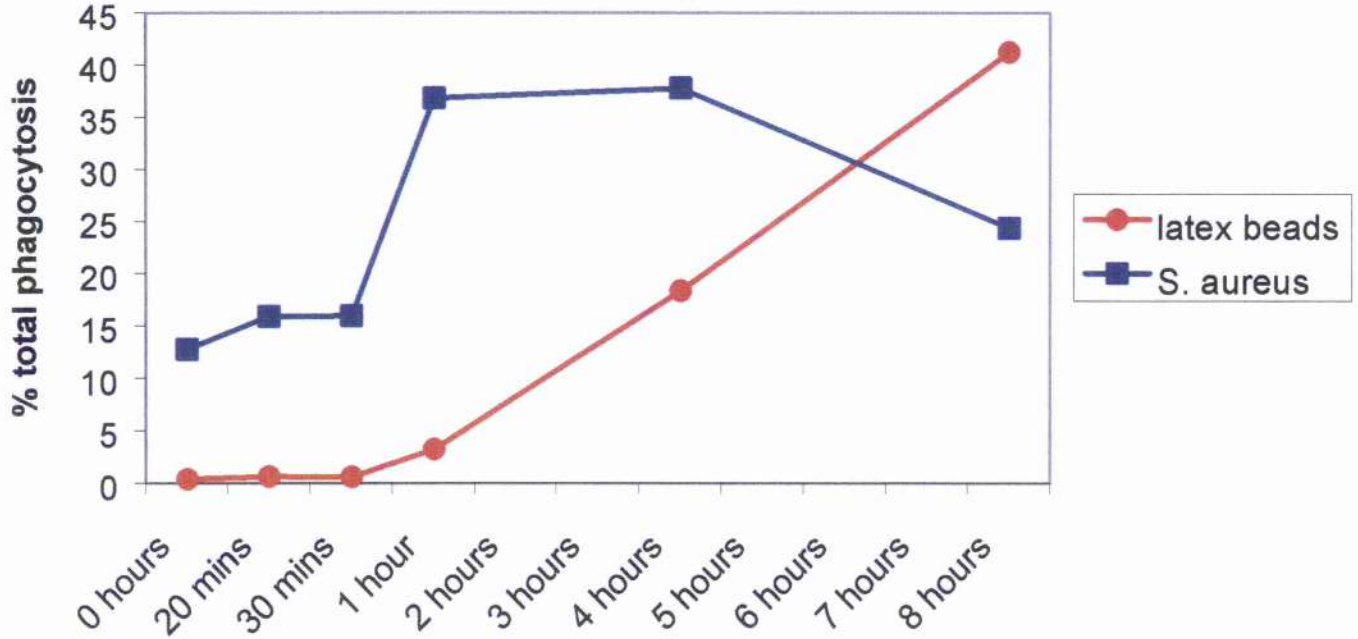


Figure 4.2: Preliminary assessment of *in vitro* PMN phagocytosis over time. Results presented are mean percentage total phagocytosis at 37°C minus mean percentage total phagocytosis at 4°C of FITC-labelled (2.0 μM) latex beads or FITC-labelled *S. aureus* by isolated PMN populations (n=3).

Cochno farm. No significant differences were seen in the level of phagocytosis in the isolated neutrophil population when the media was supplemented with heat inactivated autologous serum or heat inactivated adult bovine serum, whereas heat inactivated fetal calf serum resulted in significantly ($p<0.05$) lower levels of phagocytosis (Figure 4.3). Commercial heat inactivated adult bovine serum was chosen for all subsequent assays for reasons of low cost and ease of preparation and use.

4.1.1.4 Variation in bead size

Three different sizes of FITC-labelled latex beads; 0.1μM, 1.0μM and 2.0μM were assessed for use in the assay. Following incubation of isolated PMN and FITC-labelled beads, there was no visible indication of phagocytosis using flow cytometry of the 0.1μM and 1.0μM FITC-labelled latex beads at any of the time points compared to the control sample containing isolated PMN only at zero hours (Figure 4.4). Measurable levels of phagocytosis were only detected in the samples incubated with the FITC-labelled 2.0μM beads (Figure 4.5). The FITC-labelled 2.0μM beads were, therefore, employed subsequently in all the assays.

4.1.1.5 Assessment of the ratio of polymorphonuclear cells to FITC-labelled 2.0μM latex beads

The effect of using different ratios of PMN:FITC-labelled (2.0μM) latex beads were assessed using ratios of 1:25, 1:50 and 1:100. At six hours of incubation, lower levels of phagocytosis were seen with the 1:25 samples than the 1:50 or 1:100 samples. Very little difference was seen in the levels of phagocytosis between the 1:50 and the 1:100 ratios with the 1:100 having marginally higher levels of phagocytosis at 37°C (Figure 4.6). The background levels of non-specific adherence at 4°C (data not shown) were, however, also marginally higher for the 1:100 ratio (2.7% ± 0.8%) compared with the ratio of 1:50 (1.3% ± 0.2%). Due to there being no significant difference in phagocytosis when the ratios 1:50 or 1:100 were employed,

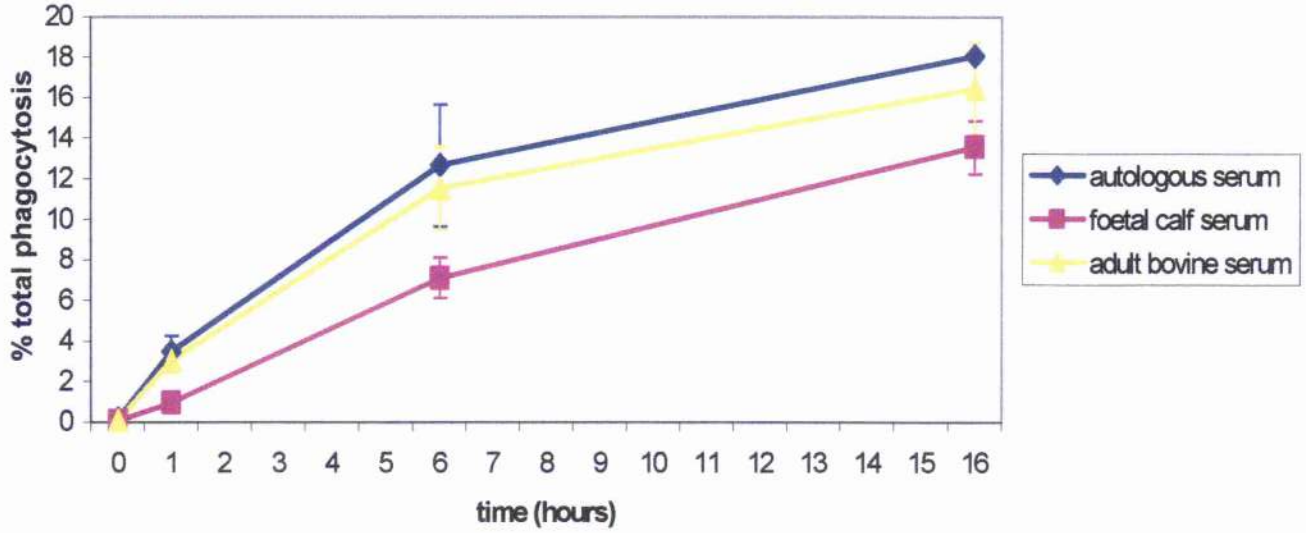


Figure 4.3: Percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by the isolated PMN population with media containing 5% (vol/vol) heat inactivated autologous bovine serum, fetal calf serum or adult bovine serum (n=3).

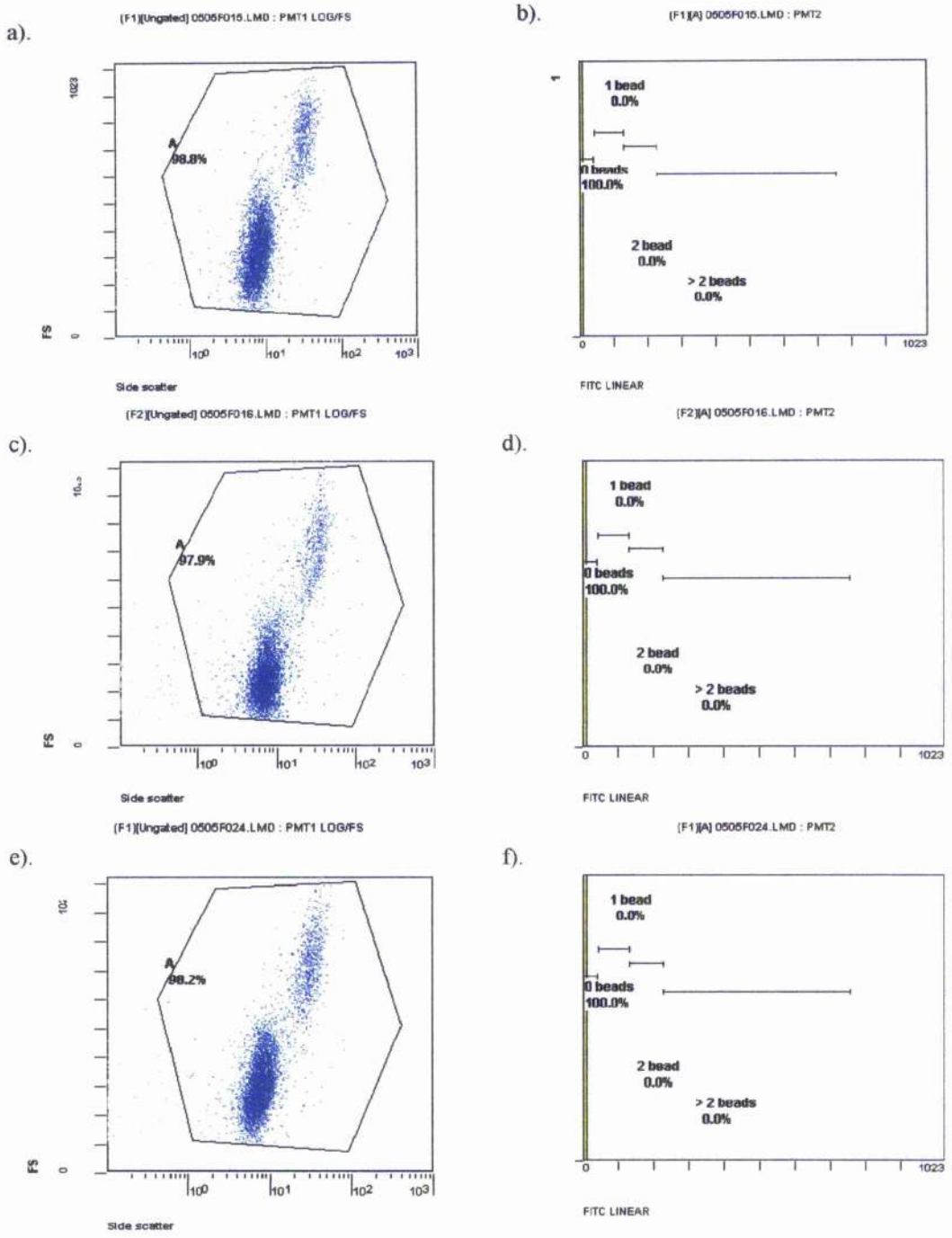
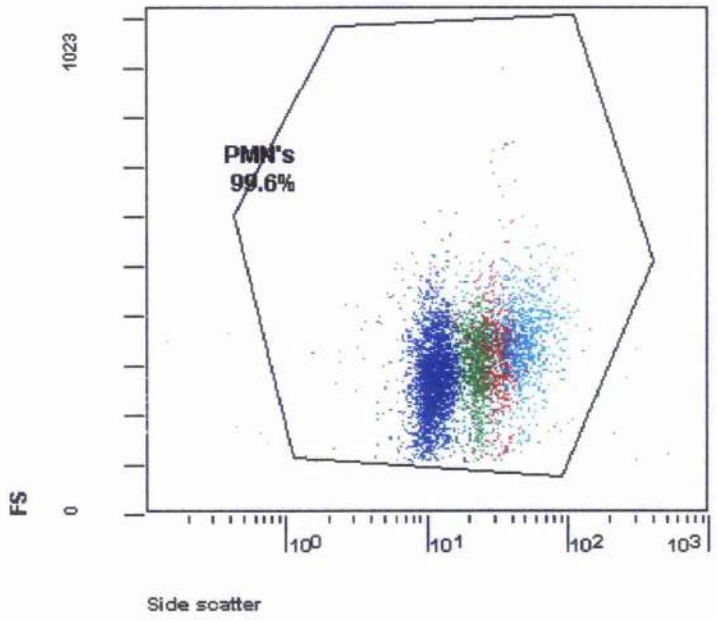


Figure 4.4: Analysis of *in vitro* PMN phagocytosis by flow cytometry with the isolated PMN population gated as A. Figures a), c), and e), show the side scatter (x-axis) and forward scatter (y-axis) of PMN only at 0 hours, PMN and FITC-labelled (0.1μM) latex beads at 6 hours and PMN and FITC-labelled (1.0μM) latex beads at 6 hours at 37°C, respectively. Figures b), d) and f) show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with gates for 0 beads (no phagocytosis), 1 bead, 2 beads and > 2 beads for the corresponding figure a), c) and e), respectively.

a)



b).

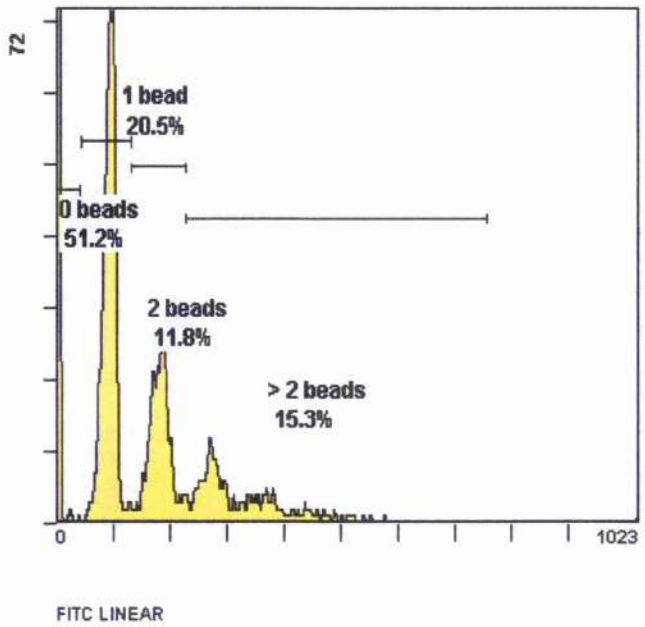


Figure 4.5: Analysis of *in vitro* PMN phagocytosis by flow cytometry. Figures a) and b) show the isolated PMN population at 6 hours incubation with 2.0 μM FITC-labelled latex beads. Figure a) shows side scatter (x-axis) and forward scatter (y-axis) with the isolated PMN population gated as PMN's. Figure b) shows a linear graph of fluorescence (x-axis) and total number of cells (y axis). On figure a), colours correspond to phagocytosis of 0 beads (blue, no phagocytosis), 1 bead (green), 2 beads (red) and > 2 beads (aqua).

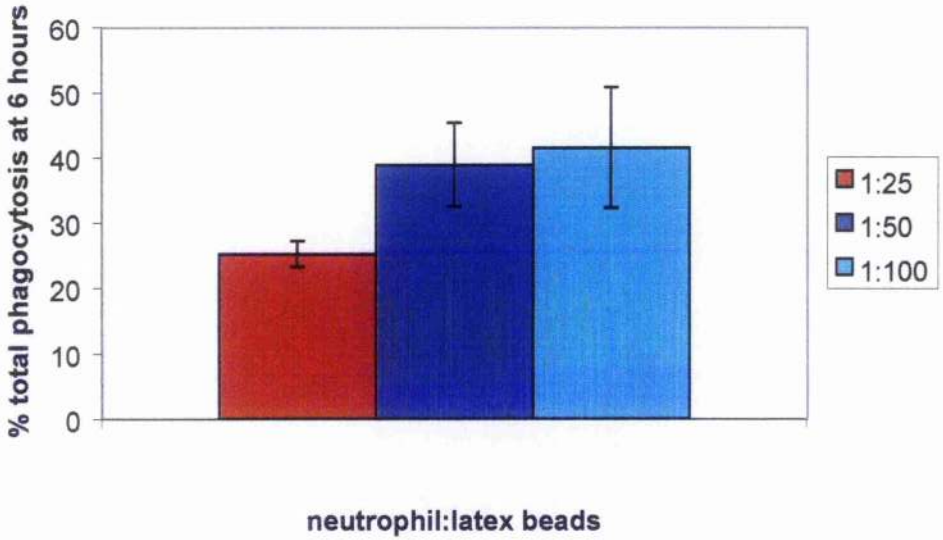


Figure 4.6: Mean (± S.E.M.) percentage total phagocytosis of FITC-labelled (2.0 μM) latex beads by the isolated PMN population at six hours incubation at 37°C at PMN:FITC-labelled latex bead ratio of 1:25, 1:50 and 1:100 (n=3).

and the slightly higher background non-specific adherence with the 1:100 ratio, the 1:50 ratio was chosen and used subsequently in the assays.

4.1.1.6 Optimal fixation of isolated polymorphonuclear population

Another part of the preliminary development of the PMN phagocytosis assay was to assess the final fixation of the cell population following incubation with FITC-labelled latex beads or *S. aureus*. Fixing of the PMN populations was assessed using ice-cold PBS or with paraformaldehyde solution (0.4% and 1% vol/vol in PBS). Higher non-specific background levels were obtained with FITC-labelled *S. aureus* when fixed with ice-cold PBS than with paraformaldehyde following incubation. No difference was observed when PBS and paraformaldehyde were used to fix FITC-labelled latex beads. Overall, fixation with paraformaldehyde following incubation was the most effective method to retain viable cells for analysis by flow cytometry and was employed subsequently in the assays.

4.1.1.7 Optimal time points for assessment of *in vitro* polymorphonuclear phagocytosis

Preliminary assessment of a range of time points to measure phagocytosis of FITC-labelled latex bead and FITC-labelled *S. aureus* was carried out over an eight-hour period. The time points assessed were 0 hours, 20 minutes, 30 minutes, one hour, four hours and eight hours. Preliminary assessment of the kinetics of phagocytosis by the isolated PMN indicated a slight variation in the peak levels of phagocytosis between the FITC-labelled latex beads and the FITC-labelled *S. aureus*. Preliminary assessment showed that the peak phagocytosis of FITC-labelled beads was in the region of four to eight hours, whereas the peak level of phagocytosis of FITC-labelled *S. aureus* was at approximately one hours incubation (Figure 4.2). Further assessment of phagocytosis of FITC-labelled (2.0μM) latex beads and FITC-labelled *S. aureus* was carried out in order to determine the optimal time point for peak phagocytosis and covered a 20-hour incubation period (Figure 4.7). Results indicated that the peak level of phagocytosis for the FITC-labelled (2.0μM) latex beads occurred between four and eight hours of incubation and the peak level of

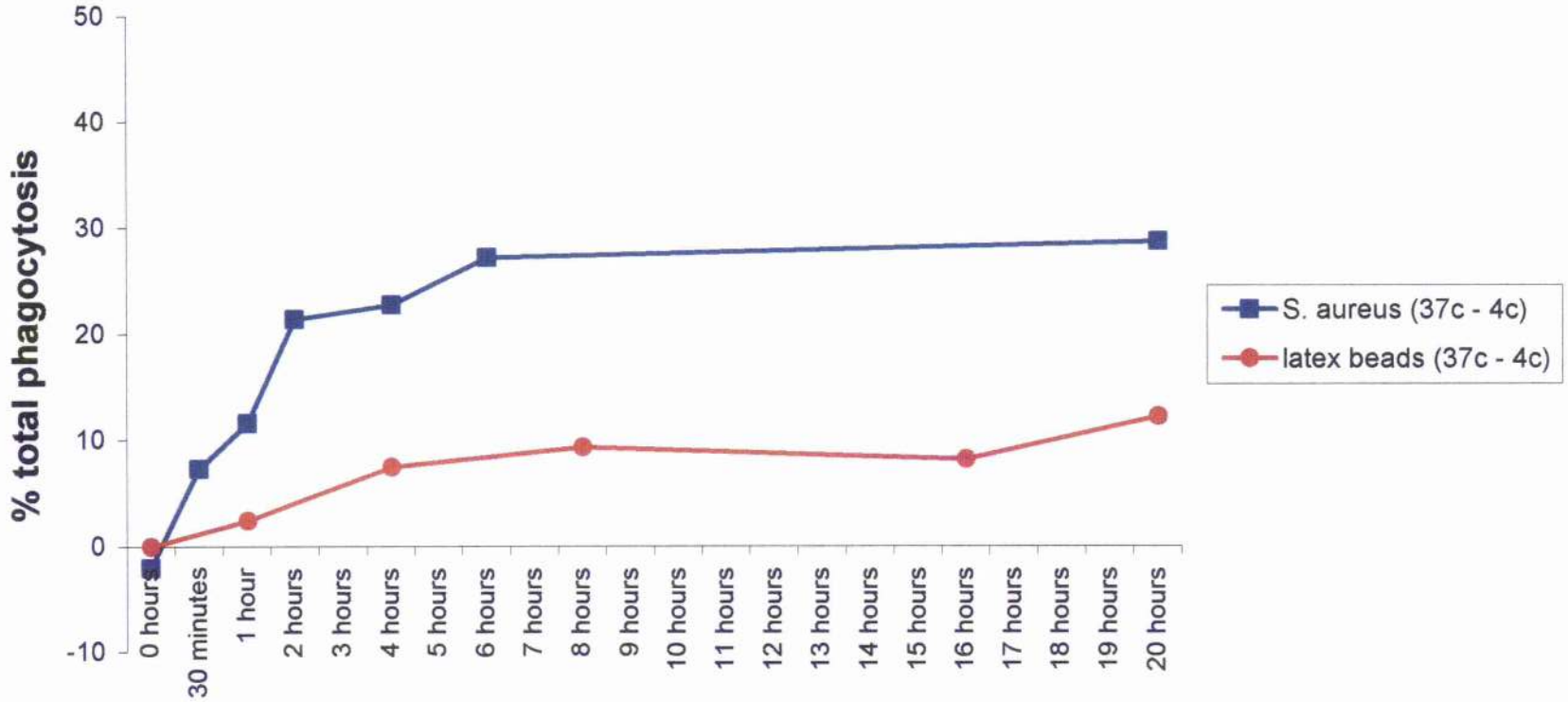


Figure 4.7: Further assessment of *in vitro* PMN phagocytosis over time. Results presented are the mean percentage total phagocytosis at 37°C minus the mean percentage total phagocytosis at 4°C of FITC-labelled (2.0 μM) latex beads or FITC-labelled *S. aureus* by isolated PMN populations (n=3).

phagocytosis of FITC-labelled *S. aureus* occurred between four and six hours. The time points 0 hour, one hour, six hours and sixteen hours were, therefore, chosen as the best indicators of phagocytosis for both FITC-labelled (2.0μM) latex beads and FITC-labelled *S. aureus* and were the time points subsequently employed in the assays.

4.1.1.8 *In vitro* phagocytosis of FITC-labelled *Staphylococcus aureus* by isolated neutrophil populations

Following the preliminary assay development which highlighted the high levels of non-specific background fluorescence with the *in vitro* phagocytosis of FITC-labelled *S. aureus*, it was considered necessary to run a duplicate set of samples at 4°C for every animal sampled. Phagocytosis of FITC-labelled *S. aureus* was assessed on approximately 20 second generation animals. Results indicated a mean non-specific background fluorescence of 64.4% ± 2.4% at zero hours, 58.2% ± 3.9% at one hour and 72.3% ± 4.2% at six hours (Figure 4.8). Levels of phagocytosis at 37°C were between 60.8% ± 2.9% at zero hours, 74.8% ± 3.3% at one hour and 94.7% ± 0.7% at six hours (Figure 4.8). The actual levels of phagocytosis calculated by subtracting the background at 4°C from the phagocytosis at 37°C, ranged from 3.2% at zero hours, 16.6% at one hour to 22.4% at six hours. Examples of the analysis by flow cytometry, of the *in vitro* phagocytosis of FITC-labelled *S. aureus*, over the 16 hour incubation period, are shown in Figures 4.9 (0 hours), 4.10 (1 hour), 4.11 (6 hours) and 4.12 (16 hours). Due to the considerable problems with background non-specific adherence and interpretation of the flow cytometry output, further optimisation of this assay is required before an accurate measure of phagocytosis of FITC-labelled *S. aureus* can be obtained.

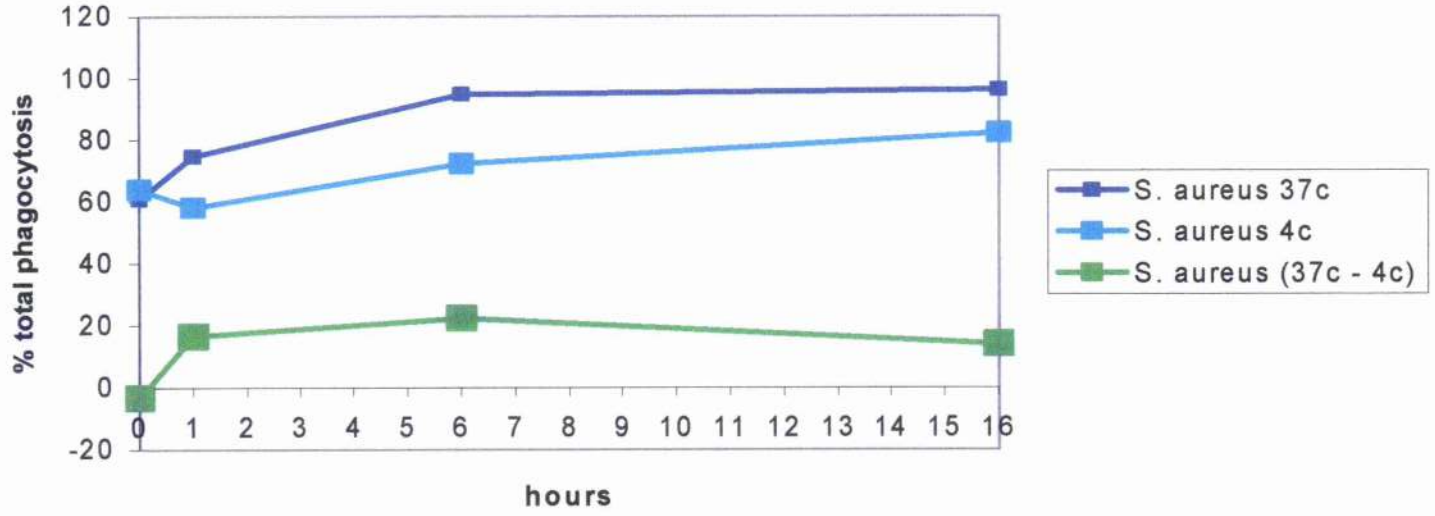


Figure 4.8: Assessment of *in vitro* PMN phagocytosis over time. Results presented are mean percentage total phagocytosis of FITC-labelled *S. aureus* by isolated PMN populations, at 37°C, 4°C and the mean percentage total phagocytosis at 37°C minus the mean percentage total phagocytosis at 4°C of FITC-labelled *S. aureus* by isolated PMN populations (n=8).

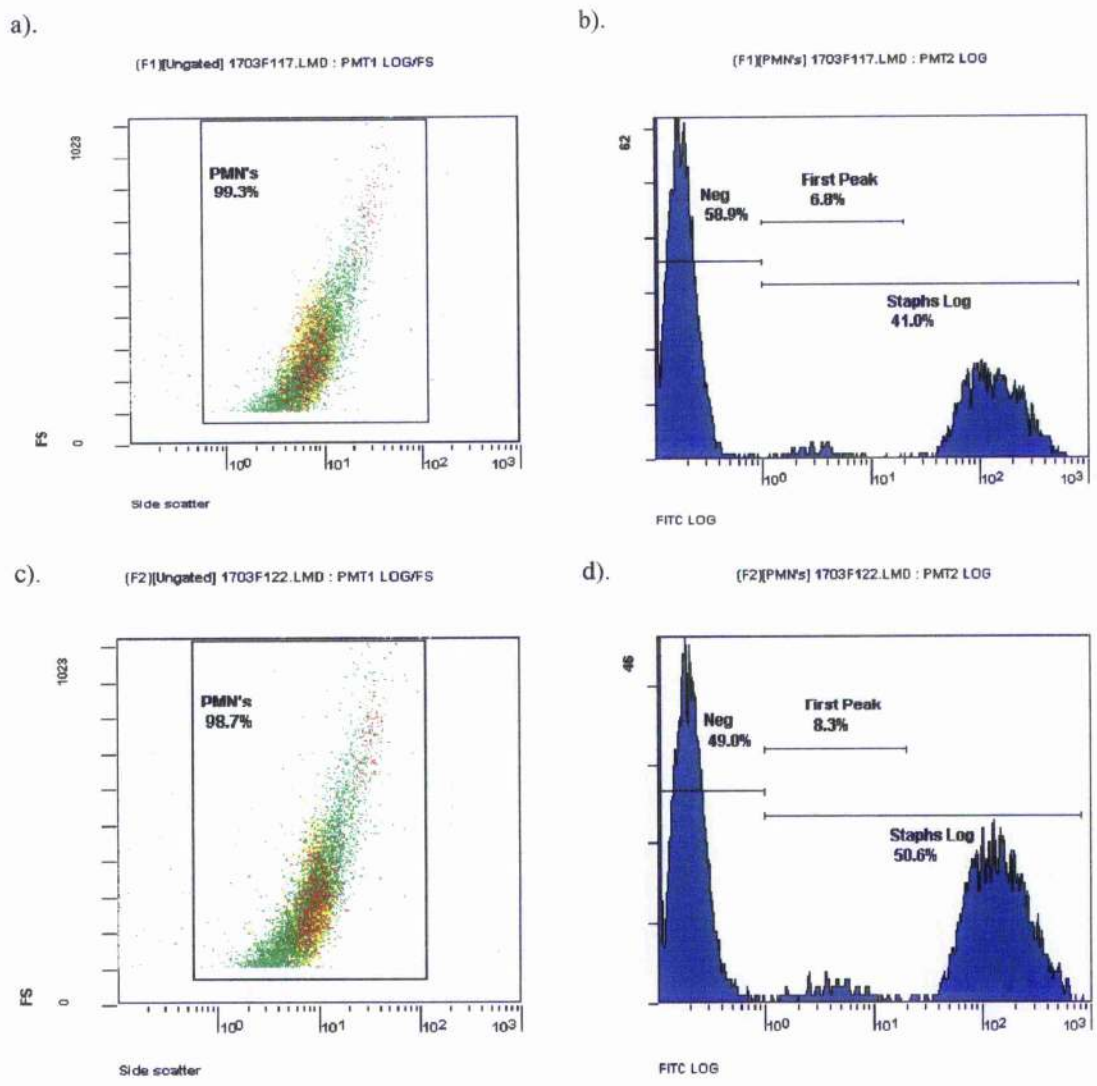


Figure 4.9: Analysis of *in vitro* PMN phagocytosis of FITC-labelled *S. aureus* by flow cytometry. Figures a) and c) show side scatter (x-axis) and forward scatter (y-axis) of the isolated PMN following incubation with the FITC-labelled *S. aureus* at 0 hours at 4°C and 37°C respectively, PMN were colour coded for no phagocytosis (green), first peak (red) and total phagocytosis (yellow). Figures b) and d) show log graphs of fluorescence (x-axis) and the total number of cells (y-axis) with gates for no phagocytosis (Neg.), first peak and total phagocytosis (Staphs log.).

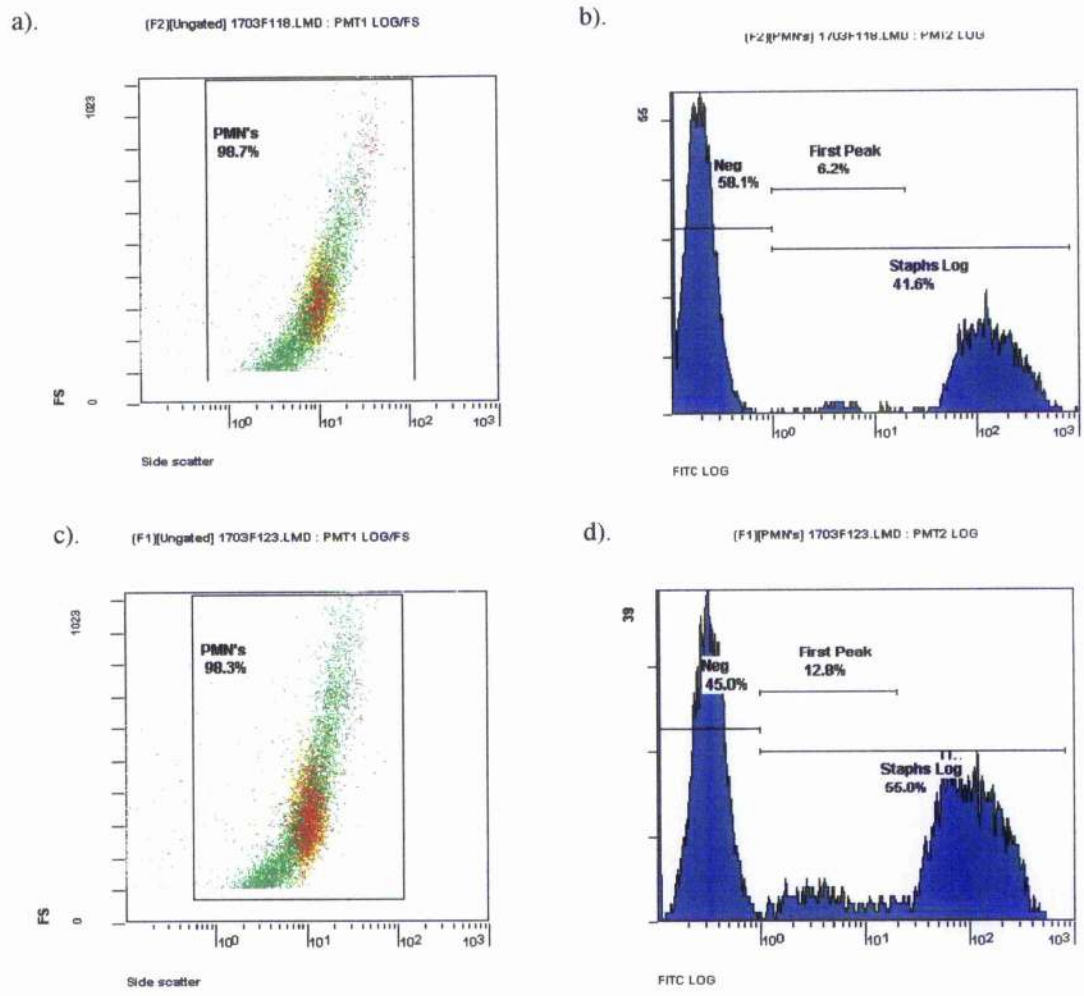


Figure 4.10: Analysis of *in vitro* PMN phagocytosis of FITC-labelled *S. aureus* by flow cytometry. Figures a) and c) show side scatter (x-axis) and forward scatter (y-axis) of the isolated PMN following incubation with the FITC-labelled *S. aureus* at 1 hour at 4°C and 37°C respectively, PMN were colour coded for no phagocytosis (green), first peak (red) and total phagocytosis (yellow). Figures b) and d) show log graphs of fluorescence (x-axis) and the total number of cells (y-axis) with gates for no phagocytosis (Neg.), first peak (red) and the total phagocytosis (Staphs Log.).

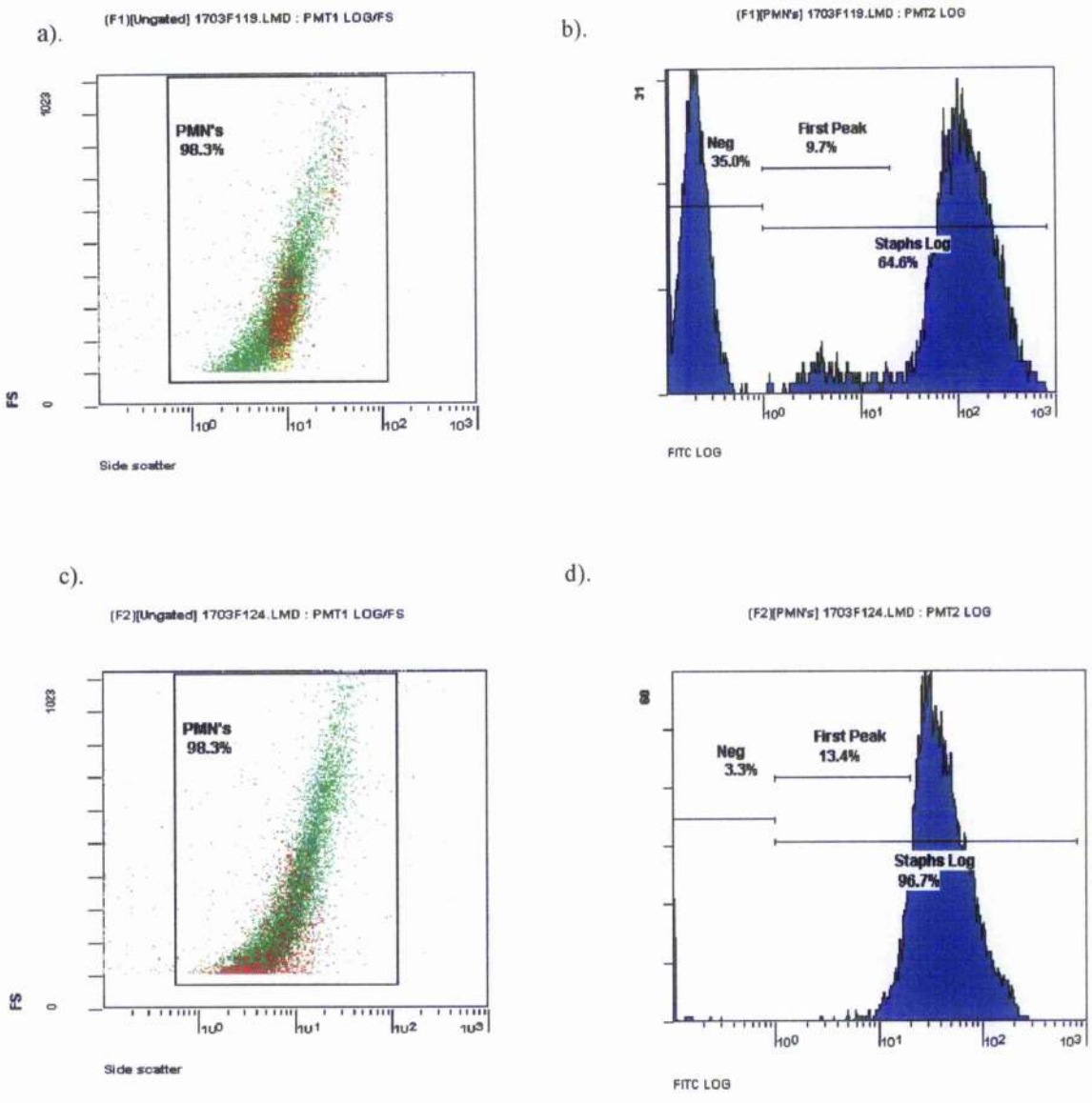


Figure 4.11: Analysis of *in vitro* PMN phagocytosis of FITC-labelled *S. aureus* by flow cytometry. Figures a) and c) show side scatter (x-axis) and forward scatter (y-axis) of the isolated PMN following incubation with the FITC-labelled *S. aureus* at 6 hours at 4°C and 37°C respectively, PMN were colour coded for no phagocytosis (green), first peak (red) and total phagocytosis (yellow). Figures b) and d) show log graphs of fluorescence (x-axis) and the total number of cells (y-axis) with gates for no phagocytosis (Neg.), first peak and total phagocytosis (Staphs Log.).

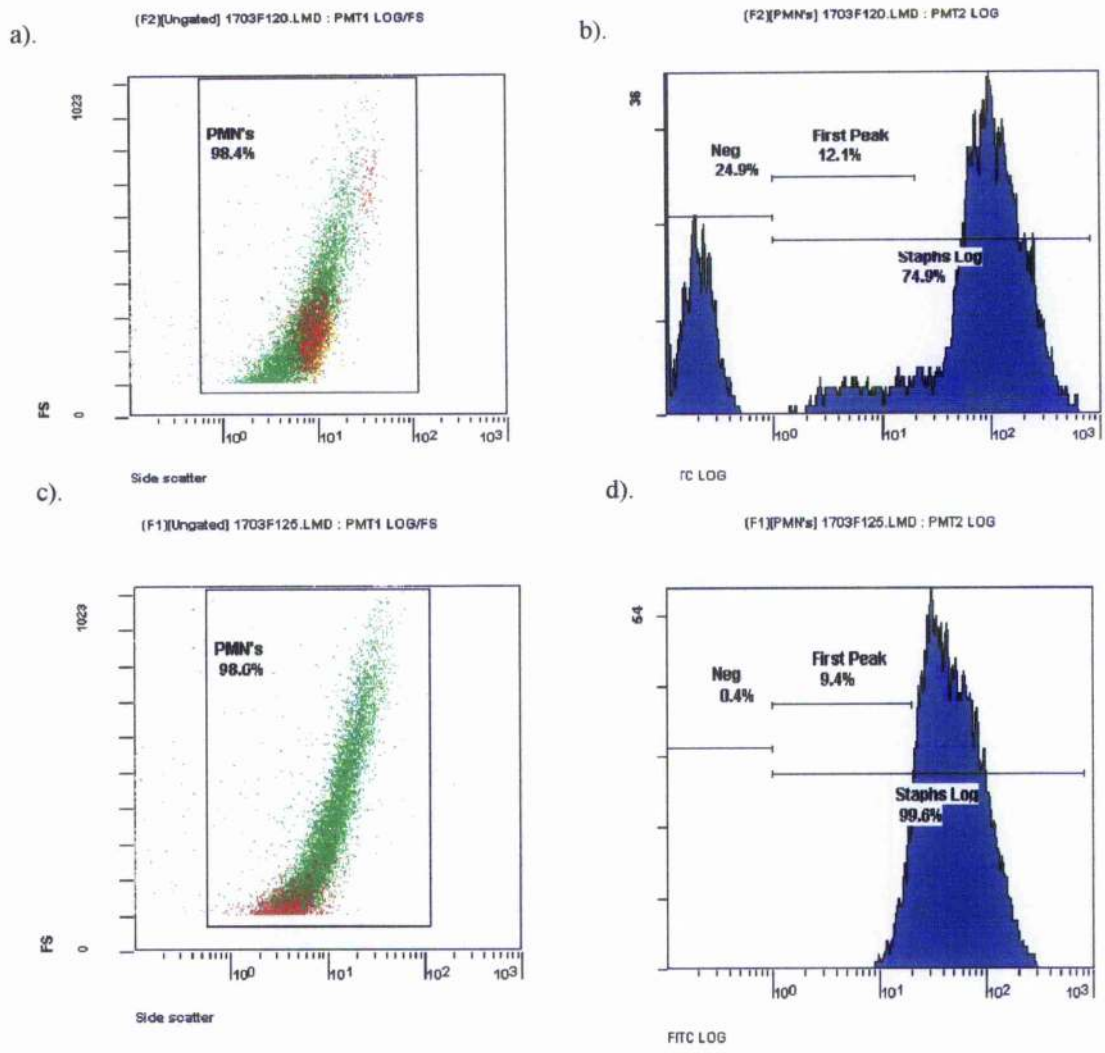


Figure 4.12: Analysis of *in vitro* PMN phagocytosis of FITC-labelled *S. aureus* by flow cytometry. Figures a) and c) show side scatter (x-axis) and forward scatter (y-axis) of the isolated PMN following incubation with the FITC-labelled *S. aureus* at 16 hours at 4°C and 37°C respectively, PMN were colour coded for no phagocytosis (green), first peak (red) and total phagocytosis (yellow). Figures b) and d) show log graphs of fluorescence (x-axis) and the total number of cells (y-axis) with gates for no phagocytosis (Neg.), first peak (red) and total phagocytosis (Staphs Log.).

4.1.2 *In vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated polymorphonuclear cell populations in second generation animals.

In vitro phagocytosis of FITC-labelled (2.0μM) latex beads was assessed in 140 second generation animals on one occasion at approximately six months of age. Animals were sampled in batches of approximately ten per week. Due to the different management and housing regimes that were required for the different sexes under other aspects of the Robogene project, males and females were housed separately. For this reason, the batches of ten animals sampled were either all male or all female. A duplicate sample containing isolated PMN and FITC-labelled (2.0μM) latex beads was incubated at 4°C for one hour was used as an indicator of non-specific background. Variation in the percentage total phagocytosis was seen among individual animals (Figure 4.13). Variation in the percentage total phagocytosis was seen among individuals at each of the time points, with the largest variation among individual animals at six and sixteen hours of incubation (Figure 4.14). The mean (± S.E.M.) percentage total phagocytosis was similar between the 0 hours time point and the non-specific adherence at one hour (4°C) (Figure 4.14). Phagocytosis of one bead, two beads and greater than two beads also increased throughout the incubation (Figure 4.15). Following flow cytometry analysis, it was observed that, as PMN phagocytosed an increasing number of FITC-labelled (2.0μM) latex beads, the size of the PMN increased. The gates were set in the linear graphs of fluorescence versus total number of cells, which represented the phagocytosis of zero beads, one bead, two beads and greater than two beads by the isolated PMN. These, were colour coded in the graphs of side scatter versus forward scatter to allow the cells within these gates to be visualised corresponding to their levels of phagocytosis, i.e. phagocytosis of zero beads (blue), one bead (green), two beads (red) or greater than two beads (aqua). This colour coding of the isolated PMN showed that the PMN which had not undergone phagocytosis were smallest in size, and that the size of the PMN increased in accordance with the phagocytosis of increasing numbers of FITC-labelled (2.0μM) latex beads. Examples of the analysis by flow cytometry, showing the kinetics of the *in vitro* phagocytosis of FITC-labelled (2.0μM) latex

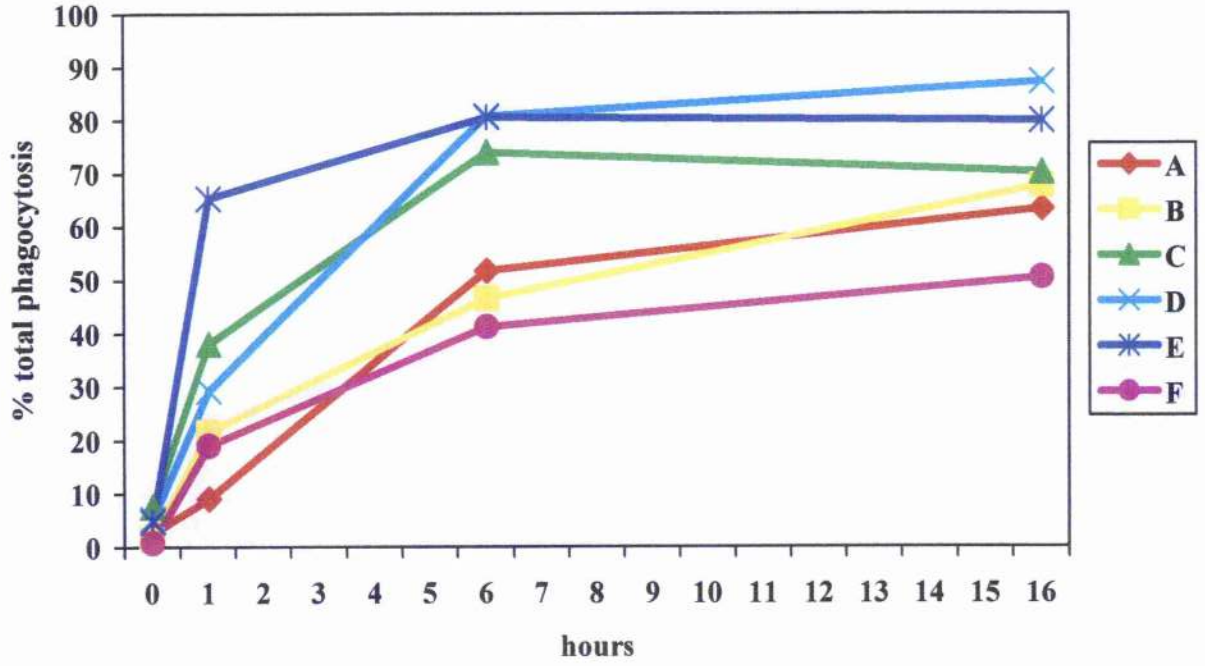


Figure 4.13: Representative example of individual variation of *in vitro* phagocytosis of FITC-labelled (2.0 μM) latex beads by the isolated PMN population in six second generation animals (A-F). Graph shows hours of incubation (x-axis) and the percentage total phagocytosis (y-axis).

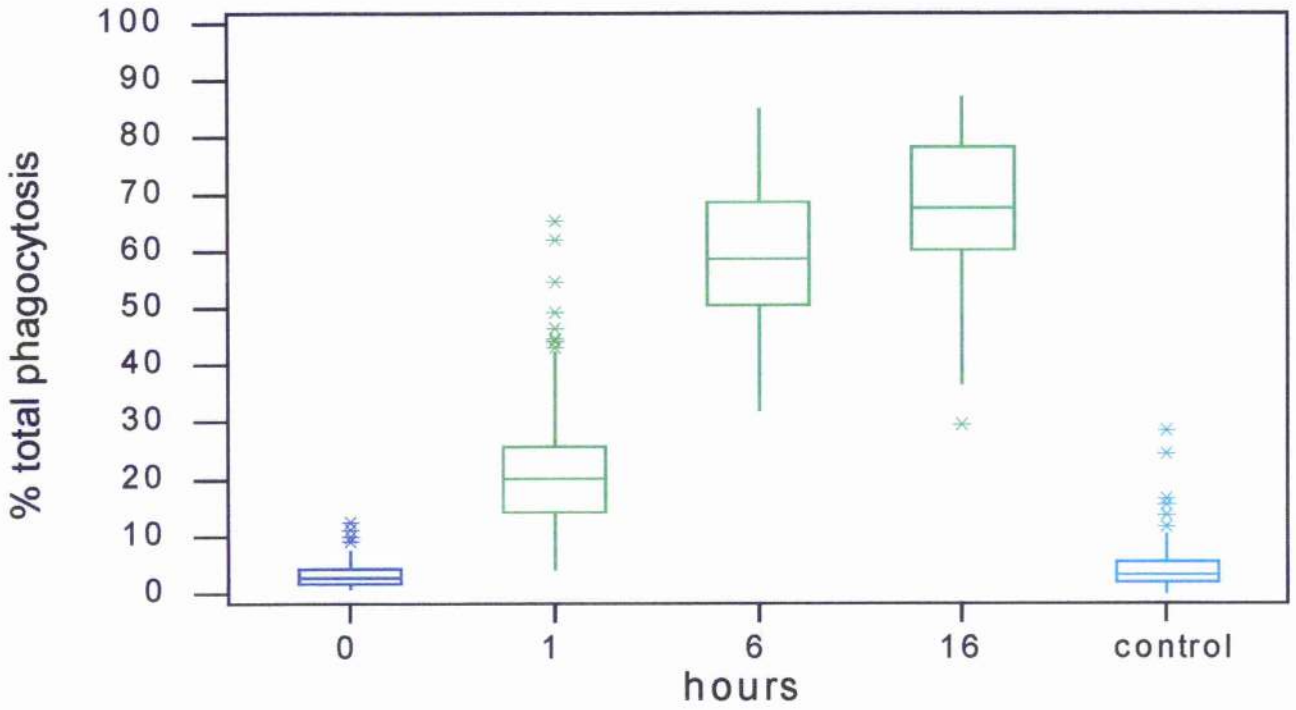
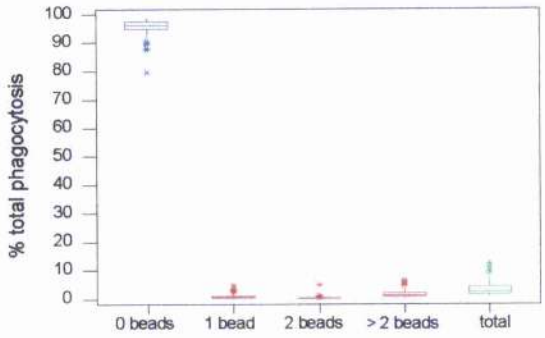
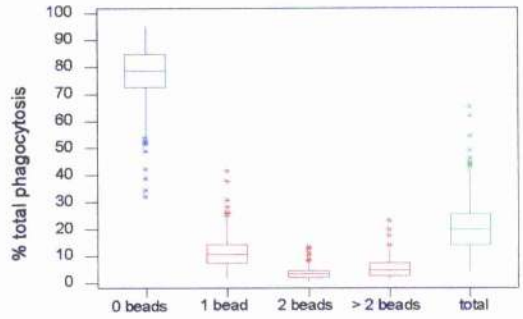


Figure 4.14: *In vitro* percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by the isolated PMN population at each time point, 0 hours (blue), one hour (green), six hours (green), and 16 hours (green) The 4°C sample at 1 hour is designated the control (aqua). The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

a).



b



c).

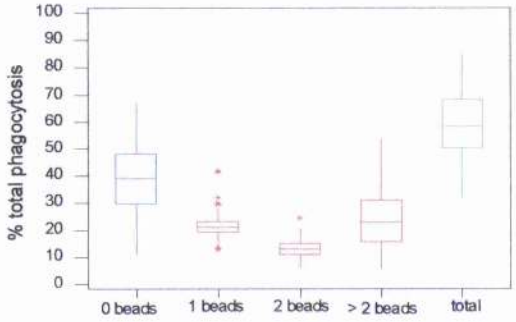


Figure 4.15: A comparison of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from second generation animals (n=140) at 37°C at 0 hours (a), 1hour (b) and 6 hours (c). Graphs shows percentage total phagocytosis of 0 beads (blue), 1 bead (red), 2 beads (red), > 2 beads (red) and the total phagocytosis (green) by isolated PMN. The plot shows the median (line within the box), 25th and 75th percentiles (whiskers) and the outliers (symbol).

beads, over the 16 hour incubation period, are shown in Figures 4.16 (0 hours at 37°C and 1 hour at 4°) and 4.17 (1, 6 and 16 hours at 37°C).

An additional large, granular, cell population was identified in the isolated PMN from some animals, which was absent in the isolated PMN of other animals (Figure 4.18). No phagocytosis of FITC-labelled (2.0μM) latex beads was observed in this population of large, granular cells in any animal possessing these additional cells, when compared to animals without these additional cells in their isolated PMN populations (Figure 4.18).

4.1.3 *In vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by polymorphonuclear cells in whole blood

Many authors have developed whole blood assays to study all aspects of PMN phagocytosis (Hirabayashi, *et al.*, 1985; Hasui *et al.*, 1989; Perticari *et al.*, 1994; Smits *et al.*, 1997). Whole blood assays require smaller volumes of blood, are generally faster than traditional assays involving cell isolation and purification, and may give a more accurate indication of cell function in the cells' natural environment i.e. in the presence of other cell populations and blood components. In this study, *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by PMN in whole blood was assessed in order to determine how much variation, if any, existed in the phagocytic ability of the PMN in whole blood when compared to the phagocytic ability of isolated PMN from the same individual animals. *In vitro* phagocytosis of FITC-labelled (2.0μM) latex beads in whole blood PMN was assessed in approximately 20 second generation animals. Three animals were removed from the analysis following flow cytometry due to poor PMN yield and individual animal samples were discounted if less than 5000 PMN were counted within the PMN gate from the total 50,000 cells counted by flow cytometry. Samples were assessed at 37°C and 4°C at all time points. Variation in the percentage total phagocytosis by PMN in whole blood was seen among individual animals (Figure 4.19). Variation in the percentage total phagocytosis by PMN in whole blood at 37°C was seen among

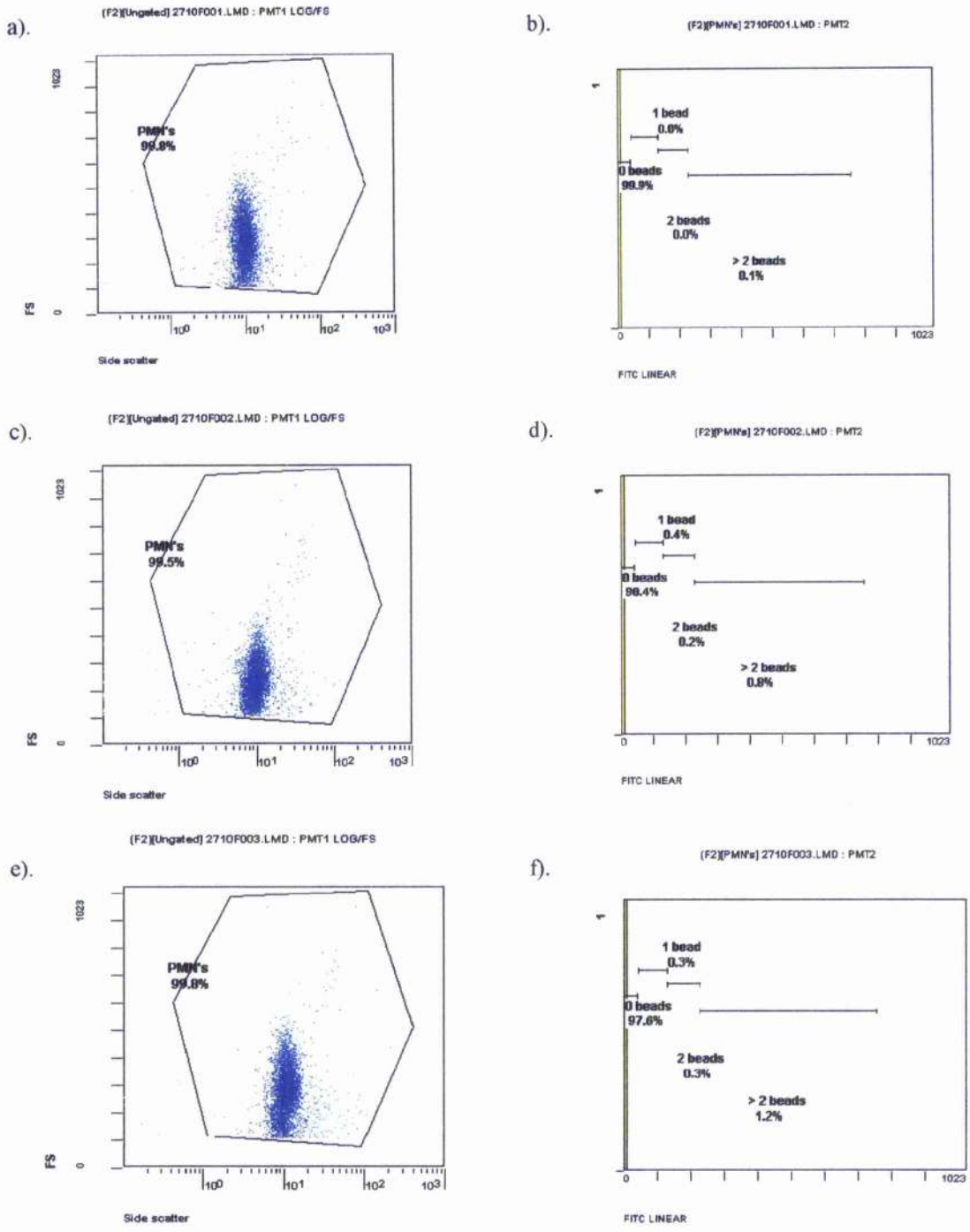


Figure 4.16: Analysis of *in vitro* PMN phagocytosis by flow cytometry. Figures a), c), and e), show the side scatter (x-axis) and forward scatter (y-axis) with the isolated PMN population as PMN only (a), PMN and FITC-labelled (2.0μM) latex beads at 0 hours at 37°C (b) and PMN and FITC-labelled (2.0μM) latex beads at 1 hour at 4°C (c). Figure b), d) and f) show linear graphs of fluorescence (x-axis) and the total number of cells (y axis) with gates for 0 beads (no phagocytosis), 1 bead, 2 beads and > 2 beads.

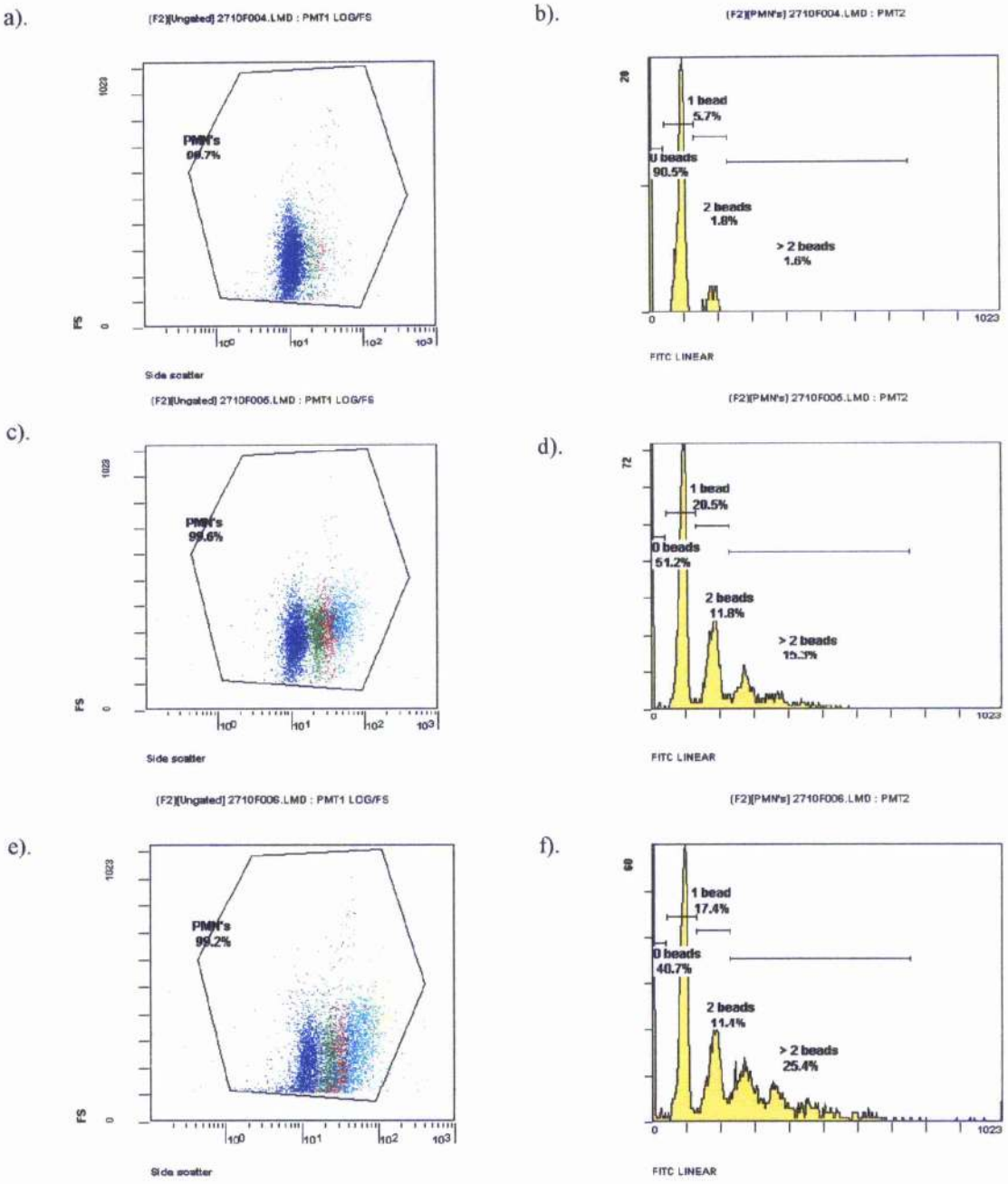


Figure 4.17: Analysis of *in vitro* PMN phagocytosis by flow cytometry. Figures a), c), and e), show the side scatter (x-axis) and forward scatter (y-axis) with the isolated PMN following phagocytosis of the FITC-labelled (2.0μM) latex beads at 1, 6 and 16 hour incubation at 37°C, respectively, PMN were colour coded for the phagocytosis of 0 beads (blue), 1 bead (green), 2 beads (red), and > 2 beads (aqua). Figures b), d) and f) show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with gates for 0 beads (no phagocytosis), 1 bead, 2 beads and > 2 beads.

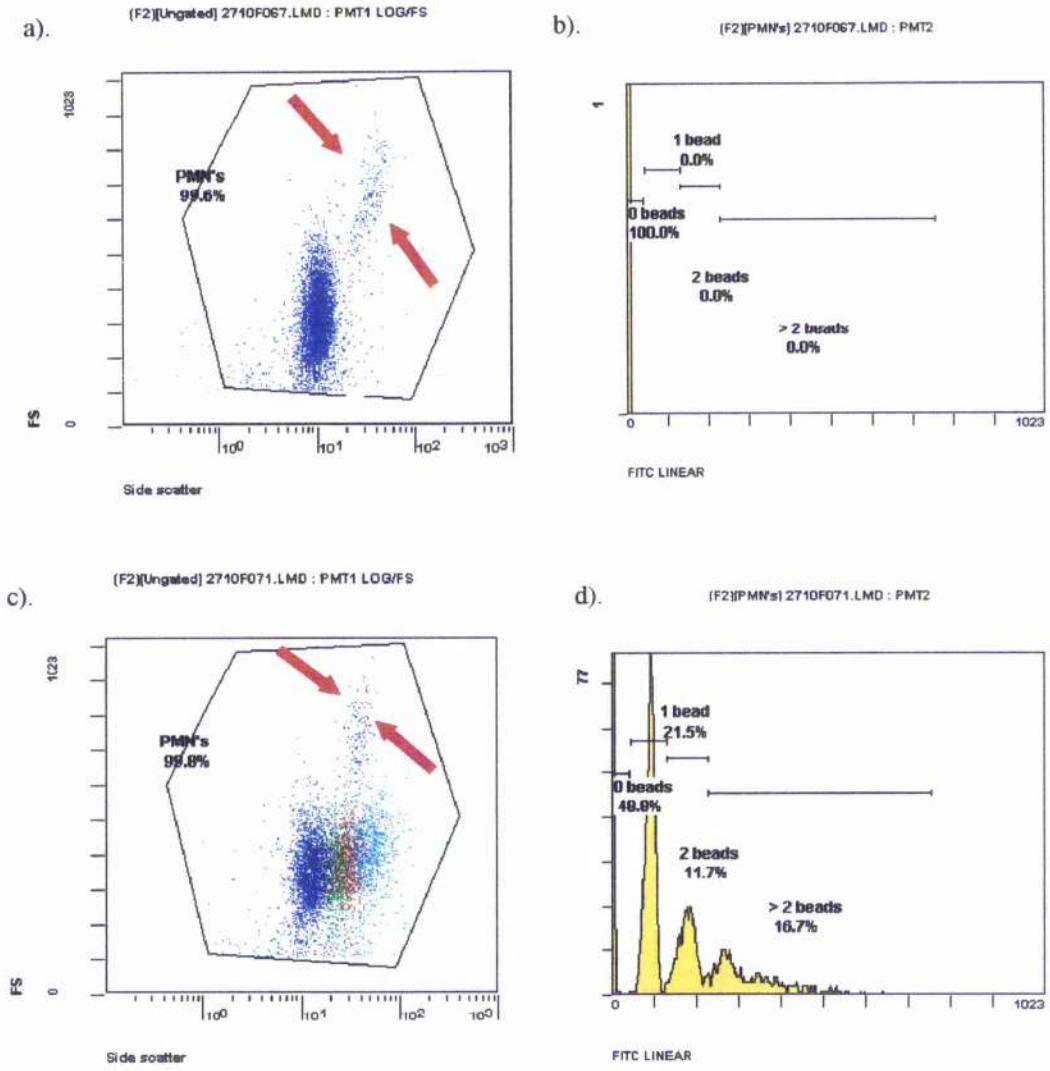


Figure 4.18: Analysis by flow cytometry of additional large, granular cells visible in some isolated PMN populations. Figures a) and c) show side scatter (x-axis) and forward scatter (y-axis) of the isolated PMN. Figure a) shows PMN only. Figure c) shows isolated PMN following incubation with FITC-labelled (2.0μM) latex beads at 6 hours, PMN were colour coded for 0 beads (blue), 1 bead (green), 2 beads (red) and > 2 beads (aqua). Figures b) and d) show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with the gates for 0 beads, 1 bead, 2 beads and >2 beads. Red arrows highlight additional cell population in figures a) and c) which show no visible signs of phagocytosis.

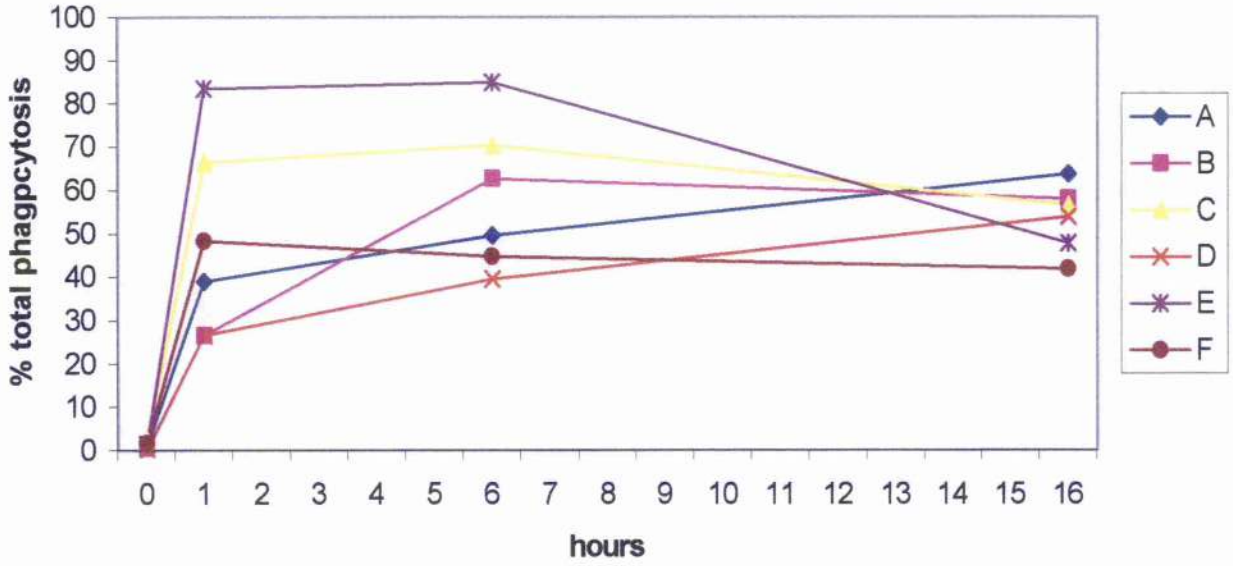


Figure 4.19: Representative example of individual variation of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by PMN cells in whole blood in six second generation animals (A-F). Graph shows hours of incubation (x-axis) and the percentage total phagocytosis (y-axis).

individual animals at each of the time points, with the largest variation among individual animals at the one hour time point (Figure 4.20). The background non-specific adherence at 4°C was less than 10% for the 0, one and six hour time points and increased to approximately 20% at 16 hours (Figure 4.21). Phagocytosis of one bead, two beads and greater than two beads by PMN in whole blood also increased throughout the incubation (Figure 4.22), with maximal phagocytosis reached by one hour of incubation. No significant variation was observed between the percentage total phagocytosis in the one bead, two beads and greater than two beads gates after the one hour incubation point. Following flow cytometry analysis, it could be seen that as PMN in whole blood phagocytosed an increasing number of FITC-labelled latex beads the size of the PMN increased in accordance with the phagocytosis of either one bead, two beads or greater than two beads. Examples of the analysis by flow cytometry, showing the kinetics of the *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by PMN in whole blood, over the 16 hour incubation period, are shown in Figures 4.23 (PMN only), 4.24 (0 hours), 4.25 (1 hour) 4.26 (6 hours) and 4.27 (16 hours).

A gate set for the MNC population was monitored to confirm that MNC did not undergo phagocytosis of the FITC-labelled (2.0μM) latex beads under these conditions. Only PMN gated linear graphs of fluorescence versus total number of cells showed any visible fluorescence and therefore, phagocytosis of FITC-labelled latex beads.

Phagocytosis of FITC-labelled (2.0μM) latex beads by PMN in whole blood was not as visually clear in the gated PMN populations in the graphs of side scatter versus forward scatter when compared visually to the phagocytosis of FITC-labelled (2.0μM) latex beads by the isolated PMN (Figure 4.28). The mean (± S.E.M.) phagocytosis of FITC-labelled (2.0μM) latex beads PMN from whole blood and isolated PMN was very similar. Polymorphonuclear cells from whole blood reached maximal levels of phagocytosis by one hours incubation, compared to the isolated PMN which did not reach peak levels of phagocytosis until approximately six hours incubation (Figure 4.29). Slightly higher levels of non-specific adherence of latex

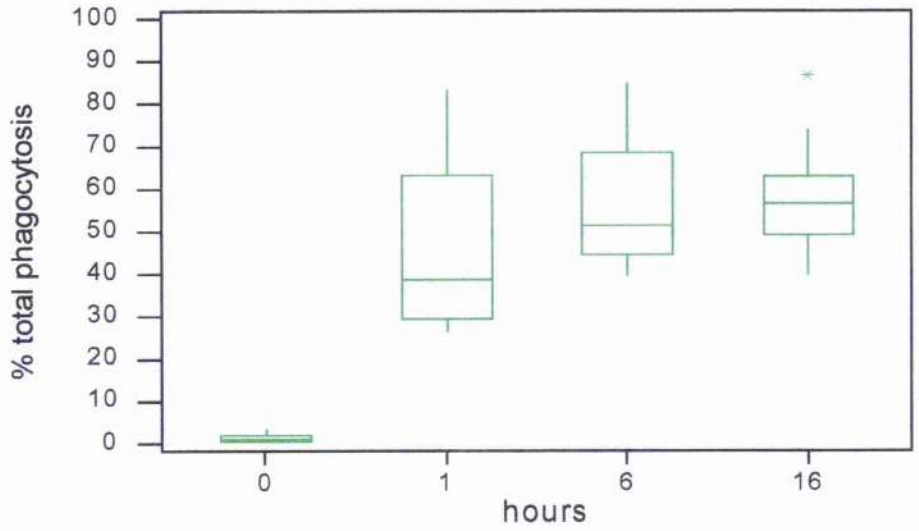


Figure 4.20: *In vitro* percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by the PMN population in whole blood at each time point at 37°C (n=17). The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

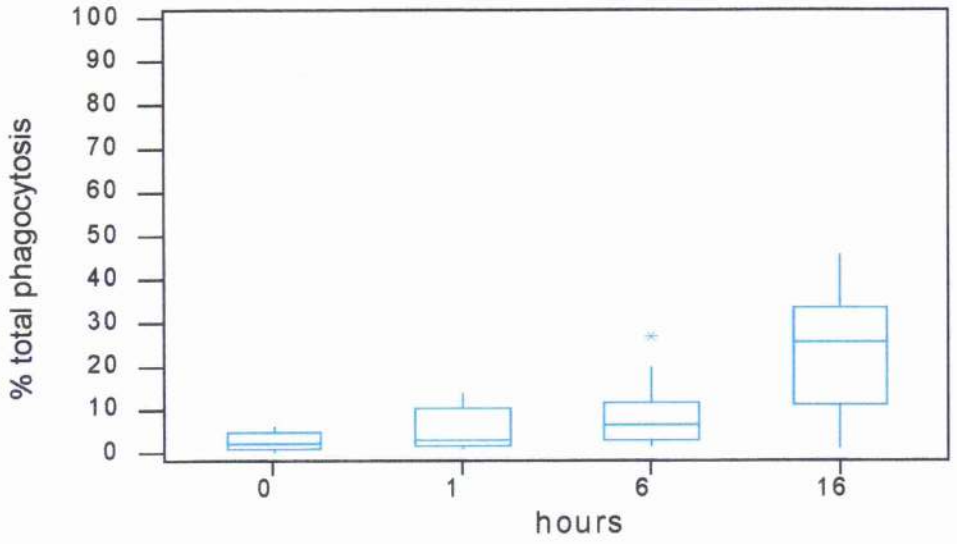
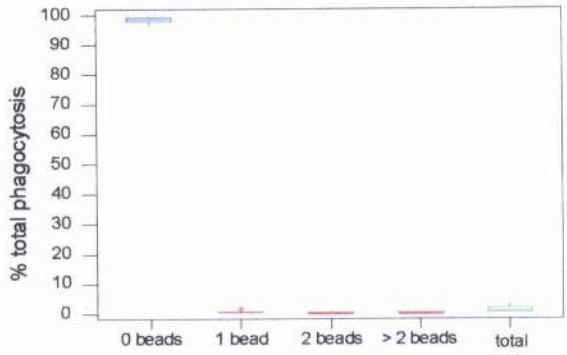
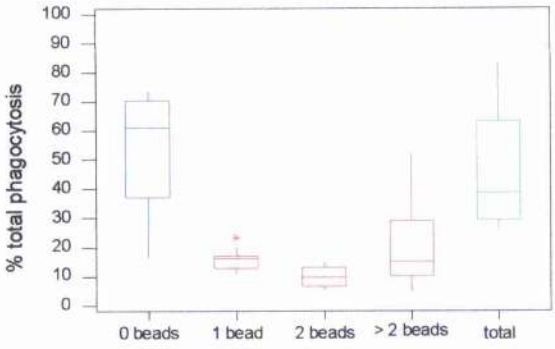


Figure 4.21: *In vitro* percentage total non-specific background adherence/phagocytosis of FITC-labelled (2.0μM) latex beads by the PMN population in whole blood at each time point at 4°C (n=17). The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

a). 0 hours



b). 1 hour



c). 6 hours

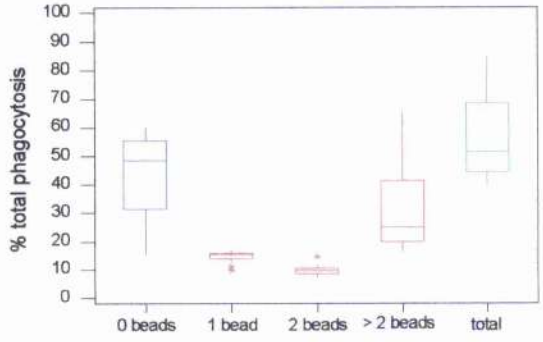


Figure 4.22: A comparison of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by PMN in whole blood in second generation animals (n=17) at 37°C at 0 hours (a), 1 hour (b) and 6 hours (c). The graph shows percentage total phagocytosis of 0 bead (blue), 1 bead (red), 2 beads (red), > 2 beads (red) and the total phagocytosis (green) by PMN in whole blood. The plot shows the median (line within the box), 25th and 75th percentiles (whiskers) and the outliers (symbol).

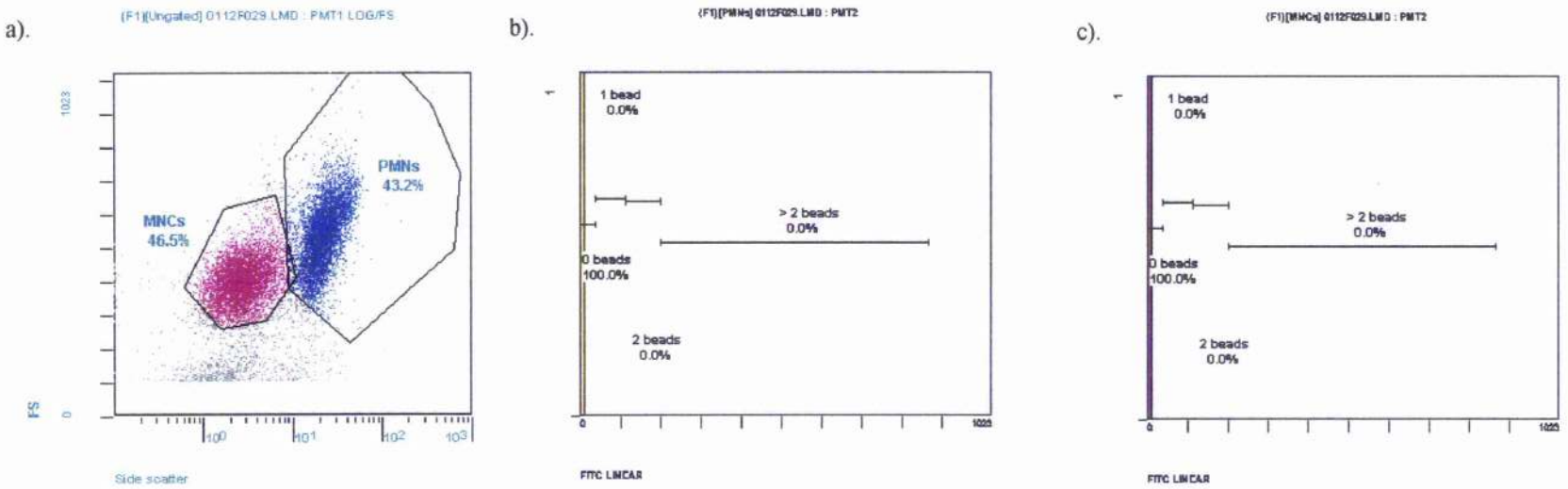


Figure 4.23: *In vitro* analysis of PMN in whole blood by flow cytometry. Figure a). shows side scatter (x-axis) and forward scatter (y-axis) of the PMN in whole blood at 37°C with gates for PMN and mononuclear cells (MNC, pink), PMN were colour coded for 0 beads (blue), 1 bead (green), 2 beads (red) and > 2 beads (aqua) . Figure b) represents the PMN gate in figure a), figure c) represents the MNC gate in figure a) and show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with the gates for 0 beads, 1 bead, 2 beads and > 2 beads.

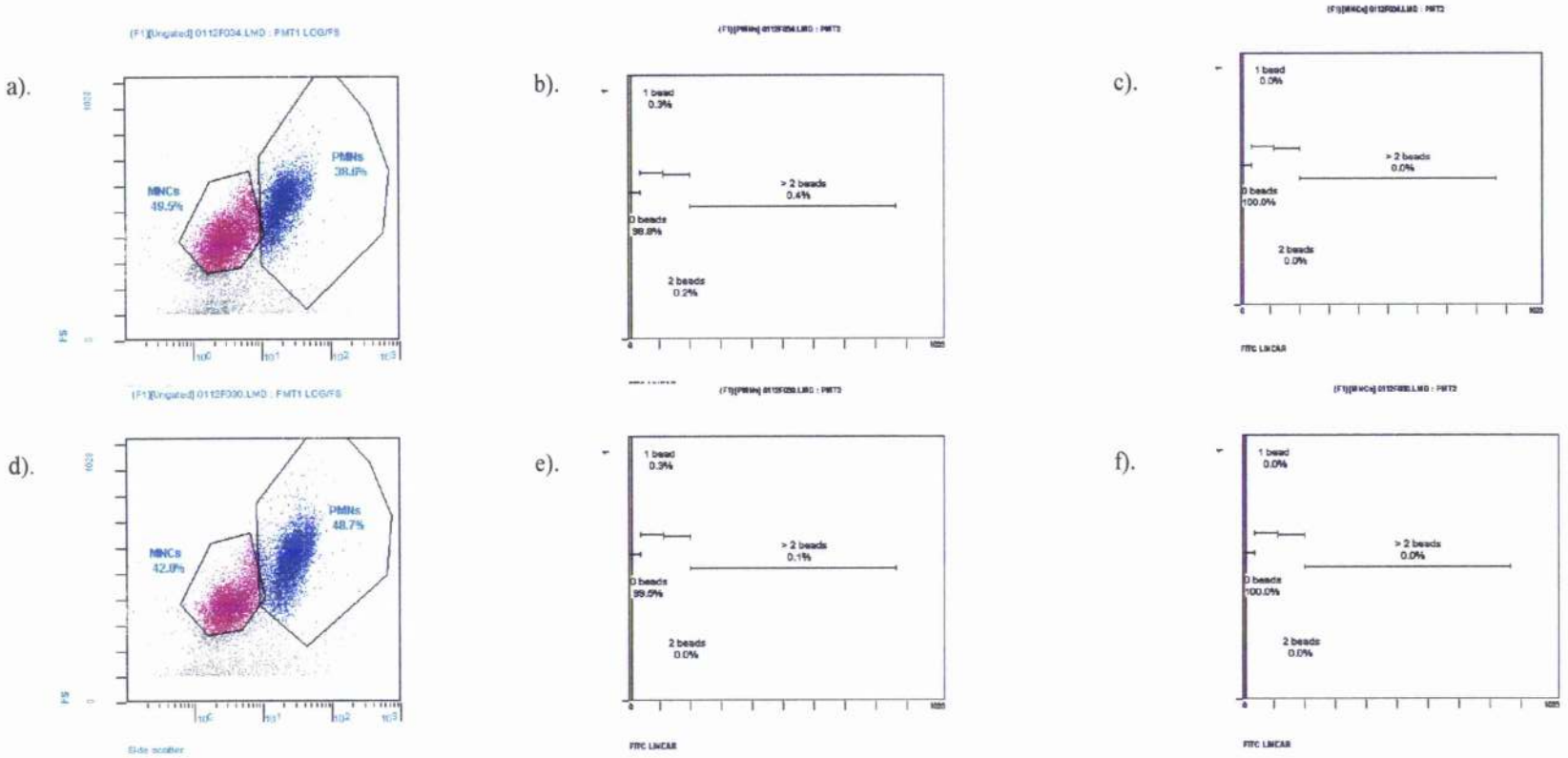


Figure 4.24: *In vitro* analysis of PMN phagocytosis in whole blood by flow cytometry. Figures a) and d) show side scatter (x-axis) and forward scatter (y-axis) of the PMN in whole blood following incubation with FITC-labelled (2.0μM) latex beads for 0 hours at 4°C and 37°C respectively, with gates for PMN and mononuclear cells (MNC, pink), PMN were colour coded for 0 beads (blue), 1 bead (green), 2 beads (red) and >2 beads (aqua). Figures b) and e) represent the PMN gates in figure a) and c), respectively, figures c) and f) represent the MNC gate in figures a) and c), respectively and show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with the gates for 0 beads, 1 bead, 2 beads and >2beads.

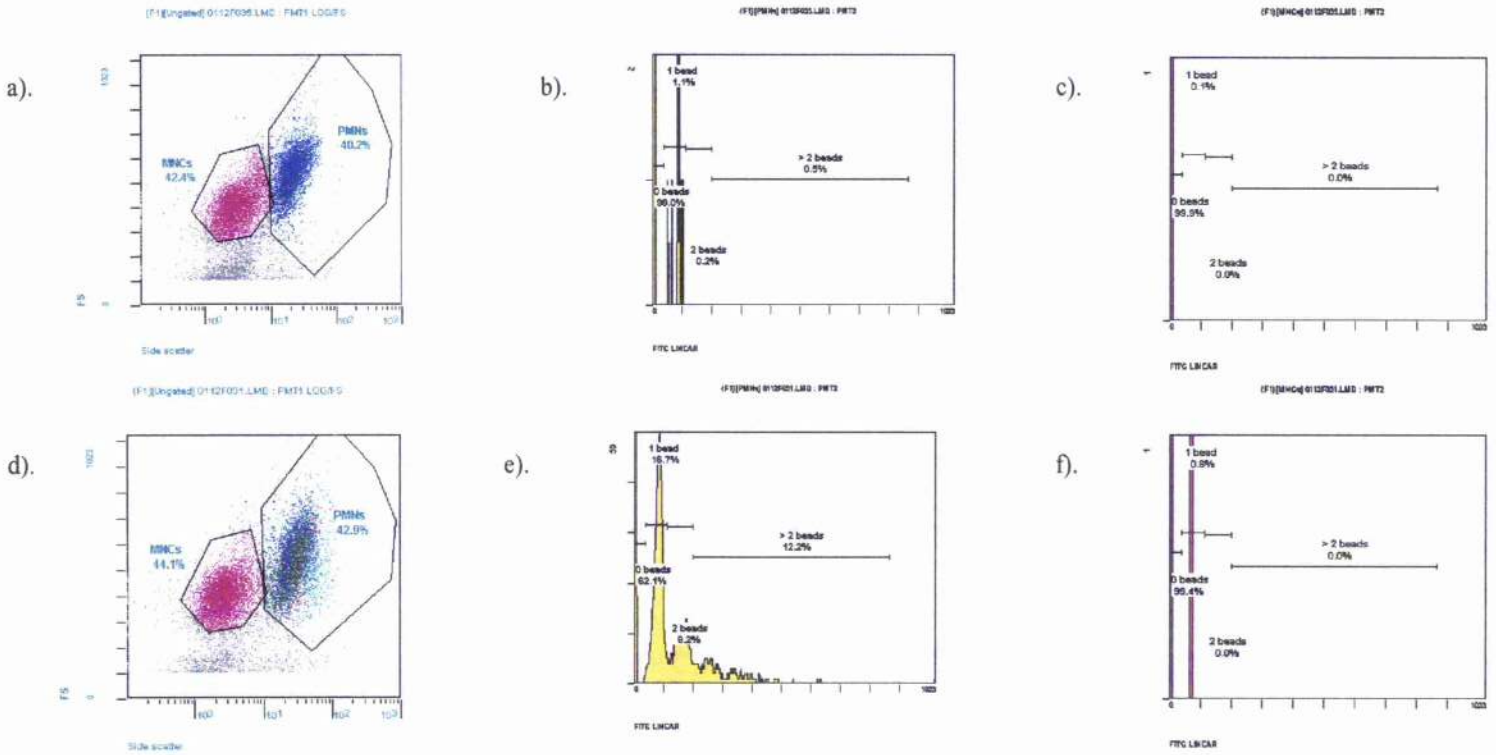


Figure 4.25: *In vitro* analysis of PMN phagocytosis in whole blood by flow cytometry. Figures a) and d) show side scatter (x-axis) and forward scatter (y-axis) of the PMN in whole blood, following incubation with FITC-labelled (2.0μM) latex beads at 1 hour at 4°C and 37°C respectively, with gates for PMN and mononuclear cells (MNC, pink), PMN were colour coded for 0 beads (blue), 1 bead (green), 2 beads (red) and > 2 beads (aqua). Figures b) and e) represent the PMN gates in figure a) and c) respectively, figures c) and f) represent the MNC gates in figures a) and c) respectively and show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with the gates for 0 beads, 1 bead, 2 beads and > 2 beads.

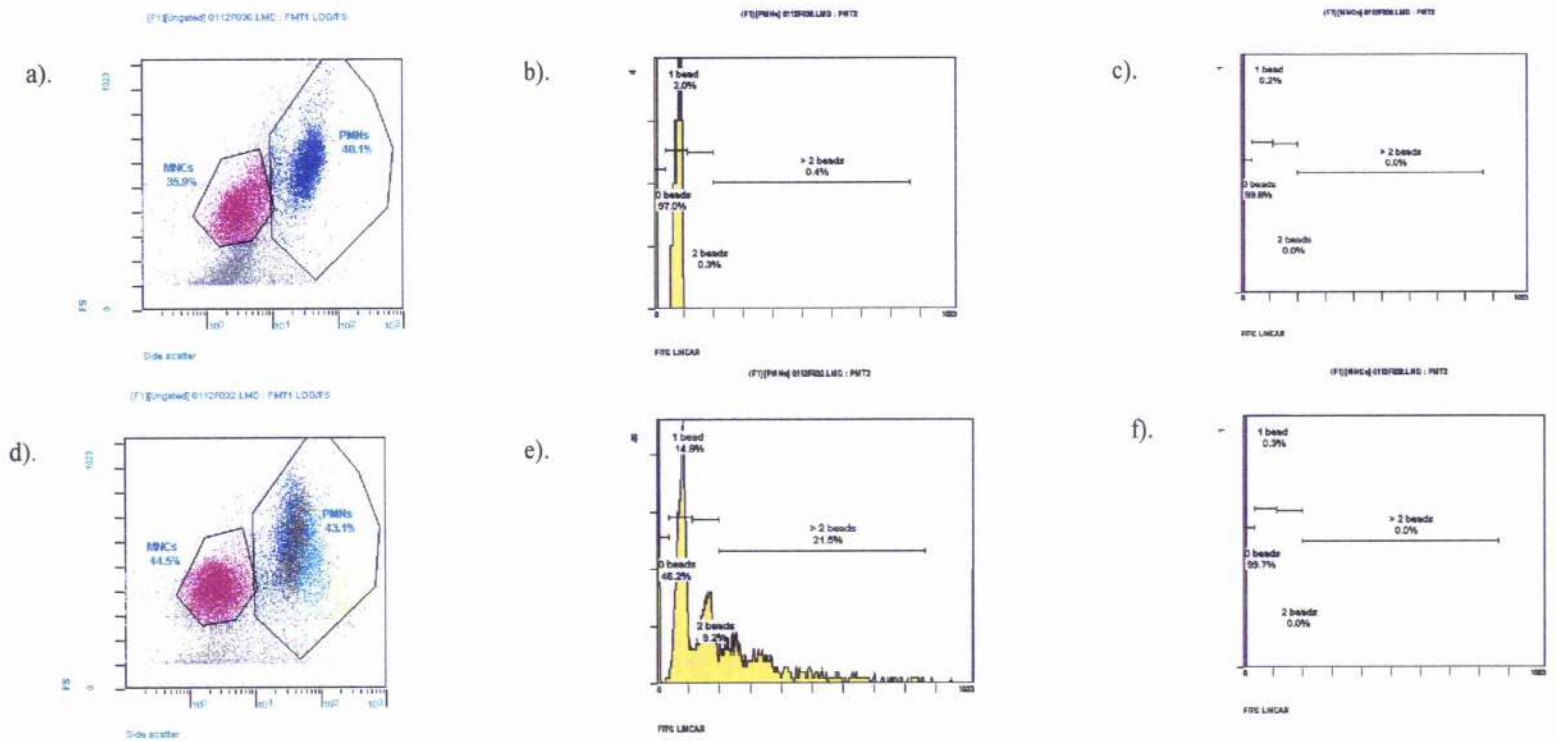


Figure 4.26: *In vitro* analysis of PMN phagocytosis in whole blood by flow cytometry. Figures a) and d) show side scatter (x-axis) and forward scatter (y-axis) of the PMN in whole blood, following incubation with FITC-labelled (2.0μM) latex beads at 6 hours at 4°C and 37°C respectively, with gates for PMN and mononuclear cells (MNC, pink), PMN were colour coded for 0 beads (blue), 1 bead (green), 2 beads (red) and >2 beads (aqua). Figures b) and e) represent the PMN gates in figure a) and c) respectively. Figures c) and f) represent the MNC gates in figures a) and c) respectively and show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with the gates for 0 beads, 1 bead, 2 beads and > 2 beads.

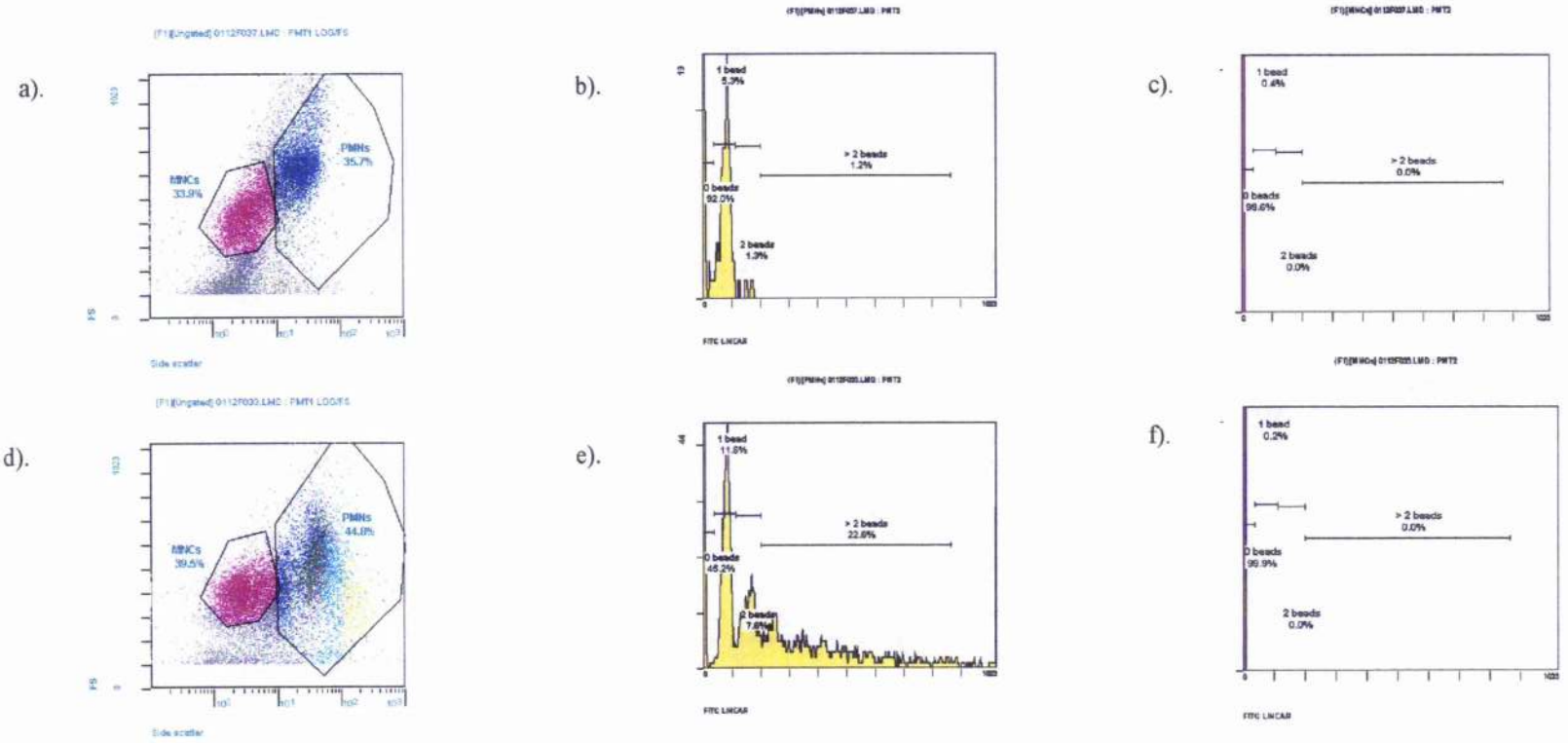


Figure 4.27: *In vitro* analysis of PMN phagocytosis in whole blood by flow cytometry. Figures a) and d) show side scatter (x-axis) and forward scatter (y-axis) of PMN in whole blood, following incubation with FITC-labelled (2.0μM) latex beads at 16 hours at 4°C and 37°C respectively, with gates for PMN and mononuclear cells (MNC, pink), PMN were colour coded for 0 beads (blue), 1 bead (green), 2 beads (red) and >2 beads (aqua). Figures b) and e) represent the PMN gates in figures a) and c) respectively. Figures c) and f) represent the MNC gates in figures a) and c) respectively and show linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with the gates for 0 beads, 1 bead, 2 beads and > 2 beads.

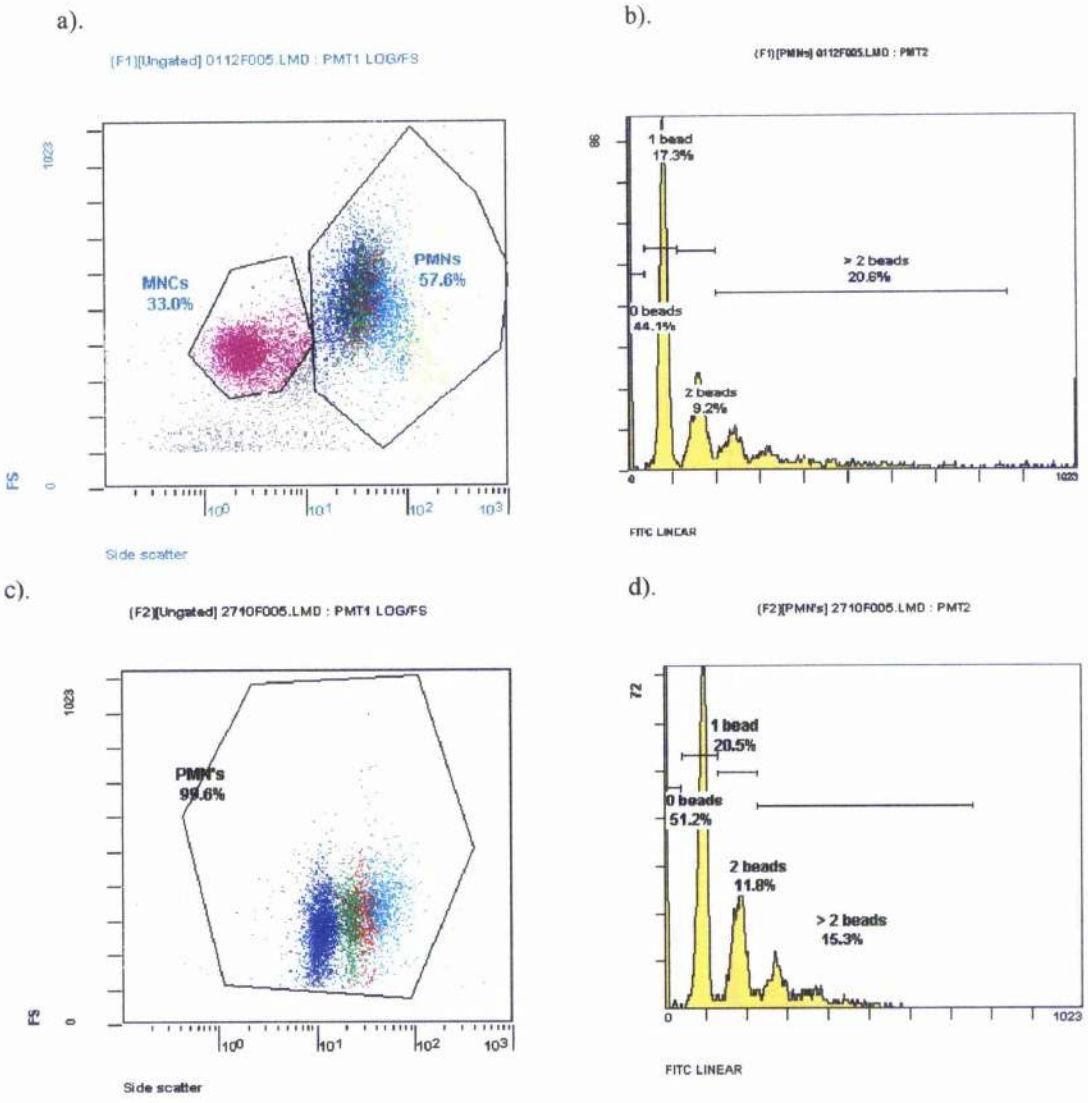


Figure 4.28: Comparison of *in vitro* PMN phagocytosis by flow cytometry of isolated PMN and whole blood PMN with gates for PMN and MNC (pink). Figures a) and c) show the side scatter (x-axis) and forward scatter (y-axis) of the whole blood and isolated PMN phagocytosis of FITC-labelled (2.0μM) latex beads at 6 hours at 37°C, respectively, PMN were colour coded for 0 beads (blue), 1 bead (green), 2 beads (red) and > 2 beads (aqua). Figures b) and d) shows linear graphs of fluorescence (x-axis) and the total number of cells (y-axis) with the gates for 0 beads, 1 bead, 2 beads and > 2 beads.

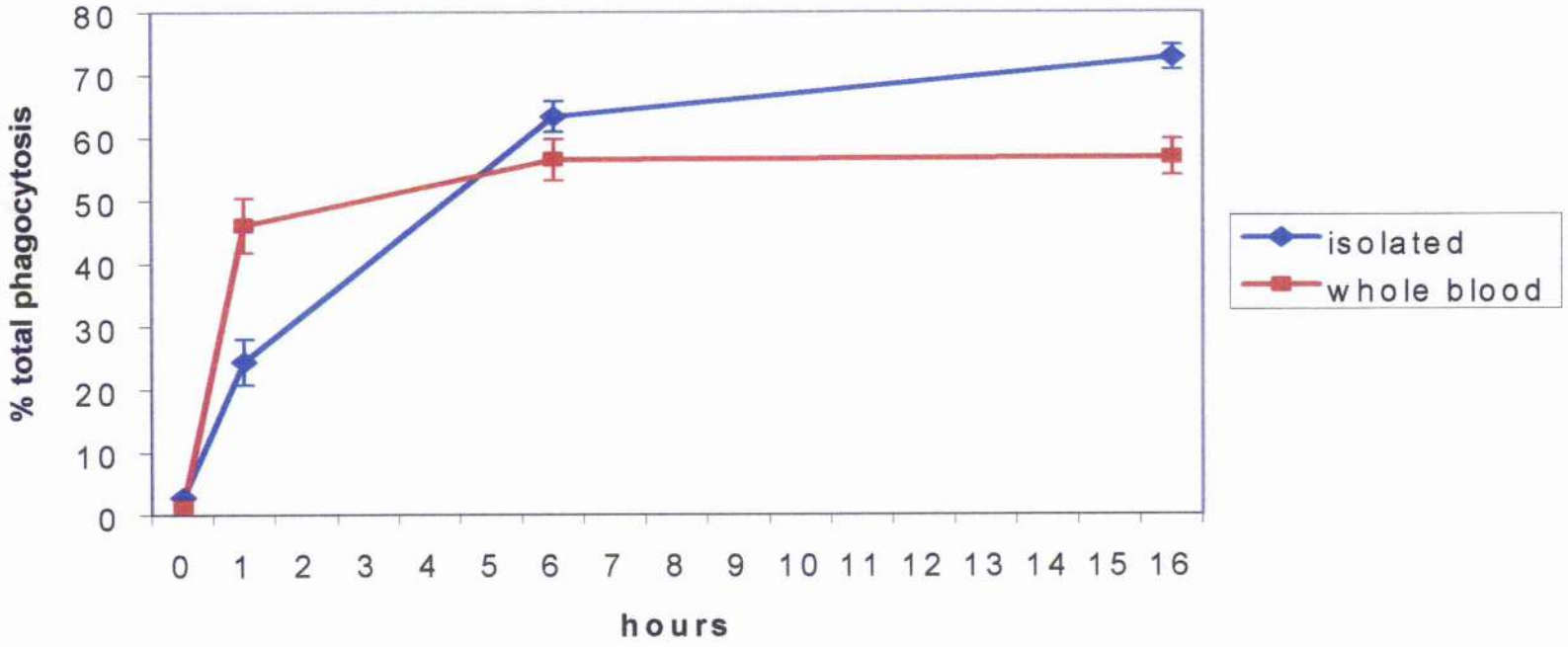


Figure 4.29: Comparison of mean (± S.E.M.) *in vitro* percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN and whole blood PMN (n=17). Graph shows hours of incubation (x-axis) and the percentage total phagocytosis (y axis).

beads to cells at 4^0C were identified in the PMN from whole blood compared to the isolated PMN.

Chapter 5

Data exploration and analyses

5.1 *In vitro* peripheral blood mononuclear cell proliferation

The main statistical analyses were carried out on data obtained from the experimental animals sampled at approximately six months of age. Basic data exploration and statistical analyses which included the assessment of correlation of individual animal responses between days of culture, correlation of individual animal responses following re-sampling (approximately three months after initial sampling), and subsequently, complex REML analysis was carried out for each parameter for all days of culture.

Only basic descriptive statistics (reported in chapter 3) were carried out on the animals over twelve months of age due to the relatively small group numbers, which prevented more detailed data exploration and statistical assessment.

5.2 Data Exploration

5.2.1 Experimental animals sampled at approximately six months of age

Three hundred and sixteen second generation animals sampled at approximately six months of age from the 1998, 1999 and 2000 cohorts were assessed for each parameter for all days of culture.

5.2.2 Distribution of data

A preliminary univariate analysis was performed using SAS statistical software. The data exploration carried out using the univariate analyses procedure generated basic parameters such as Mean, Variance, Minimum, Maximum, Skewness and Kurtosis for each data set studied.

In general the data was positively skewed (Figure 5.1, Table 5.1). A subjective determination of the positive skewness was obtained from the histograms of the raw data for each parameter i.e. *S. aureus*-induced, PHA-induced or control proliferation, on each of the days of culture (Figure 5.1). The positive skewness indicated that most animals had relatively low levels of *in vitro* proliferation, whilst some animals had considerably higher levels of *in vitro* proliferation compared to the majority of the population. The skewness of the data increased considerably in the PHA-induced and control proliferation on days nine and ten of culture (Table 5.1). The *S. aureus*-induced proliferation also increased in skewness on days nine and ten of culture, however this increase was approximately a two-fold increase compared to a five to ten fold increase for the PHA-induced and control proliferation (Table 5.1). This increase in the skewness on days nine and ten of culture in all of the parameters may correspond with increased numbers of dead and dying cells in culture at this point in the assay. This results in the majority of the cells having very low levels of proliferation due to their lack of viability and only a few of the live cells which remain in culture are able to proliferate.

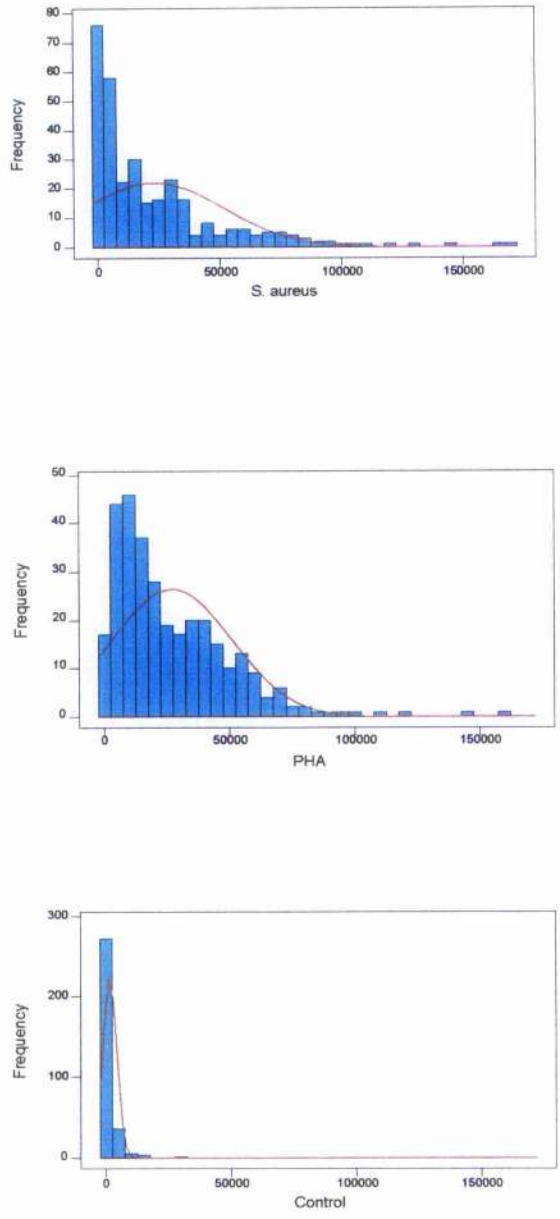


Figure 5.1: Graphical representation of positive skewness of *in vitro* PBMC proliferation on day four of culture. Figures a) *S. aureus*-induced (skewness=2.05), b) PHA-induced (skewness=1.73) and c) control proliferation (skewness=5.4). Red line represents a normal distribution.

PBMC PROLIFERATION	DAY 2		DAY 3		DAY 4		DAY 5		DAY 9		DAY 10	
	Skew	Prob (norm)	Skew	Prob (norm)	Skew	Prob (norm)	Skew	Prob (norm)	Skew	Prob (norm)	Skew	Prob (norm)
***S. aureus* induced**	1.94	0.0001	1.95	0.0001	2.05	0.0001	1.79	0.0001	3.39	0.0001	4.08	0.0001
PHA induced	1.75	0.0001	0.99	0.0001	1.73	0.0001	3.19	0.0001	11.58	0.0001	10.17	0.0001
Control (PBMC only)	2.26	0.0001	3.14	0.0001	5.40	0.0001	4.98	0.0001	11.65	0.0001	12.44	0.0001

Table 5.1: Univariate analyses of *in vitro* PBMC proliferation data from all second generation animals sampled at approximately six months (n=316) performed using SAS statistical software. Skew = Skewness, Prob (Norm) indicates the significance of the data being significantly different from a normal distribution calculated using the Shapiro-Wilk W test. The skewness of *S. aureus*-induced, PHA-induced or control (PBMC only) proliferation is indicated for each of the assay days i.e. days two, three, four, five, nine and ten of culture.

The normal probability plot was used as a measure of the distribution of the data: a straight line indicates a normally distributed data set. The Shapiro-Wilk W test was used to give an objective measurement of the distribution and returned a significant value if the data was non-normally distributed. The raw data for *in vitro* PBMC proliferation was non-normal for all the parameters measured i.e. *S. aureus*-induced, PHA-induced or control proliferation, for all days of culture (Table 5.1).

5.2.3 Box and Cox transformation

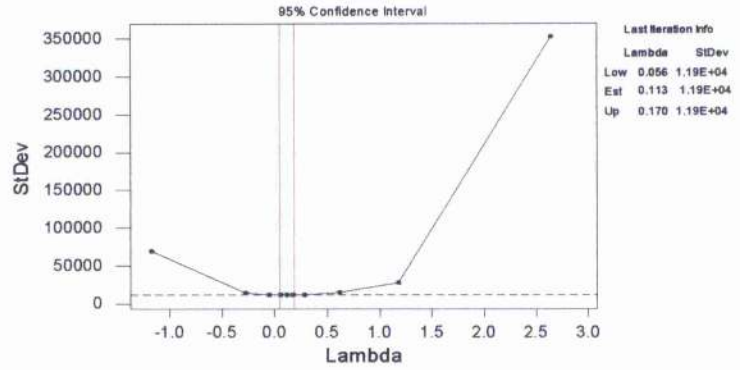
Before further statistical analyses could be undertaken, the skewness of the data indicated that statistical manipulations were required to bring it closer to a normal distribution. The optimal transformation of the data was determined using Box and Cox analysis in Minitab (Release 13). Optimal transformations were assessed for each parameter i.e. *S. aureus*-induced, PHA-induced or control PBMC proliferation, for all days of culture. Some variation was seen in the optimal transformation suggested between the days of culture for each of the individual parameters (Table 5.2). Variation in the optimal transformation suggested was also seen between the parameters within specific days of culture. A natural logarithm (ln) transformation was chosen as the optimal transformation for the data set overall due to the identification that a ln transformation was found to be near optimal for all the parameters on day four of culture, the day of peak *S. aureus*-induced proliferation (Figure 5.2). The use of a common transformation for all the parameters allowed easier biological interpretation of the data analyses for all the parameters, i.e. *S. aureus*-induced, PHA-induced and control proliferation, on each day of culture.

All subsequent statistical procedures carried out on the data from the second generation animals sampled at approximately six months of age required that the data was normally distributed, and were thus carried out using ln-transformed data.

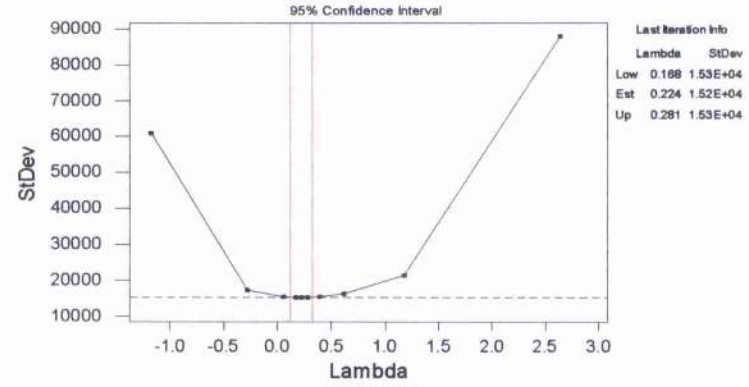
PBMC PROLIFERATION	DAY 2		DAY 3		DAY 4		DAY 5		DAY 9		DAY 10	
	λ	Trans.	λ	Trans.	λ	Trans.	λ	Trans.	λ	Trans.	λ	Trans.
***S. aureus* induced**	0.113	$\log_e Y$	0.113	$\log_e Y$	0.113	$\log_e Y$	0.113	$\log_e Y$	0.112	$\log_e Y$	0.000	$\log_e Y$
PHA induced	0.337	$\sqrt{Y}$	0.337	$\sqrt{Y}$	0.224	$\log_e Y$	0.000	$\log_e Y$	0.000	$\log_e Y$	-0.225	$\log_e Y$
Control (PBMC only)	0.000	$\log_e Y$	-0.112	$\log_e Y$	-0.225	$\log_e Y$	-0.337	$1/\sqrt{(Y)}$	-0.449	$1/\sqrt{(Y)}$	-0.449	$1/\sqrt{(Y)}$

Table 5.2: Box and Cox transformation of *in vitro* PBMC proliferation data from all second generation animals sampled at approximately six months of age (n=316) performed using Minitab (Release 13). λ = mean estimated lambda value, Trans.= optimal recognised transformation for the data (Table 2.3).

a).



b).



c).

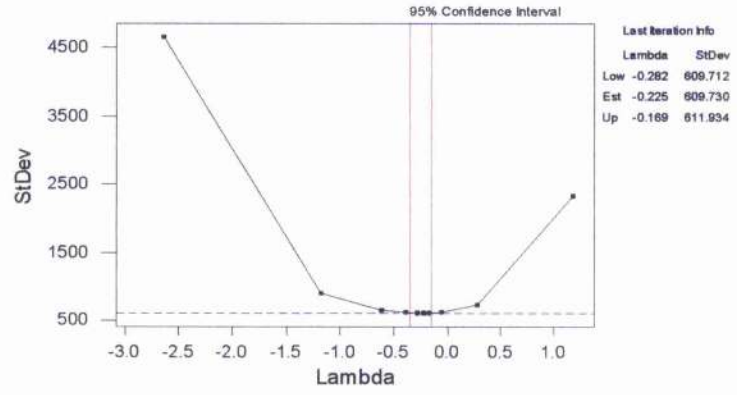


Figure 5.2: Box and Cox transformation of *in vitro* PBMC proliferation on day four of culture. Figures a), b) and c) show *S. aureus*-induced, PHA-induced and control proliferation, respectively.

5.2.4 Correlations

5.2.4.1 Correlation of individual animal responses between days culture

The Pearson's Product Moment Correlation Coefficient in Minitab (Release 13) was used to calculate the correlation of individual animal responses between days of culture, i.e. the correlation between day two and day three, day three and day four, day four and day five, day five and day nine, and day nine and day ten of a single assay. This was carried out to assess if individual animals showed any trend in the ability of their PBMC to proliferate *in vitro* e.g. if animals had relatively high levels of *S. aureus*-induced proliferation on day two of culture did they remain relatively high responders throughout the time-course of the assay; did animals with low levels of *S. aureus*-induced proliferation on day two of culture remain poor responders or did animals change from low to high responders or *vice versa* during *in vitro* culture.

A significant positive correlation was identified between the time points for *S. aureus*-induced, PHA-induced and control proliferation (Figures 5.3–5.5). This positive correlation confirmed the validity of the assay and showed that individual animals do not have major fluctuations in the levels of *in vitro* PBMC proliferation between days of culture in a single assay, indicating that if animals had relatively high levels of proliferation on day two of culture then they still had relatively high levels of proliferation on day three and subsequent days of culture when compared to the rest of the population.

Pearson's Product Moment Correlation Coefficients were also carried out to determine if any relationship existed between *S. aureus*-induced proliferation and control proliferation, PHA-induced proliferation and control proliferation, and *S. aureus*-induced and PHA-induced proliferation. This was carried out to assess if any correlation existed between: 1) the levels of *in vitro* PBMC proliferation in the presence of a specific immune stimulus when compared with the PBMC proliferation in the absence of any immune stimulus; 2) the levels of *in vitro* PBMC proliferation in the presence of a non-specific immune stimulus when compared with the PBMC

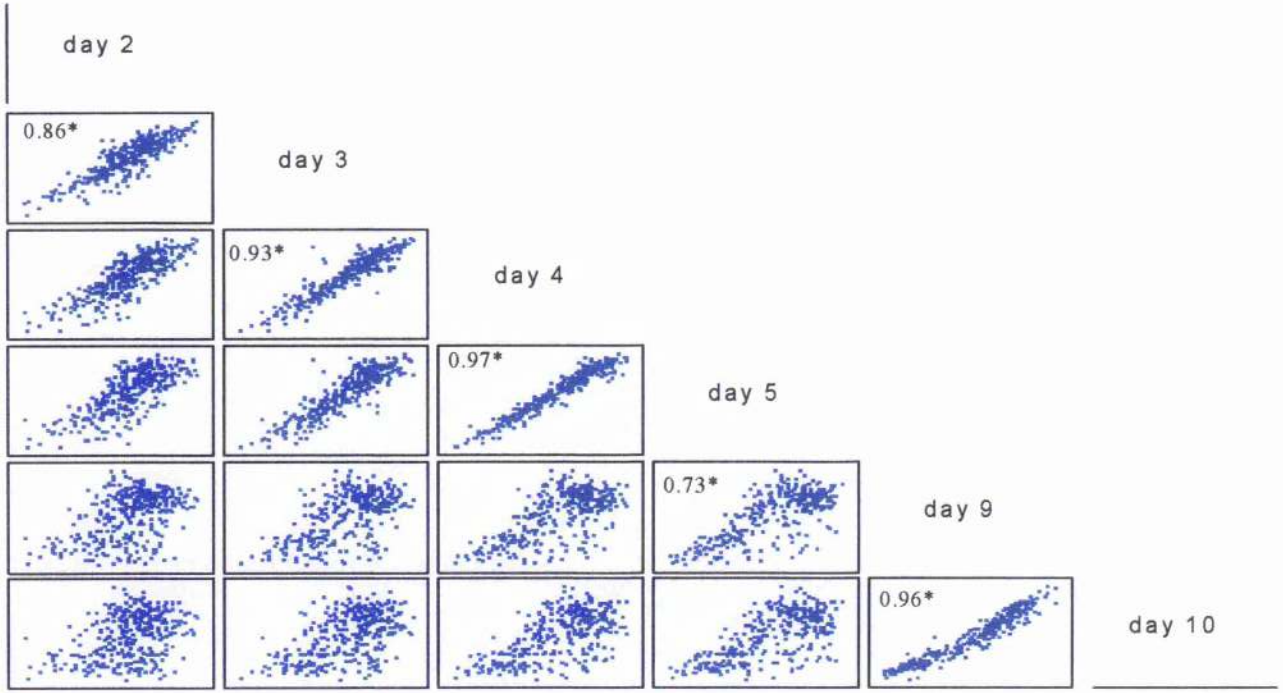


Figure 5.3: Matrix scatter plot showing correlation of individual animal responses between day of culture for *S. aureus*-induced *in vitro* PBMC proliferation. Correlations are shown between days. * indicates a significant correlation of $p<0.001$.

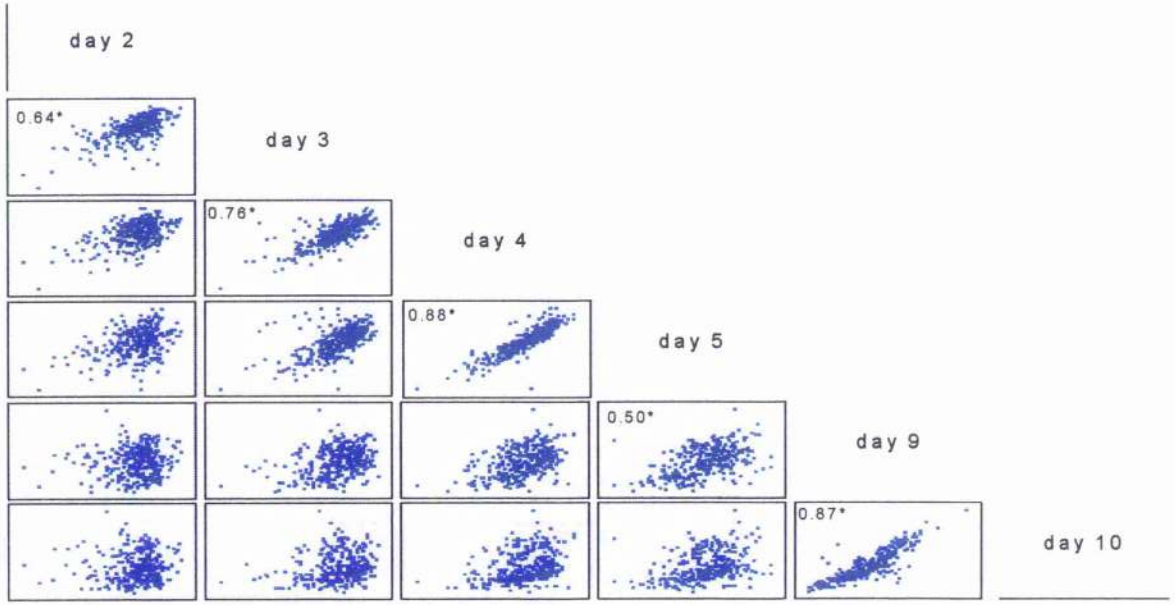


Figure 5.4: Matrix scatter plot showing correlation of individual animal responses between day of culture for PHA-induced *in vitro* PBMC proliferation. Correlations are shown between days. * indicates a significant correlation of $p<0.001$.

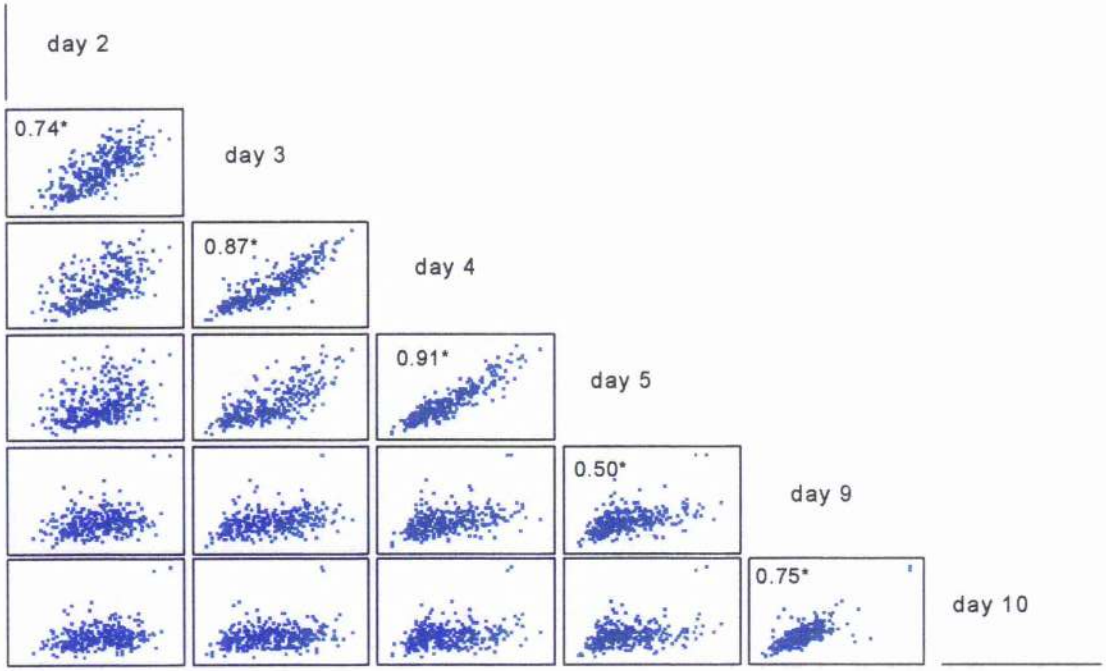


Figure 5.5: Matrix scatter plot showing correlation of individual animal responses between day of culture for control *in vitro* PBMC proliferation. Correlations are shown between days. * indicates a significant correlation of $p<0.001$.

in the absence of any immune stimulus; 3) the levels of *in vitro* PBMC proliferation in the presence of a specific or a non-specific stimulus. This assessment may be used to confirm the validity of using both specific and non-specific immune stimuli in the assessment of an individual animals *in vitro* PBMC function.

Significant positive correlations were identified between *S. aureus*-induced proliferation and control proliferation on all days of culture, however, the positive correlation decreased over time, from 0.68 ($p<0.001$) on day two, to 0.27 ($p<0.001$) on day four, and to 0.24 ($p<0.001$) on day ten of culture. Significant positive correlations were identified between PHA-induced proliferation and control proliferation on all the days of culture. The positive correlation was low 0.19 ($p<0.001$) on day two, peak PHA-induced proliferation, and increased to 0.34 ($p<0.001$) on day four, but reduced again by day ten of culture 0.18 ($p<0.01$) culture. Overall, the correlations observed were lower between control PBMC proliferation and PHA-induced PBMC proliferation than between control PBMC proliferation and *S. aureus*-induced PBMC proliferation. No significant correlations were found between *S. aureus*-induced PBMC proliferation and PHA-induced PBMC proliferation on any of the days of culture. In general, for both the *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation, the correlation between control PBMC and PBMC which received either a specific or non-specific immune stimulus decreased around peak proliferation. This confirmed the validity of assessing *in vitro* antigen or mitogen stimulated PBMC as a measure of variation in PBMC proliferation among individual animals.

5.2.4.2 Correlation of individual animal responses at re-sampling (approximately three months after initial sampling)

One hundred and twelve (approximately 35% of the total animals sampled at approximately six months of age, Table 2.1) second generation animals were re-sampled approximately three months after the initial sampling i.e. at nine months of age, from the sampling years 1998, 1999 and 2000. This was carried out to assess the correlations of individual animal responses at re-sampling. This was used as an

indicator of the accuracy of the assay as a measure of variation in the proliferative ability among animals and not just variation in the assay over time.

Repeatability was assessed using the Pearson's Product Moment Correlation Coefficient in Minitab (Release 13). A significant positive correlation was seen for *S. aureus*-induced and for control proliferation on days four and five of culture ($p<0.05$). This indicated that the *S. aureus*-induced and the control proliferation on days of peak proliferation was repeatable in individual animals re-sampled within three months. An almost significant ($p<0.1$) positive correlation for PHA-induced proliferation was seen on day four of culture, which indicated that variation existed in the PHA-induced response in individual animals when re-sampled within three months. No significant correlations were found for any of the other assay days (Table 5.3, Figure 5.6).

5.3 Data analyses

5.3.1 Linear Mixed Models – Residual Maximum Likelihood

5.3.1.1 Animals and model

A total of 302 animals were analysed, a small number of the animals (14) were removed from the total data set (316) due to inaccurate sire identification. Initially, the data was assessed as a complete data set analysing all the animals, males and females together. However, following the analyses of the males and females together, there was evidence of interactions between sexes, which prompted repeating the analyses on single sex subsets.

Preliminary analyses were carried out on all days of culture, i.e. day two, day three, day four, day five, day nine and day ten. Due to the lack of biological viability of the

PBMC PROLIFERATION	DAY 2		DAY 3		DAY 4		DAY 5		DAY 9		DAY 10	
	Corr.	*p* value	Corr.	*p* value	Corr.	*p* value	Corr.	*p* value	Corr.	*p* value	Corr.	*p* value
***S. aureus* induced**	0.124	0.196	0.170	0.075	**0.296**	**0.002**	**0.346**	**0.000**	0.005	0.955	0.005	0.960
PHA induced	0.125	0.195	0.120	0.212	0.178	0.062	0.095	0.332	-0.016	0.866	0.136	0.156
Control (PBMC only)	0.024	0.800	0.127	0.185	**0.317**	**0.001**	**0.384**	**0.000**	0.037	0.701	-0.027	0.781

Table 5.3: Correlation of individual animal responses when re-sampled (approximately three months after initial sampling) assessed using the Pearson's Product Moment Correlation Coefficient in Minitab (Release 13). One hundred and twelve (approximately 35% of the total animals sampled at six months of age, Table 2.1) second generation animals were re-sampled from the sampling years (1998, 1999 and 2000). Repeatability correlations are shown for *S. aureus*-induced, PHA-induced or control *in vitro* PBMC proliferation on days two, three, four, five, nine and ten of culture. Corr=Pearson's Product Moment Correlation Coefficient, *p* value=significance level. Significant repeatability correlations are shown in red ($p<0.01$), almost significant repeatability correlations are shown in green ($p<0.1$).

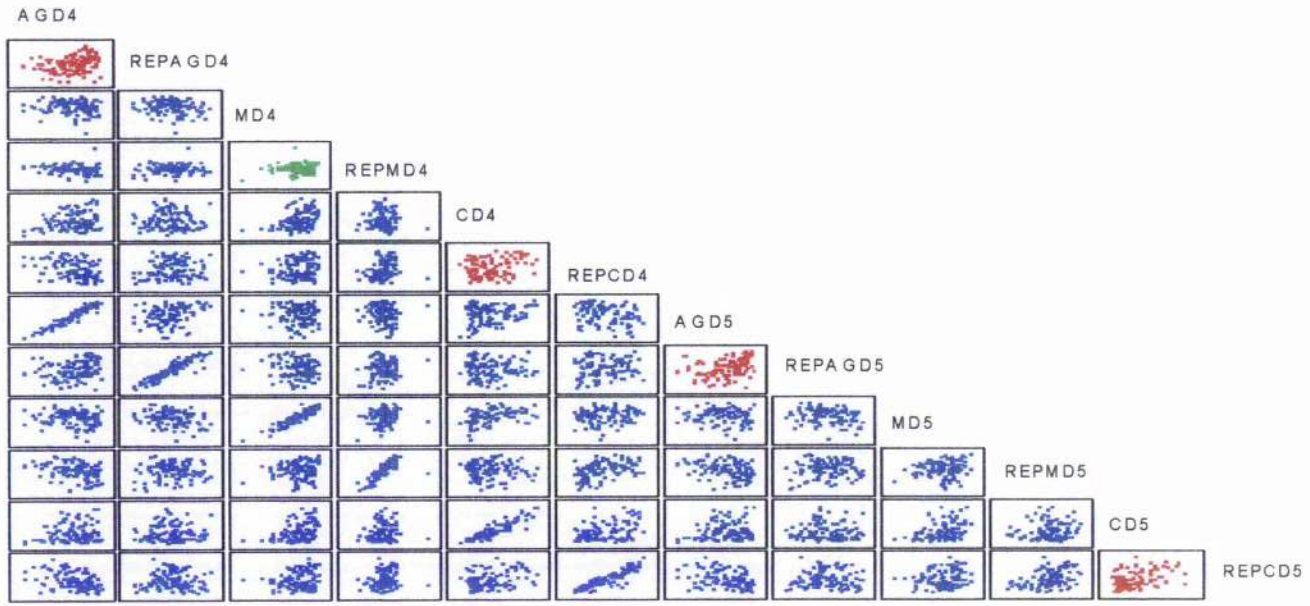


Figure 5.6: Matrix scatter plot showing correlation of individual animal responses at re-sampling (approximately three months after initial sampling) for *S. aureus*-induced, PHA-induced, and control *in vitro* PBMC proliferation on days four and five of culture. Significant positive repeatability correlations are shown in red ($p<0.05$). Almost significant positive repeatability correlations are shown in green ($p<0.1$). AG = *S. aureus*-induced proliferation, D = day of culture, M = PHA-induced proliferation, C = control proliferation and REP = repeat results on the same individuals approximately three months after the initial sampling.

cells by days nine and ten of culture for all the parameters assessed, it was considered appropriate to exclude days nine and ten from any subsequent interpretation of the results. Complete analyses of all parameters for all days of culture is shown in appendices C.1 to C.18. The remainder of this chapter reports results from days two, three four and five of culture only as these were considered to be of most biological significance.

5.3.1.1.1 Fixed factors used in the model

The fixed factors used in the final model were: the sex of the individual which had two levels, male and female; the cross of the individual, which had three levels, backcross Charolais, backcross Holstein-Friesian or F2 (50:50 Charolais:Holstein-Friesian); the year of birth, which had three levels, 1998, 1999 or 2000; the date the animal was sampled, which had 27 levels, and the sample age, a covariate, that was the age in days of the individual animal at the point of sampling.

Following preliminary analyses, the regression coefficient for the control was found to be less than one and greater than zero, indicating that the control proliferation was significant for each individual animal and, thus, the use of Δ counts per minute or stimulation indices in the analyses would be inappropriate. The control *in vitro* PBMC proliferation for each individual animal, i.e. the level of PBMC proliferation in the absence of any immune stimuli was, therefore, retained within the model, and the significant effect of the control as a fixed factor in the *S. aureus*-induced and PHA-induced models and as the Y variate was assessed subsequently.

5.3.1.1.2 Random factor used in the model

The sire of the individual animal was assessed as the random factor in the model. There were a total of 12 sires used in this study: eight of the sires were F1 (50:50, Holstein-Friesian: Charolais) sires (RO1, RO2, RO5, R11, R12, R15, R18 and R21); and four of the sires were F0 Charolais (CH108, CH158, CH200 and CH219).

5.3.1.1.3 Complete data set model

For the complete data set, the *S. aureus*-induced and PHA-induced and control proliferation was assessed as the Y variate in the model. The REML model used in the analyses of the complete data set (except in the analyses of the control as the Y variate, where control was removed as a fixed factor) was:

Fixed sex + cross + year of birth + sample date + sample age + control
Random sire

5.3.1.1.4 Subset model

The REML model used for the analyses of the subsets of males and females, except in the analyses of the control as the Y variate (where control was removed as a fixed factor) was:

Fixed cross + year of birth + sample date + sample age + control
Random sire

The date the individual animal was sampled on, was open to a wide range of influences including environmental influences such as seasonal variation, housing conditions and the sampling procedures employed in the study which resulted in the animals being sampled in single sex groups. Due to the significant number of factors that may have influenced the effect of sample date on the proliferative response, it was considered appropriate to exclude any significant effect of sample date from the remainder of the analyses.

5.3.1.2 Significant factors affecting control proliferation

5.3.1.2.1 Sex

Plotting the raw data for the control proliferation showed that the male animals had consistently higher levels of control proliferation than the females throughout the

assay (Table 5.4, Figure 5.7). The smallest variation between males and females occurred on day two when the mean male control proliferation was 28.5% higher than the mean female control proliferation (Table 5.4). The largest variation between males and females occurred on days four and five when the mean male control proliferation was 66.1% and 64% higher, respectively, than the mean female control proliferation (Table 5.4).

On REML analysis of the whole data set, sex was found to have a significant effect on the levels of control PBMC proliferation. These significant effects were identified as a fixed factor on day two of culture ($p<0.01$), and as a significant sex by cross two-way interaction on days three, four and five of culture ($p<0.001$).

5.3.1.2.2 Cross

Plotting the raw data for the whole data set showed that the backcross Charolais animals had significantly higher levels of control proliferation than the backcross Holstein-Friesian and the F2 animals (Figure 5.8). For example, the mean (± S.E.M.) control proliferation on day four of culture for the backcross Charolais, backcross Holstein-Friesian and the F2 animals was 1710.74 ± 630.55, 1145.43 ± 171.44 and 1408.19 ± 217.05 counts per minute, respectively.

On REML analysis of the whole data set, a significant sex by cross two-way interaction was found on days three, four and five of culture ($p<0.001$).

When the males and females were analysed separately, the mean control proliferation for each of the crosses was found to differ between the sexes (Table 5.5, Figure 5.9). In the males, the mean control proliferation for the F2 animals was higher than the mean backcross Holstein-Friesian control proliferation or the mean backcross Charolais control proliferation. For example, the predicted mean ± standard error of the difference (± S.E.D.) control proliferation on day five of culture for the F2, backcross Holstein-Friesian and backcross Charolais males was 383.75 ± 1.33, 279.42 ± 1.33 and 200.33 ± 1.33 counts per minute, respectively.

PBMC Proliferation	DAY 2		DAY 3		DAY 4		DAY 5		DAY 9		DAY 10	
	Males (±S.E.M)	Females (±S.E.M)	Males (±S.E.M)	Females (±S.E.M)	Males (±S.E.M)	Females (±S.E.M)	Males (±S.E.M)	Females (±S.E.M)	Males (±S.E.M)	Females (±S.E.M)	Males (±S.E.M)	Females (±S.E.M)
S. aureus induced	6755.8 (± 441.0)	5761.2 (± 500.5)	17330.4 (± 1347.4)	13296.1 (± 1321.4)	26572.1 (± 2187.0)	19650.1 (± 1609.4)	29698.1 (± 2538.5)	21504.1 (± 2748.4)	7505.6 (± 766.9)	5285.9 (± 432.9)	4603.3 (± 532.0)	2938.9 (± 517.6)
PHA induced	42993.7 (± 2004.8)	37948.7 (± 2682.6)	48472.6 (± 2609.4)	43872.3 (± 2739.0)	29411.0 (± 1982.3)	24714.6 (± 1773.5)	13019.1 (± 1313.1)	9909.8 (± 878.6)	1363.7 (± 259.0)	1080.6 (± 90.4)	905.5 (± 187.9)	639.5 (± 62.6)
Control (PBMC only)	1161.9 (± 77.0)	830.9 (± 60.2)	1967.6 (± 187.3)	905.8 (± 100.7)	2005.0 (± 284.2)	679.3 (± 90.3)	1038.52 (± 152.4)	374.2 (± 47.7)	298.54 (± 80.4)	146.5 (± 11.21)	267.6 (± 90.1)	132.1 (± 10.8)

Table 5.4: *In vitro* PBMC proliferation data from second generation animals sampled at approximately six months of age (n=316). The table shows mean (± S.E.M.) *S. aureus*-induced, PHA-induced or control proliferation for males (n=167) and females (n=149) on days two, three, four, five, nine and ten of culture. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

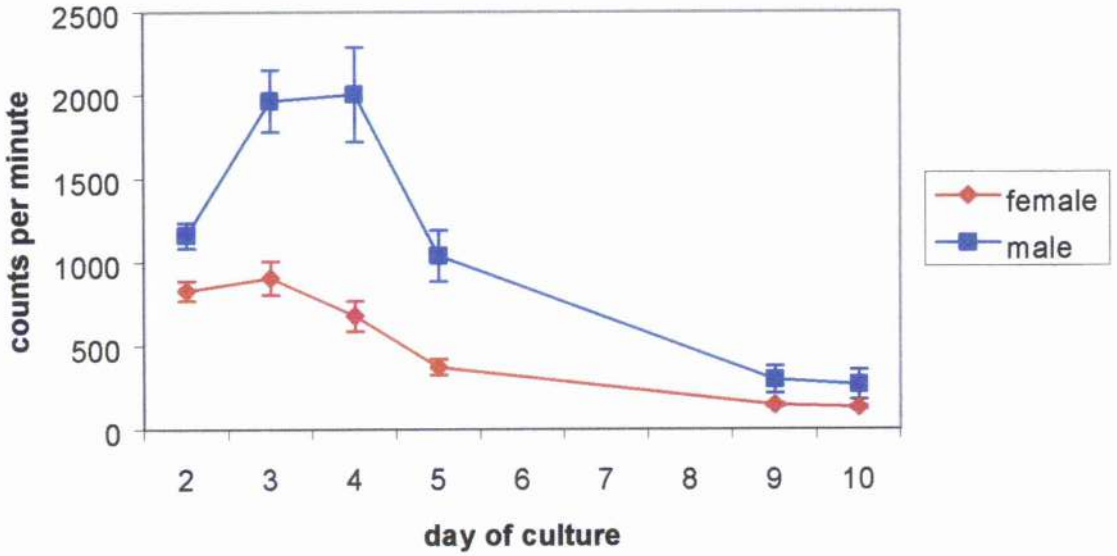


Figure 5.7: Control *in vitro* PBMC proliferation in second generation animals sampled at approximately six months of age (n=316). Graph represents mean (± S.E.M.) counts per minute control *in vitro* PBMC proliferation for females (◆, n=149) and males (■, n=167). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

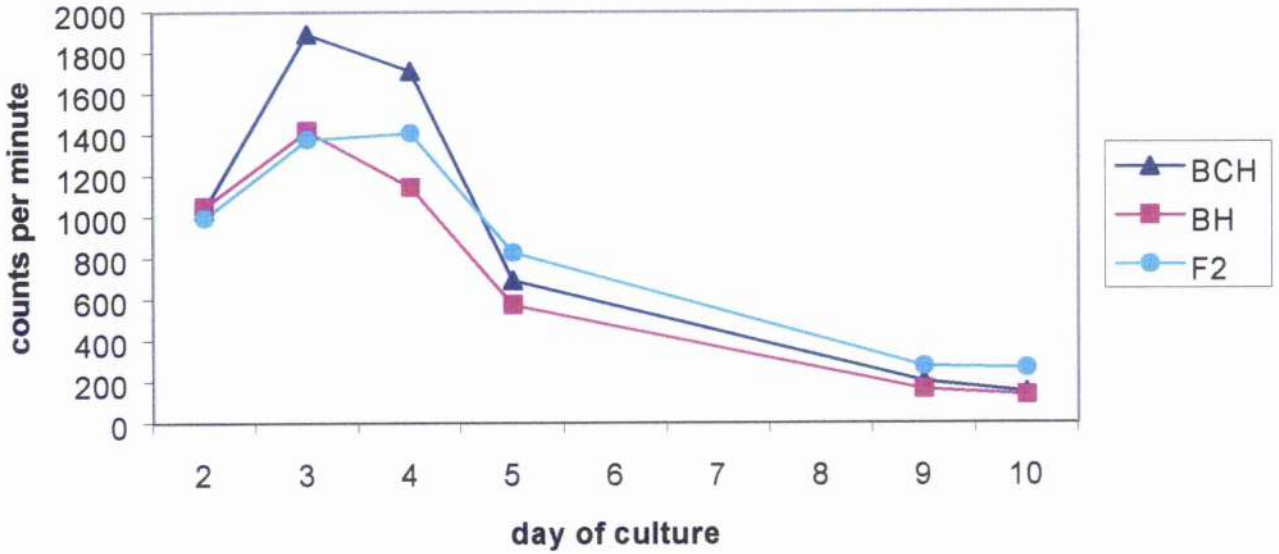


Figure 5.8: Control *in vitro* PBMC proliferation in second generation animals sampled at approximately six months of age (n=316), categorised by cross. Graph represents mean counts per minute control *in vitro* PBMC proliferation for BCH = backcross Charolais (▲, n=51), BH = backcross Holstein-Friesian (■, n=82) and F2 = Holstein-Friesian : Charolais (●, 50:50, n=166). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

Control PBMC Proliferation	BACK CROSS CHAROLAIS	BACK CROSS HOLSTEIN	F2
Male	0.000	0.298	0.646
Female	0.000	-0.330	-0.686

Table 5.5: Data output following REML analysis (Genstat, 5th Edition) for control *in vitro* PBMC proliferation on day five of culture in second generation animals sampled at approximately six months of age (n=316). Table shows table of effects of cross on control *in vitro* PBMC proliferation in males (n=149) and females (n=167).

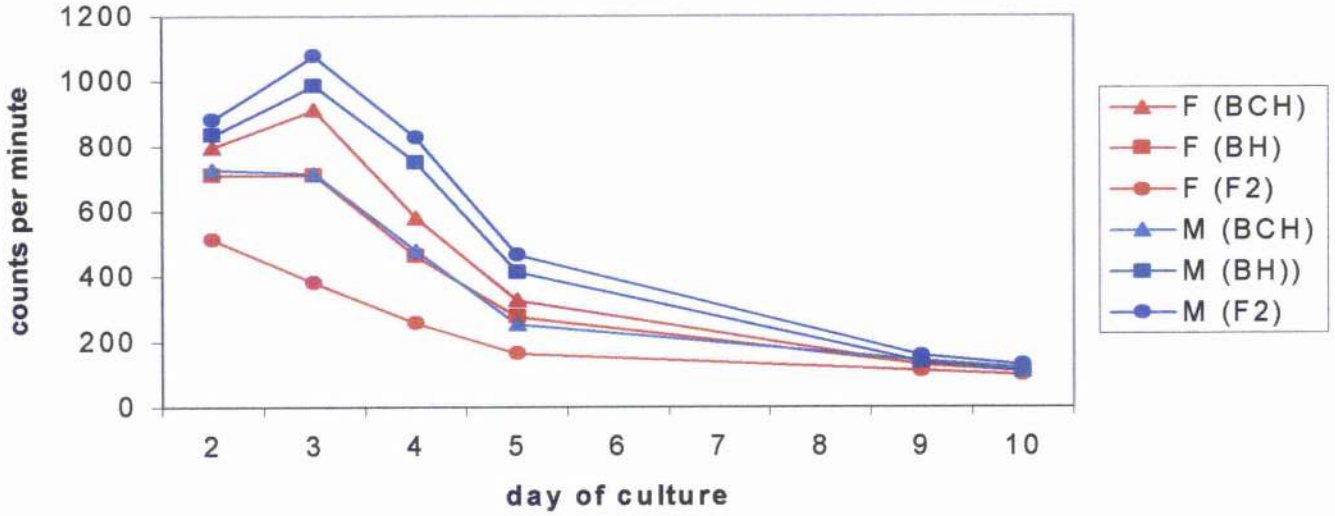


Figure 5.9: Control *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=316), showing males in blue and females in red and categorised by cross: BCH = backcross Charolais (▲, n=51), BH = backcross Holstein-Friesian (■, n=82), F2 = Holstein-Friesian : Charolais (●, 50:50, n=166). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

In contrast to the males, in the females, the backcross Charolais animals had the highest level of mean control proliferation, followed by the backcross Holstein-Friesian, with the F2 females showing the lowest levels of mean control proliferation (Table 5.5, Figure 5.9). For example, the predicted mean (± S.E.D.) control proliferation on day five of culture for the backcross Charolais, backcross Holstein-Friesian and F2 females was 317.66 ± 1.22, 228.37 ± 1.22 and 159.97 ± 1.22 counts per minute, respectively.

On REML analyses of the males, cross was identified as a significant effect as a fixed factor on control proliferation on day five of culture only ($p<0.05$), whereas, in the females, cross was identified as a significant effect as a fixed factor on control proliferation on days three, four and five of culture ($p<0.01$).

5.3.1.2.3 Year of birth

Plotting the raw data for the whole data set showed that the mean control proliferation was lower overall in the animals born and sampled in the 1998 cohort than in the animals from the 1999 or 2000 cohorts (Figure 5.10).

On REML analysis, the table of effects for day three of culture confirmed this finding (Table 5.6). On day three of culture, the animals from the 1998, 1999, and 2000 cohorts had predicted mean (± S.E.D.) control proliferation of 411.17 ± 1.22, 820.57 ± 1.22 and 690.21 ± 1.22 counts per minute, respectively.

On REML analysis, the year of birth was identified as being a significant effect as fixed factor on the control proliferation only on day two of culture ($p<0.001$).

No significant effect of the year of birth was identified when the males and females were analysed separately.

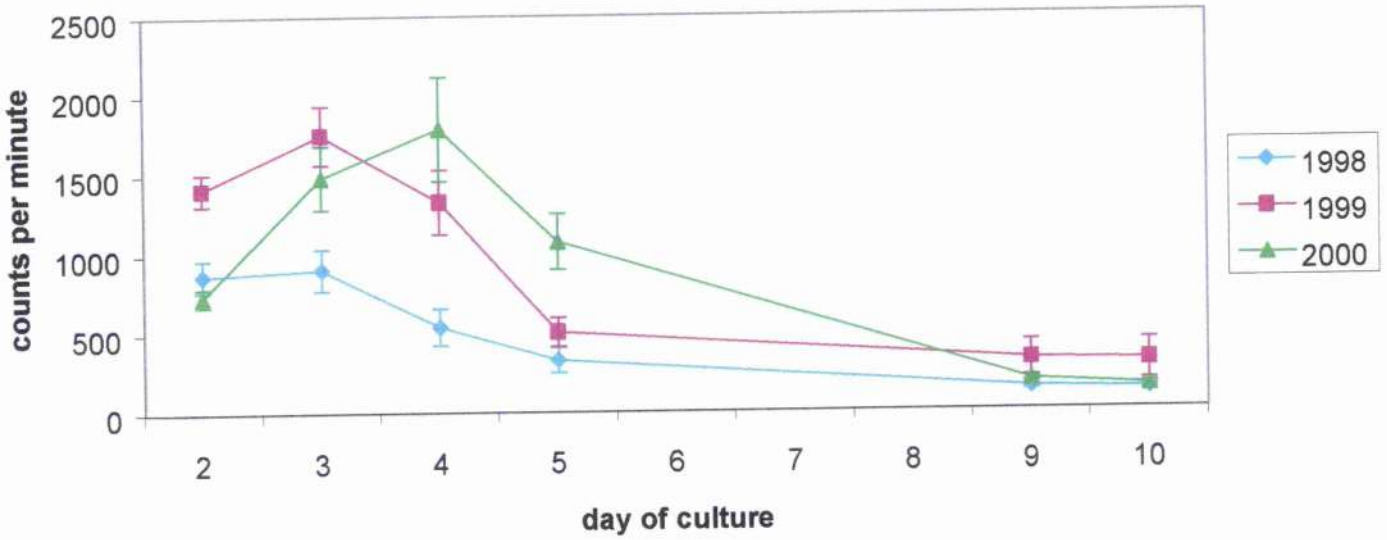


Figure 5.10: Control *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=316), categorised by year of birth: 1998 = ◆ (n=55), 1999 = ■ (n=118) and 2000 = ▲ (n=132) cohorts. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

Control PBMC Proliferation	YEAR OF BIRTH		
	1998	**1999**	**2000**
	0.000	0.692	0.518

Table 5.6: Data output following REML analysis (Genstat, 5th Edition) for control *in vitro* PBMC proliferation on day three of culture in second generation animals sampled at approximately six months of age (n=316). Table shows table of effects of year of birth on control *in vitro* PBMC proliferation in the whole data set.

5.3.1.2.4 Sire

Plotting the raw data showed considerable variation in the mean control proliferation among sires throughout the assay, especially on days three and four of culture (Figure 5.11). Less variation was observed on days two and five of culture with very little variation towards the end of the assay on day nine and ten of culture (Figure 5.11).

On REML analyses of the males only, a significant effect of sire was identified on the mean control proliferation on day two of culture ($p<0.05$) (Figure 5.12).

5.3.1.3 Significant factors affecting *Staphylococcus aureus*-induced proliferation

5.3.1.3.1 Sex

Plotting the raw data for the *S. aureus*-induced proliferation indicated that males had consistently higher levels of *S. aureus*-induced proliferation than the females, especially at peak levels of proliferation i.e. days four and five of culture (Table 5.4, Figure 5.13). The difference between sexes was generally lower for the *S. aureus*-induced proliferation than for control proliferation. The *S. aureus*-induced proliferation in males was 14% higher on day two and 26.1% and 27.6% on days four and five, respectively, than the *S. aureus*-induced proliferation of females (Table 5.4).

On REML analysis of the whole data set, sex was found to have a significant effect on the levels of *S. aureus*-induced PBMC proliferation as part of a significant two-way interaction on days two and three of culture. There was a significant sex by control interaction on day two ($p<0.001$), and a significant sex by cross ($p<0.050$) interaction identified on day three.

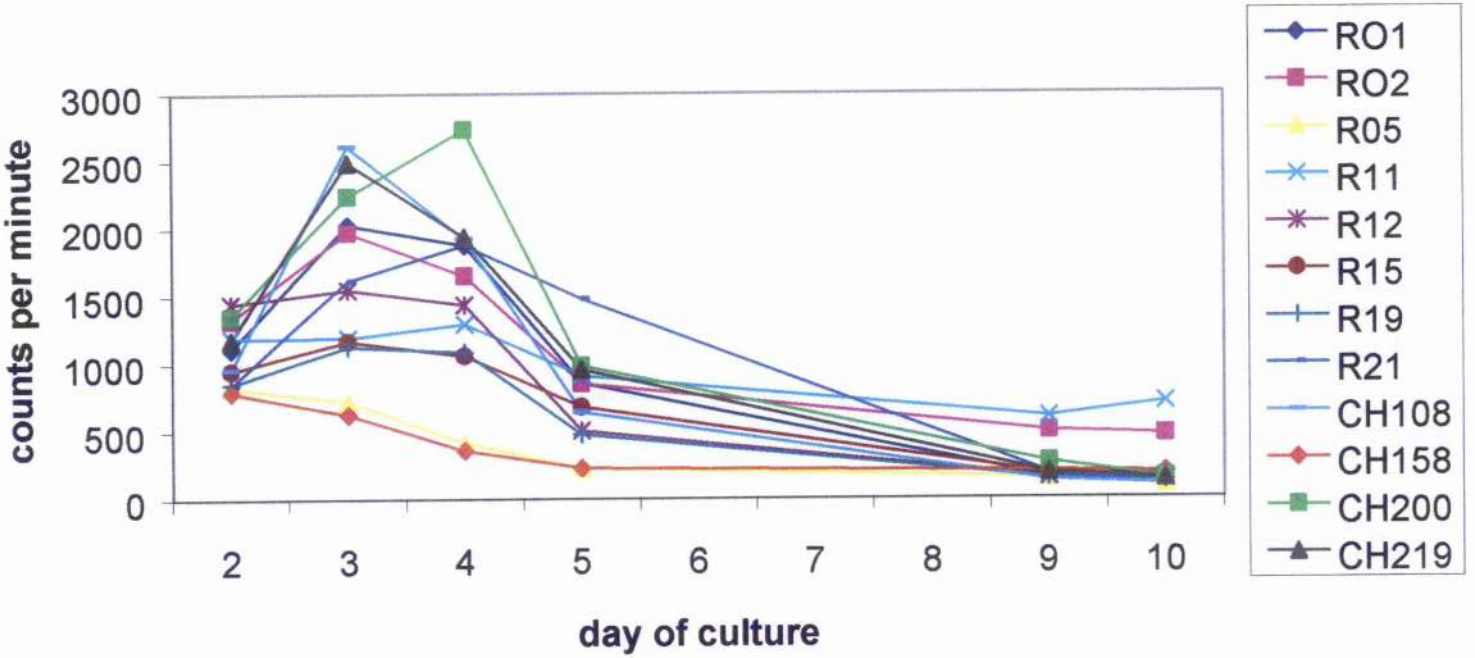


Figure 5.11: Mean control *in vitro* PBMC proliferation throughout the assay showing all second generation animals sampled at approximately six months of age (n=316), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire.

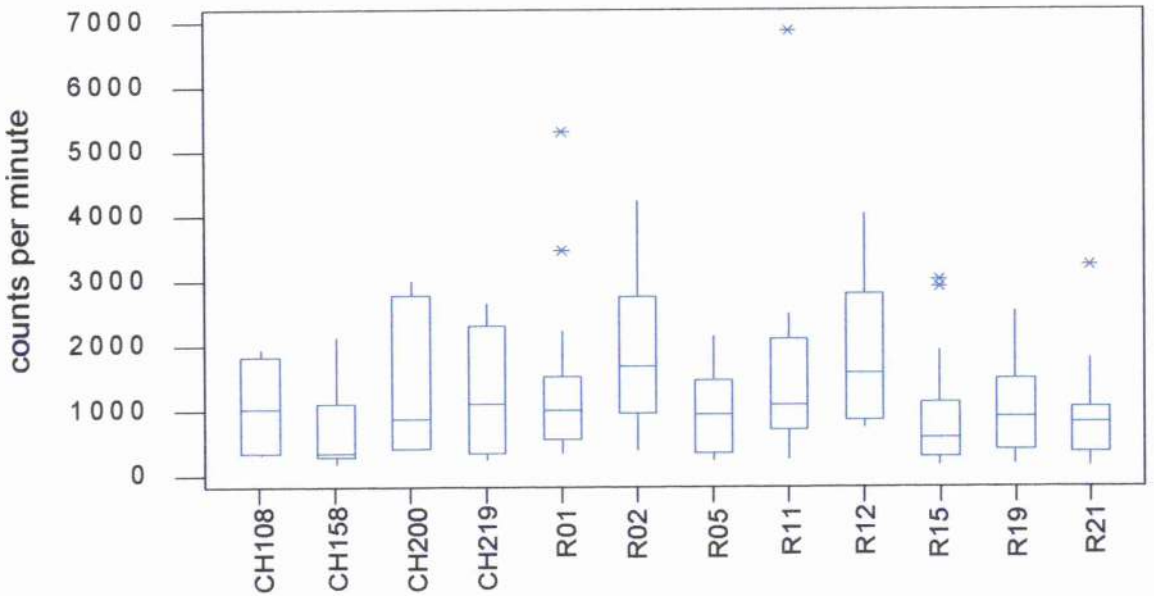


Figure 5.12: Mean control *in vitro* PBMC proliferation on day two of culture showing all second generation males sampled at approximately six months of age (n=167), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire. The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

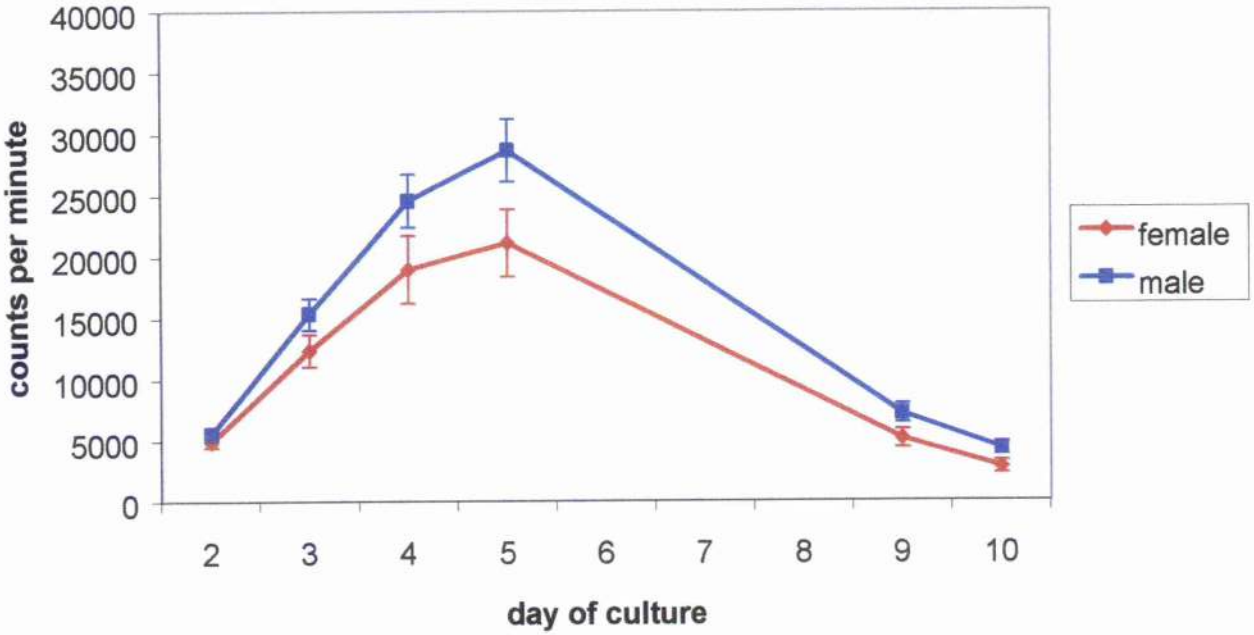


Figure 5.13: *Staphylococcus aureus*-induced *in vitro* PBMC proliferation in second generation animals sampled at approximately six months of age (n=316). Graph represents mean (± S.E.M.) counts per minute *S. aureus*-induced *in vitro* PBMC proliferation for females (◆, n=149) and males (■, n=167). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

5.3.1.3.2 Cross

Plotting the raw data for the whole data set showed that up to, and including day four of culture, the backcross Holstein-Friesian animals had marginally higher levels of *S. aureus*-induced proliferation than the backcross Charolais animals, with the F2 animals having the lowest levels of *S. aureus*-induced proliferation (Figure 5.14). On day five of culture, however, the backcross Charolais animals had marginally higher levels of *S. aureus*-induced proliferation than the F2 animals or the backcross Holstein-Friesian animals which had the lowest levels of *S. aureus*-induced proliferation of the three crosses on day five of culture (Figure 5.14).

On REML analysis cross was found to be significant as a fixed factor on day two of culture ($p<0.001$) and as significant cross by sample age, and sex by cross two-way interactions on day three of culture ($p<0.01$). In addition, cross was almost significant as a fixed factor on day four of culture ($p<0.1$) and showed that the backcross Holstein-Friesian had higher levels of *S. aureus*-induced proliferation than the F2 or the backcross Charolais. For example, the predicted mean (± S.E.D.) *S. aureus*-induced PBMC proliferation on day four of culture for the backcross Charolais, backcross Holstein-Friesian and the F2 were 9154.49 ± 1.25, 15244.90 ± 1.25, and 10394.17 ± 1.25 counts per minute, respectively.

When data from the male and females were plotted separately, the mean *S. aureus*-induced proliferation for each of the crosses was found to differ between the sexes (Figure 5.15). In the males, the mean *S. aureus*-induced proliferation for the F2 animals was higher than for the backcross Holstein-Friesian or the backcross Charolais cattle.

On REML analysis, the table of effects (Table 5.7) and table of predicted means on day four of culture, allowing for confounding, showed for *S. aureus*-induced *in vitro* PBMC proliferation that the backcross Holstein-Friesian had the highest levels of proliferation and the backcross Charolais had the lowest levels of proliferation. For

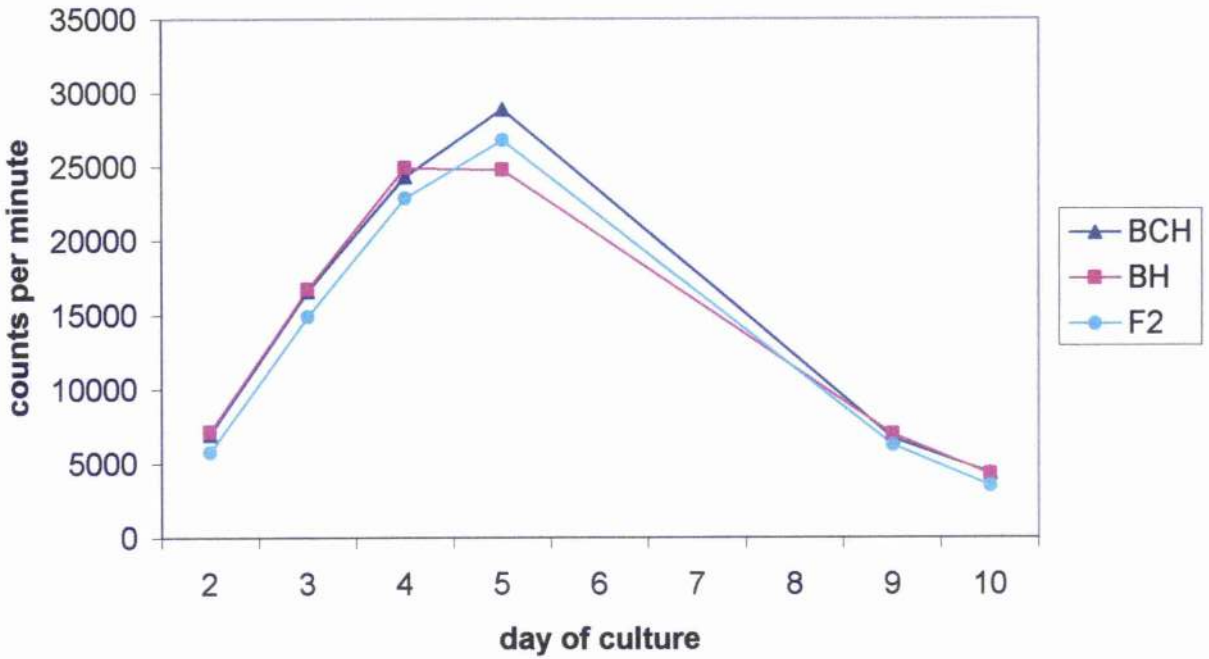


Figure 5.14: *Staphylococcus aureus*-induced *in vitro* PBMC proliferation in second generation animals sampled at approximately six months of age (n=316), categorised by cross. Graph represents mean counts per minute *S. aureus*-induced *in vitro* PBMC proliferation for BCH = backcross Charolais (▲, n=51), BH = backcross Holstein-Friesian (■, n=82) and F2 = Holstein-Friesian : Charolais (●, 50:50, n=166). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

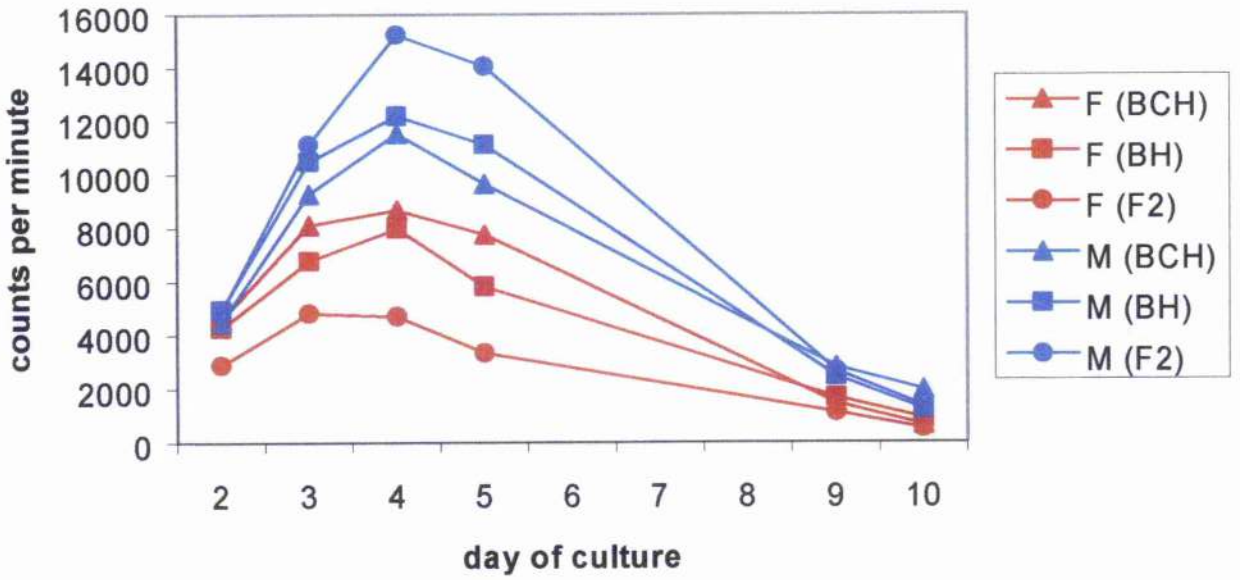


Figure 5.15: *Staphylococcus aureus*-induced *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=319), showing males in blue and females in red and categorised by cross: BCH = backcross Charolais (▲, n=51), BH = backcross Holstein-Friesian (■, n=82), F2 = Holstein-Friesian : Charolais (●, 50:50, n=166). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

S. aureus-induced PBMC Proliferation	BACK CROSS CHAROLAIS	BACK CROSS HOLSTEIN	F2
Male	0.000	0.714	0.276
Female	0.000	0.370	-0.210

Table 5.7: Data output following REML analysis (Genstat, 5th Edition) for *S. aureus*-induced *in vitro* PBMC proliferation on day four of culture in second generation animals sampled at approximately six months of age (n=316). Table shows table of effects of cross on *S. aureus*-induced *in vitro* PBMC proliferation in males (n=149) and females (n=167).

example, the predicted mean (± S.E.D.) for the backcross Charolais, backcross Holstein-Friesian and the F2 on day four of culture was 11373.02 ± 1.31, 23225.36 ± 1.31 and 15002.92 ± 1.31 counts per minute, respectively.

In contrast to the males, when data from the females was plotted, the backcross Charolais animals had marginally higher levels of *S. aureus*-induced proliferation than the backcross Holstein-Friesian, with the F2 females showing the lowest levels of mean *S. aureus*-induced proliferation (Figure 5.15).

On REML analysis, the table of effects (Table 5.7) and table of predicted means on day four of culture, allowing for confounding, showed for *S. aureus*-induced *in vitro* PBMC proliferation, that the backcross Holstein-Friesian animals had the highest level of proliferation and the F2 animals had the lowest level of proliferation. For example, the predicted mean (± S.E.D.) *S. aureus*-induced proliferation on day four of culture for the backcross Charolais, backcross Holstein-Friesian and F2 females was 10006.59 ± 1.42, 14617.89 ± 1.42 and 8103.08 ± 1.42 counts per minute, respectively.

On REML analysis, in the males, cross was identified as being significant cross by sample age by control ($p<0.001$) three-way interaction and cross by year of birth ($p<0.01$) two-way interactions on day three of culture.

5.3.1.3.3 Year of birth

Plotting the data for the whole data set showed that the mean *S. aureus*-induced proliferation was highest in the animals born and sampled in the 1998 cohort than the animals from the 1999 cohort, with the animals from the 2000 cohort having the lowest levels of mean *S. aureus*-induced proliferation (Figure 5.16).

On REML analysis, both the table of effects (Table 5.8) and the table of predicted means confirm this finding, for example, on day four of culture, the animals from the 1998, 1999 and 2000 cohorts had predicted mean (± S.E.D.) *S. aureus*-induced

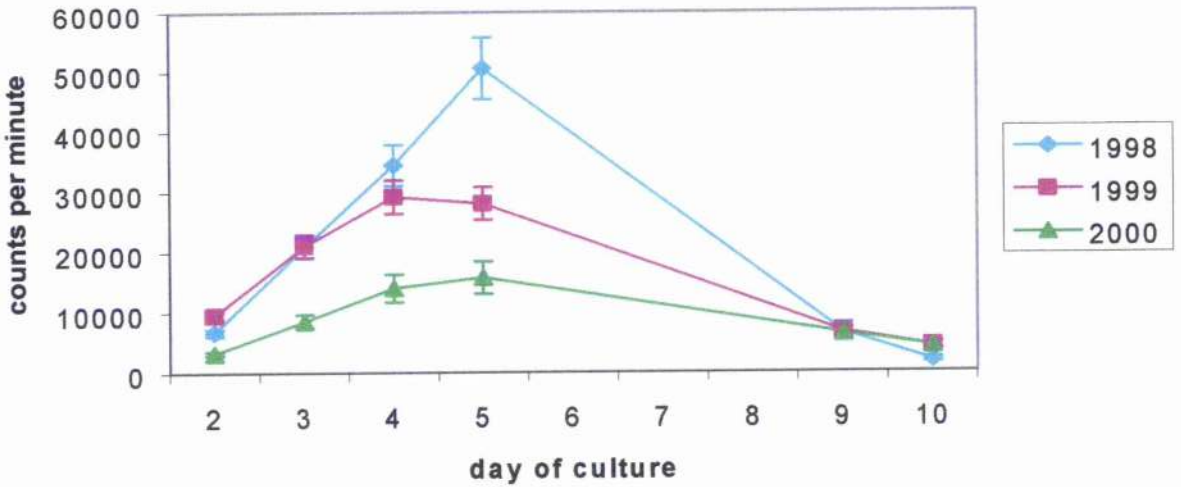


Figure 5.16: *Staphylococcus aureus*-induced *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=316), categorised by year of birth: 1998 = ◆ (n=55), 1999 = ■ (n=118) and 2000 = ▲ (n=132) cohorts. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

S. aureus-induced PBMC Proliferation	YEAR OF BIRTH		
	1998	**1999**	**2000**
	0.000	-1.304	-2.703

Table 5.8: Data output following REML analysis (Genstat, 5th Edition) for *S. aureus*-induced *in vitro* PBMC proliferation on day four of culture in second generation animals sampled at approximately six months of age (n=316). Table shows table of effects of year of birth on *S. aureus*-induced *in vitro* PBMC proliferation in the whole data set.

proliferation of 43044.94 ± 1.25, 11684.28 ± 1.25 and 2887.08 ± 1.25 counts per minute, respectively.

On REML analysis, the year of birth was identified as being a significant effect as a fixed factor on the *S. aureus*-induced proliferation on days two ($p<0.01$), four ($p<0.001$) and five ($p<0.001$) of culture and as a significant year of birth by sample age two-way interaction on day three ($p<0.05$).

When the males and females were plotted separately, the overall mean *S. aureus*-induced proliferation followed the same pattern in males and females, with the 1998 cohort having higher levels of *S. aureus*-induced proliferation than the 1999 cohort, and the 2000 cohort having the lowest levels of *S. aureus*-induced proliferation overall (Figure 5.17). One observation was that, overall, the females from the 1998 cohort had higher levels of *S. aureus*-induced proliferation compared to the females from the 1999 and 2000 cohorts and also compared to the males from all three cohorts.

On REML analysis, in both the table of effects (Table 5.9) and table of predicted means, for example, on day five of culture, in the males from the 1998, 1999 and 2000 cohorts, the predicted mean (± S.E.D.) *S. aureus*-induced proliferation was 59944.00 ± 3.57, 24100.79 ± 3.57 and 2100.64 ± 3.57 counts per minute, respectively.

On REML analysis in the females from the 1998, 1999 and 2000 cohorts, on day five of culture, the predicted mean (± S.E.D.) *S. aureus*-induced proliferation was 80338.16 ± 2.87, 6204.31 ± 2.87 and 2421.16 ± 2.87 counts per minute, respectively.

In the males, the year of birth was identified as being a significant effect on the *S. aureus*-induced proliferation on days three and four of culture. On day three, year of birth is significant as part of the significant two-way interactions: year of birth by control ($p<0.001$), year of birth by sample age ($p<0.001$) and cross by year of birth, whereas, year of birth was identified as being significant as a fixed factor only, on day four of culture ($p<0.01$).

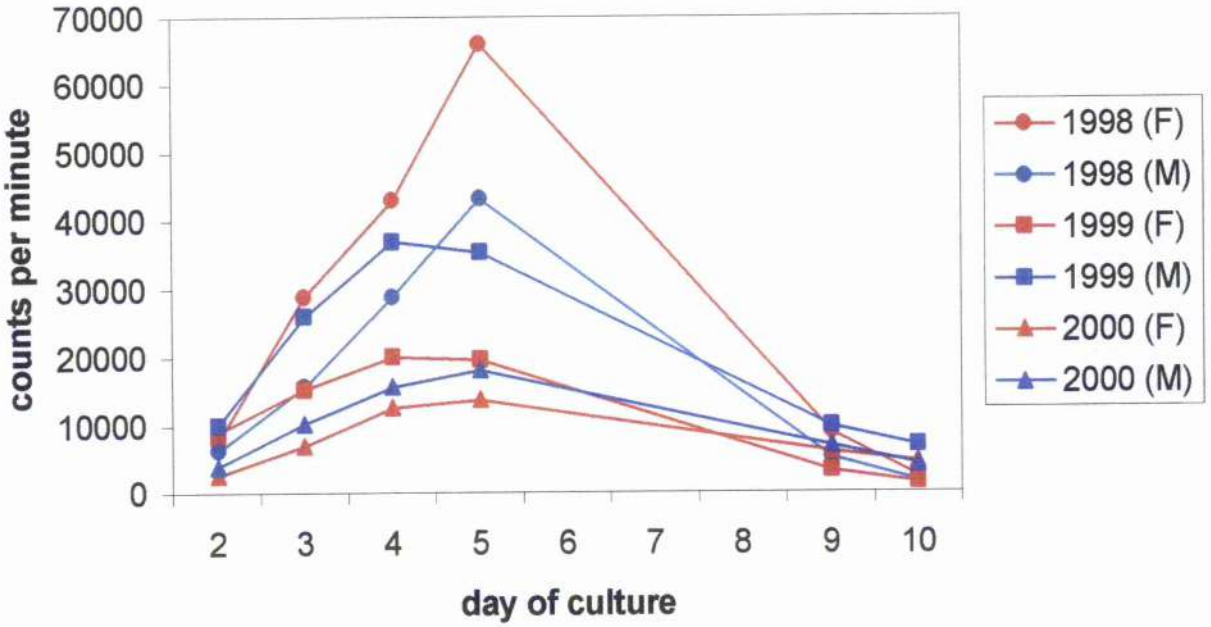


Figure 5.17: *Staphylococcus aureus*-induced *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=316), showing males in blue and females in red and categorised by year of birth: 1998 (●), 1999 (■), 2000 (▲) cohorts. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

S. aureus-induced PBMC Proliferation	YEAR OF BIRTH		
	1998	**1999**	**2000**
Male	0.000	-0.911	-3.352
Female	0.000	-2.561	-3.502

Table 5.9: Data output following REML analysis (Genstat, 5th Edition) for *S. aureus*-induced *in vitro* PBMC proliferation on day five of culture in second generation animals sampled at approximately six months of age (n=316). Table shows table of effects of year of birth on *S. aureus*-induced *in vitro* PBMC proliferation in males (n=149) and females (n=167).

In the females, the year of birth had a significant effect on the *S. aureus*-induced proliferation on days three, four and five of culture. On day three, year of birth is significant as part of the significant two-way interaction, year of birth by sample age interaction ($p<0.01$), whereas, year of birth was significant as a fixed factor on days four ($p<0.05$) and five ($p<0.01$) of culture.

5.3.1.3.4 Sample age

On REML analysis, in general, the levels of *S. aureus*-induced proliferation increased with the age of the individual animal assessed in the current study. On day four of culture, in the whole data set, an increase of seven days in the age of the individual animals sampled resulted in a mean ± standard error (± S.E.) increase of 6.7 ± 3.2% in the levels of *S. aureus*-induced proliferation.

On REML analysis, the sample age was identified as being a significant effect as a fixed factor on the *S. aureus*-induced proliferation on day four ($p<0.05$) of culture, and as the significant two way interactions, cross by sample age and year of birth by sample age ($p<0.05$) on day three of culture.

In the males, on day four of culture, an increase of seven days in the age of the individual animals sampled resulted in a mean (± S.E.) increase of 9.8 ± 4.0% in the levels of *S. aureus*-induced proliferation.

In contrast, in the females, on day four of culture, an increase of seven days in the age of the individual animals sampled resulted in a mean (± S.E.) decrease of 1.0 ± 6.0% in the levels of *S. aureus*-induced proliferation.

In the males, sample age was identified as having a significant effect on *S. aureus*-induced proliferation on day four of culture ($p<0.05$), whereas, in the females, sample age was identified as being a significant effect as the two way interaction year of birth by sample age on day three of culture ($p<0.01$).

5.3.1.3.5 Sire

On plotting the raw data, considerable variation was observed in the mean *S. aureus*-induced proliferation among sires throughout the duration of the assay, especially on days four and five of culture (Figure 5.18). Slightly less variation was observed on day three of culture with very little variation observed on days two, nine and ten of culture (Figure 5.18).

On REML analysis, in the whole data set, a significant effect of sire was identified on the mean *S. aureus*-induced proliferation on day two of culture ($p<0.05$) (Figure 5.19). When the males and females were analysed separately, a significant effect of sire was identified in the females only, on day two of culture ($p<0.05$) (Figure 5.20).

5.3.1.4 Significant factors affecting Phytohaemagglutinin-induced proliferation

5.3.1.4.1 Sex

On plotting the raw data of the PHA-induced proliferation, the males had marginally higher levels of PHA-induced proliferation than the females throughout the assay (Figure 5.21), although the difference between sexes was generally lower than for the control proliferation described above. The PHA-induced proliferation of males was 12% higher on day two and 16% and 23.9% on days four and five respectively than the PHA-induced proliferation of females (Table 5.4, Figure 5.21).

On REML analysis, in the whole data set, sex had a significant effect on the levels of PHA-induced proliferation as a fixed factor ($P<0.001$) and the two-way interaction sex by sample age ($P<0.01$) on day two of culture.

5.3.1.4.2 Cross

On plotting the raw data for the whole data set, overall, the backcross Charolais animals had marginally higher levels of PHA-induced proliferation than the

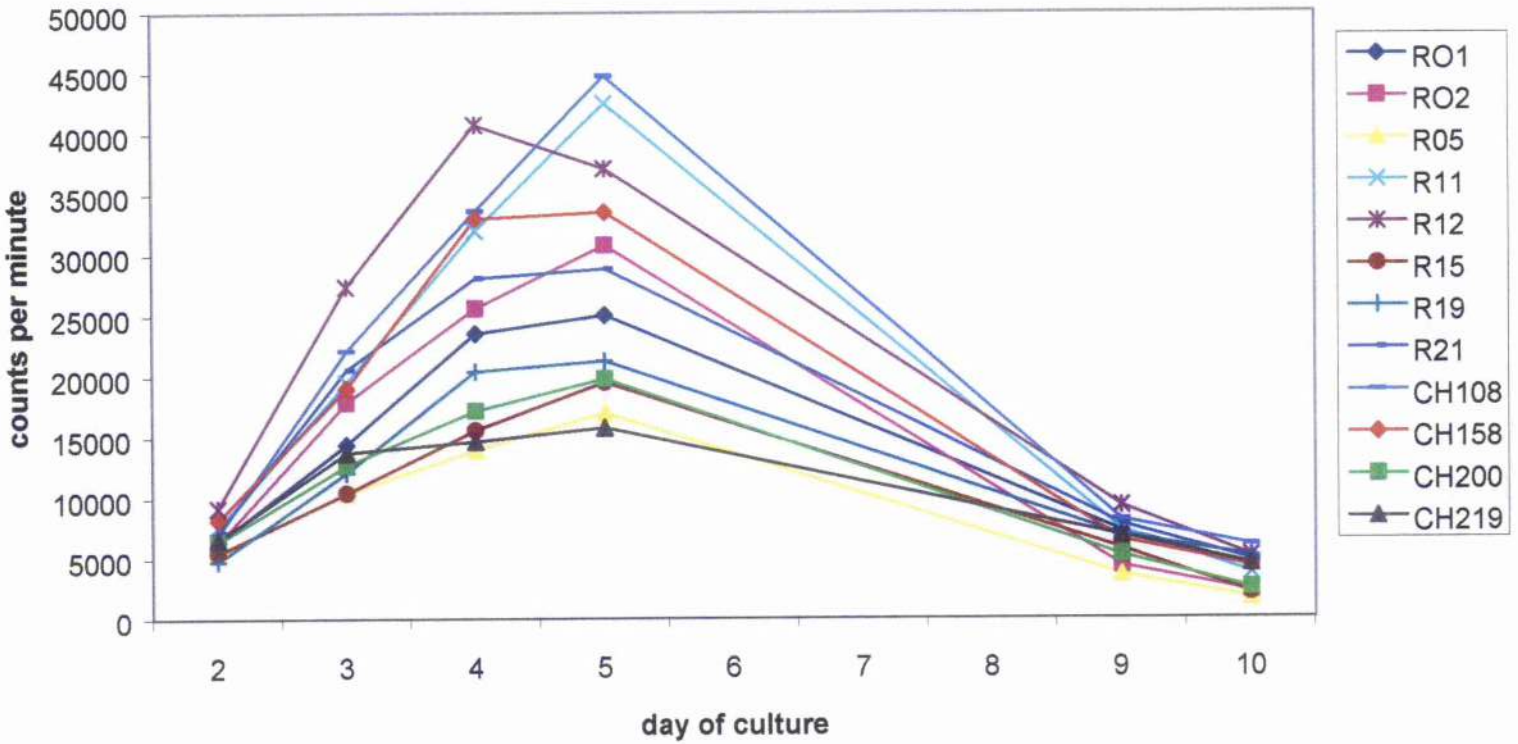


Figure 5.18: Mean *S. aureus*-induced *in vitro* PBMC proliferation throughout the assay showing all second generation animals sampled at approximately six months of age (n=316), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire.

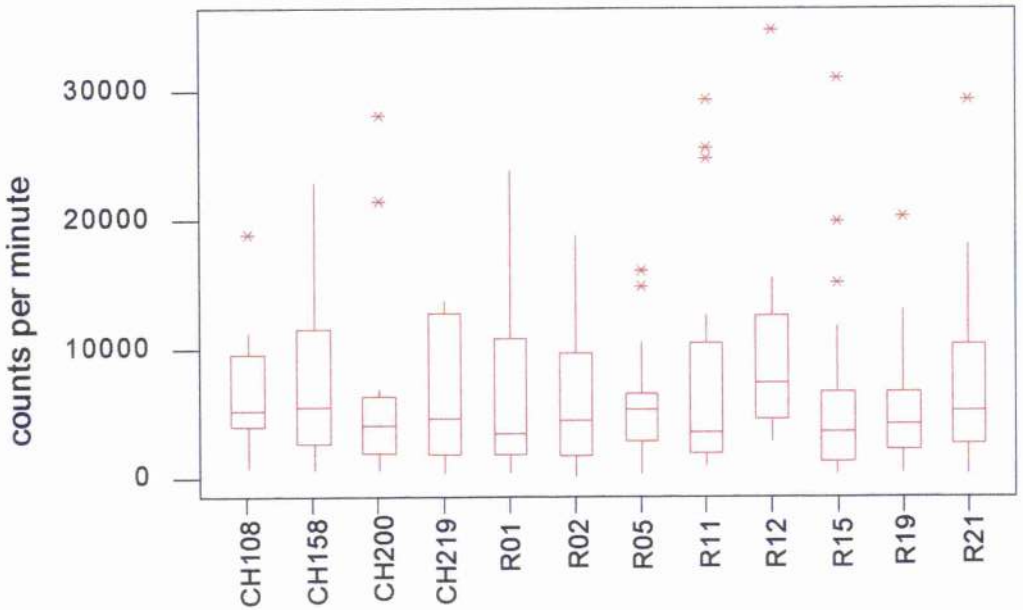


Figure 5.19: Mean *S. aureus*-induced *in vitro* PBMC proliferation on day two of culture showing all second generation animals sampled at approximately six months of age (n=316), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire. The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

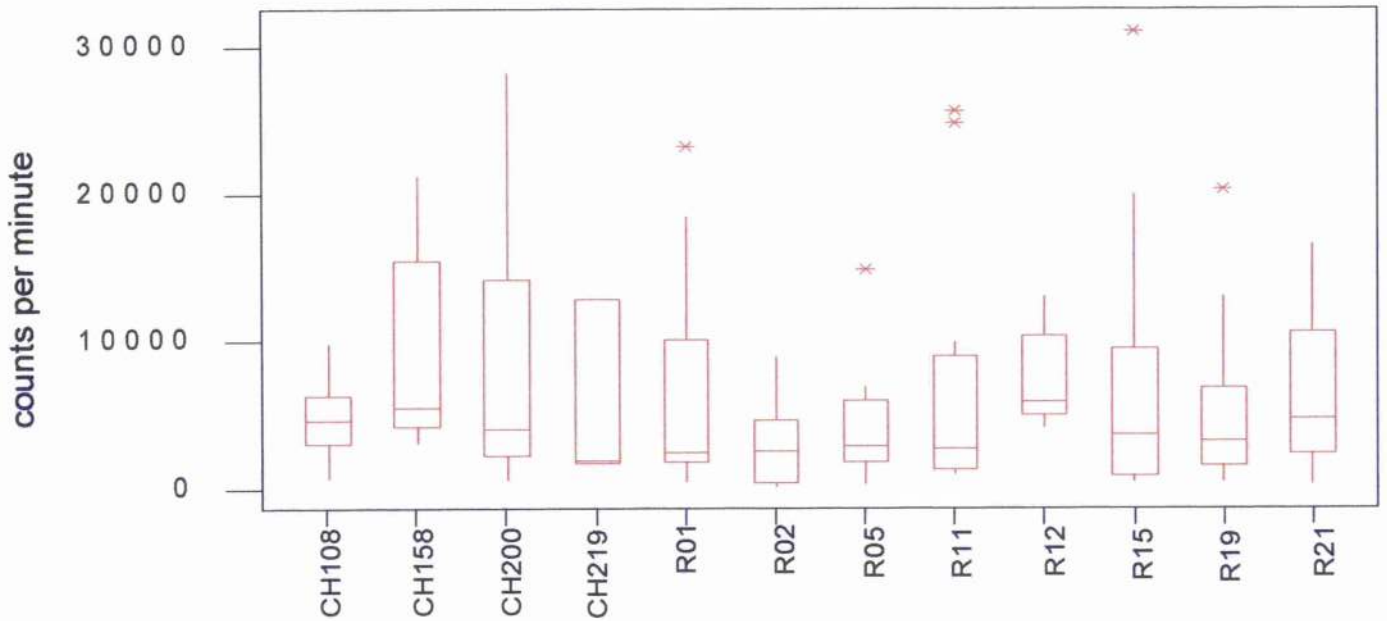


Figure 5.20: Mean *S. aureus*-induced *in vitro* PBMC proliferation on day two of culture showing all second generation females sampled at approximately six months of age (n=149), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire. The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

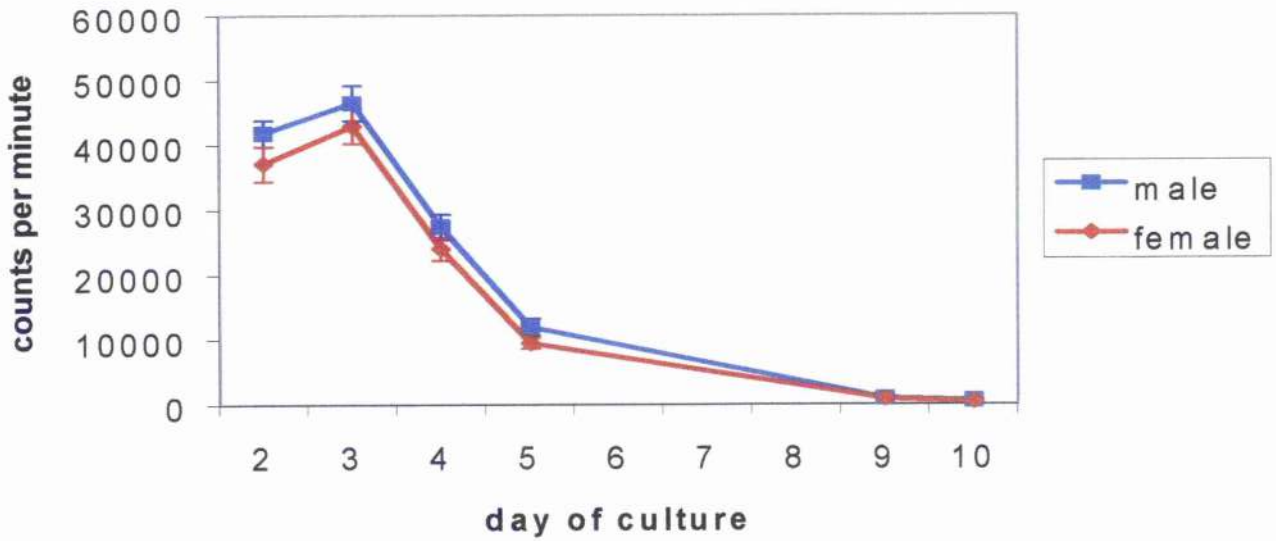


Figure 5.21: Phytohaemagglutinin-induced *in vitro* PBMC proliferation in second generation animals sampled at approximately six months of age (n=316). Graph represents mean (± S.E.M.) counts per minute PHA-induced *in vitro* PBMC proliferation for females (◆, n=149) and males (■, n=167). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation).

backcross Holstein-Friesian, with the F2 animals having the lowest levels of PHA-induced proliferation (Figure 5.22).

On REML analysis, the table of effects (Table 5.10) and table of predicted means confirmed this, for example, on day three of culture, the predicted mean (± S.E.D.) PHA-induced proliferation for the backcross Charolais, backcross Holstein-Friesian and F2 animals was 41772.77 ± 1.16, 31888.48 ± 1.16 and 30946.03 ± 1.16 counts per minute, respectively.

On REML analysis in the whole data set, the cross of the individual animal had a significant effect on the levels of PHA-induced proliferation as a fixed factor on day five ($p<0.05$) of culture, and was almost significant on day four ($p<0.1$) of culture.

When data from the males and females were plotted separately, the mean PHA-induced proliferation for each of the crosses was found to differ between the sexes (Figure 5.23), however, the overall mean PHA-induced proliferation followed the same pattern between males and females. The backcross Charolais animals had higher levels of PHA-induced proliferation than the backcross Holstein-Friesian animals, with the F2 animals having the lowest levels of proliferation in both the males and females (Figure 5.23).

On REML analysis, in the males, cross was identified as being a significant effect as a fixed factor on the PHA-induced proliferation on days two and five of culture ($p<0.05$).

5.3.1.4.3 Year of birth

On plotting the raw data for the whole data set, the mean PHA-induced proliferation was lower overall in the animals born and sampled in the 1998 cohort than in the animals in the 1999 or 2000 cohorts. Overall, the animals in the 1999 cohort had the highest mean level of PHA-induced proliferation on days two and three of culture (Figure 5.24).

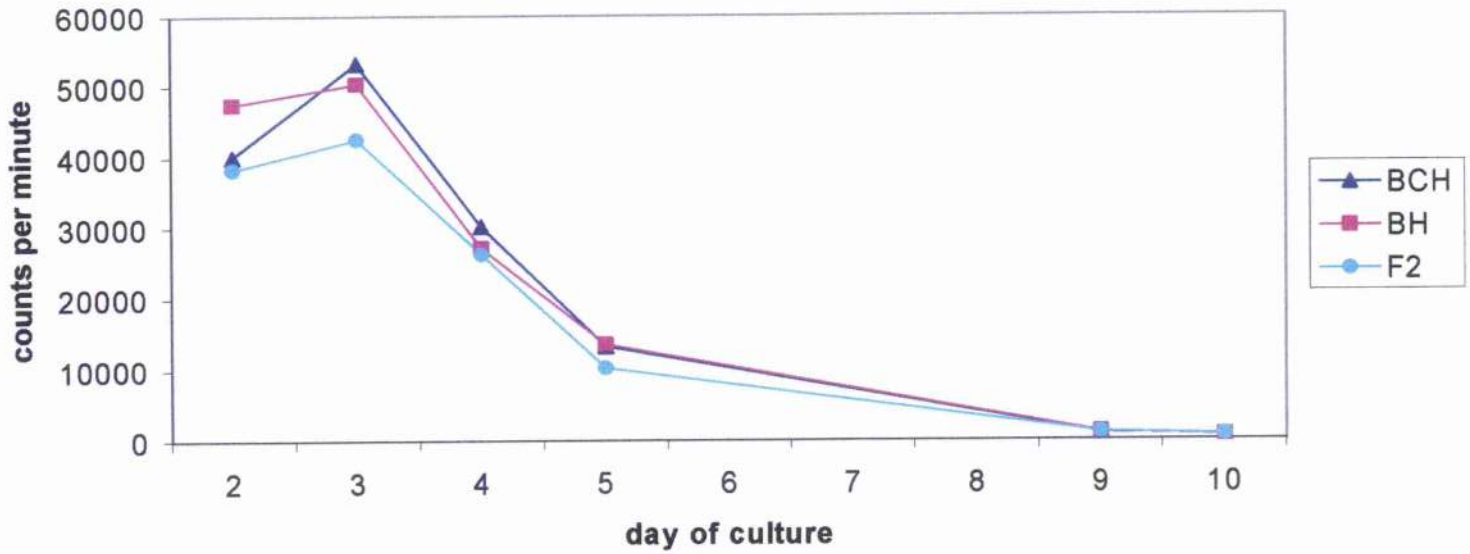


Figure 5.22: Phytohaemagglutinin-induced *in vitro* PBMC proliferation in second generation animals sampled at approximately six months of age (n=316), categorised by cross. Graph represents mean counts per minute control *in vitro* PBMC proliferation for BCH = backcross Charolais (▲, n=51), BH = backcross Holstein-Friesian (■, n=82) and F2 = Holstein-Friesian : Charolais (●, 50:50, n=166). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

PHA-induced PBMC Proliferation	BACK CROSS CHAROLAIS	BACK CROSS HOLSTEIN	F2
	0.000	-2.702	-0.245

Table 5.10: Data output following REML analysis (Genstat, 5th Edition) for PHA-induced *in vitro* PBMC proliferation on day three of culture in second generation animals sampled at approximately six months of age (n=316). Table shows table of effects for cross for PHA-induced *in vitro* PBMC proliferation in the whole data set.

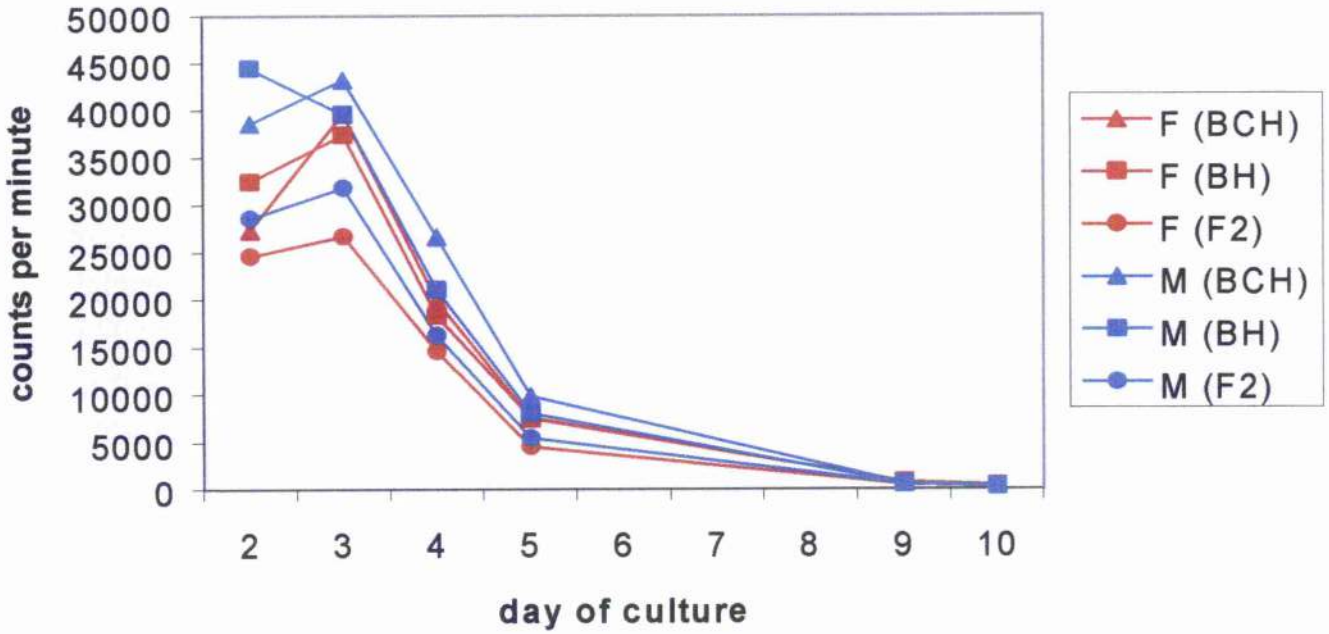


Figure 5.23: Phytohaemagglutinin-induced *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=319), showing males in blue and females in red and categorised by cross: BCH = backcross Charolais (▲, n=51), BH = backcross Holstein-Friesian (■, n=82), F2 = Holstein-Friesian : Charolais (●, 50:50, n=166). Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

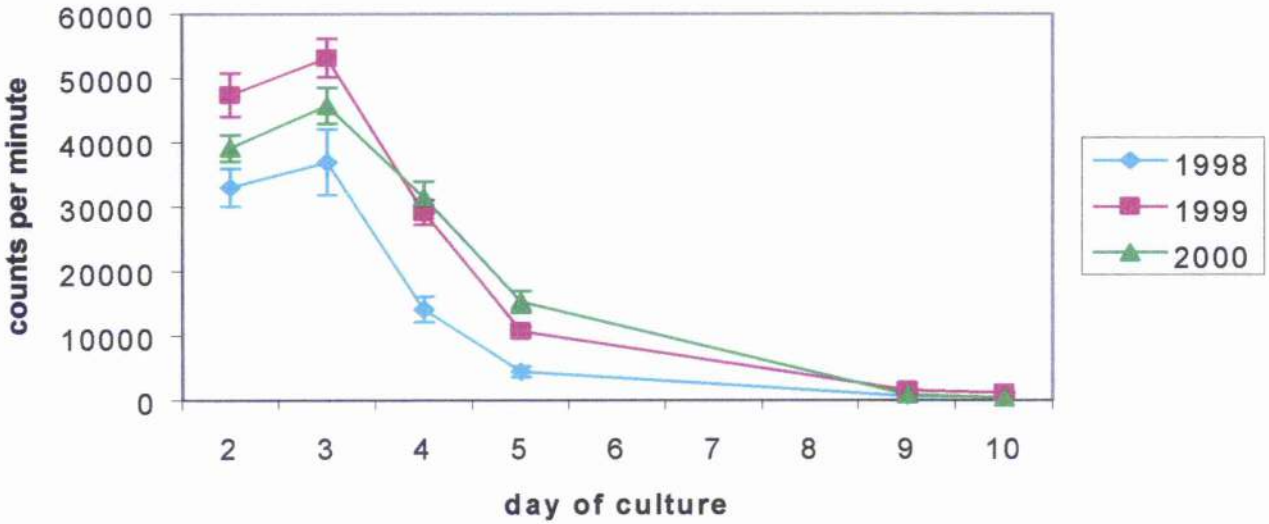


Figure 5.24: Phytohaemagglutinin-induced *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=316), categorised by year of birth: 1998 = ◆ (n=55), 1999 = ■ (n=118) and 2000 = ▲ (n=132) cohorts. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

On REML analysis, on day three of culture the animals from the 1998, 1999 and 2000 cohorts had predicted means (± S.E.D.) for PHA-induced proliferation of 12581.72 ± 1.16, 76879.92 ± 1.16 and 36315.50 ± 1.16, counts per minute, respectively. By day four of culture, the animals from the 1998 cohort had the lowest level of PHA-induced proliferation, followed by the 1999 cohort, with the animals from the 2000 cohort having the highest levels of PHA-induced proliferation (Figure 5.24).

On REML analysis, in the whole data set, the year of birth was identified as being a significant effect as a fixed factor on the PHA-induced proliferation days two and four of culture ($p<0.001$).

When raw data from the males and females were plotted separately, the overall mean PHA-induced proliferation pattern differed between the sexes, with the 1998 females having higher levels of proliferation than the 1999 and 2000 cohort animals, whereas, the 1998 males had the lowest level of PHA-induced proliferation when compared to the 2000 cohort males, with the 1999 cohort males having the highest levels of proliferation (Figure 5.25). On day four of culture, a similar pattern was seen between the males and females with the 1998 cohort animals having the lowest levels of PHA-induced proliferation (Figure 5.25).

On REML analysis, a significant effect of the year of birth was identified in the PHA-induced response in both the males and females when analysed separately.

For males, the year of birth was significant as a fixed factor on days two ($p<0.001$), four ($p<0.01$), and five ($p<0.01$) of culture.

For females, the year of birth was significant as a fixed factor only on day two of culture ($p=0.05$).

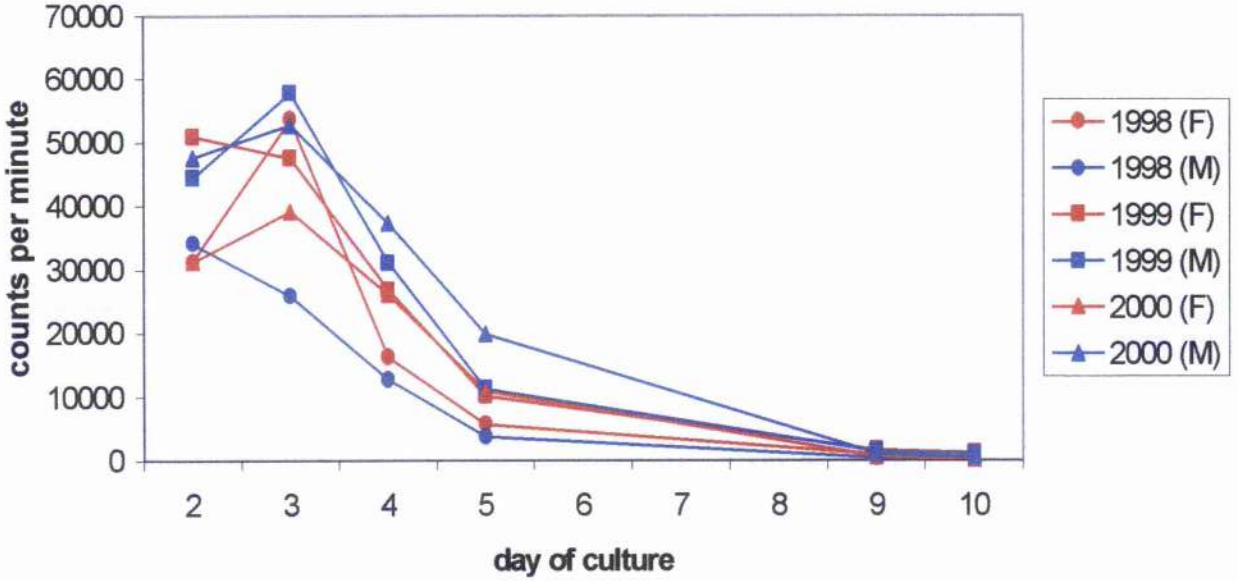


Figure 5.25: Phytohaemagglutinin-induced *in vitro* PBMC proliferation showing second generation animals sampled at approximately six months of age (n=316), showing males in blue and females in red and categorised by year of birth: 1998 (●), 1999 (■), 2000 (▲) cohorts. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H-thymidine incorporation).

5.3.1.4.4 Sample age

On REML analysis, in general, the levels of PHA-induced proliferation decreased as the age of the animals increased in the current study. On day four of culture, in the whole data set, an increase of seven days in the age of the individual animals sampled resulted in a mean (±S.E.) decrease of 6.4 ± 2.3 % in the levels of PHA-induced proliferation.

On REML analysis, the sample age had a significant effect as a fixed factor on PHA-induced proliferation on days two, four and five of culture, and as the significant two-way interaction sex by sample age on day two of culture ($p<0.01$).

In the males, on day four of culture, an increase of seven days in the age of the individual animals sampled resulted in a mean (±S.E.) decrease of 4.8 ± 3.5% in the levels of PHA-induced proliferation.

In the females, on day four of culture, every increase of seven days in the age of the individual animals sampled resulted in a mean (±S.E.) decrease of 13.9 ± 3.8% in the levels of PHA-induced proliferation.

On REML analysis a significant effect of sample age as a fixed factor on the PHA-induced proliferation in males was identified on day five ($p<0.05$) of culture.

On REML analysis, the sample age was significant on PHA-induced proliferation in the females on all days of culture. The sample age was significant as a fixed effect on days two ($p<0.05$), three ($p<0.05$), four ($p<0.001$) and five ($p<0.001$) of culture.

5.3.1.4.5 Sire

On plotting the raw data, considerable variation was observed in the mean PHA-induced proliferation among sire throughout the assay, especially on days two and three of culture (Figure 5.26). Slightly less variation was observed on days four and

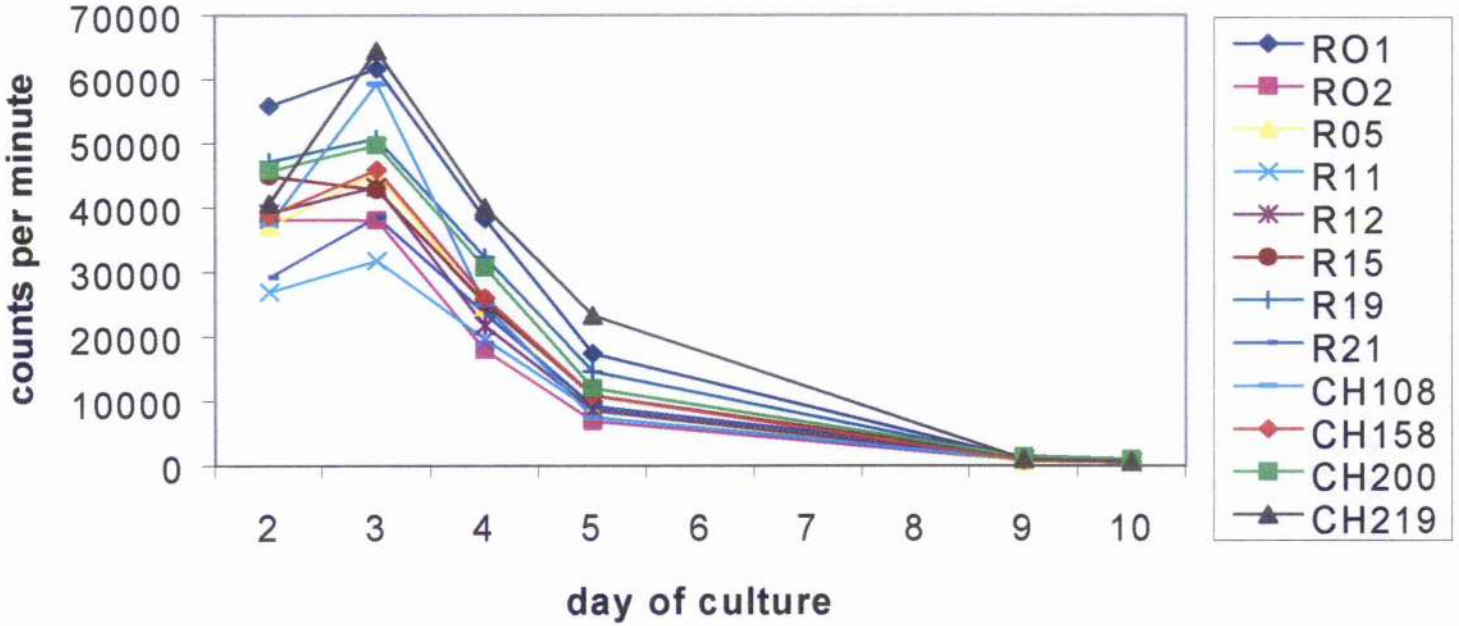


Figure 5.26: Mean PHA-induced *in vitro* PBMC proliferation throughout the assay showing all second generation animals sampled at approximately six months of age (n=316), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire.

five of culture with very little variation observed on days nine and ten of culture (Figure 5.26).

On REML analysis, in the whole data set, significant effect of sire on PHA-induced proliferation was found on day two ($0.01<p>0.001$) of culture (Figure 5.27).

In the males, a significant effect of sire on the PHA-induced proliferation was identified on days two ($0.01<p>0.001$) and three ($0.05<p>0.01$) of culture (Figure 5.28).

In the females, there was no significant effect of sire on the PHA-induced proliferation on any day of culture.

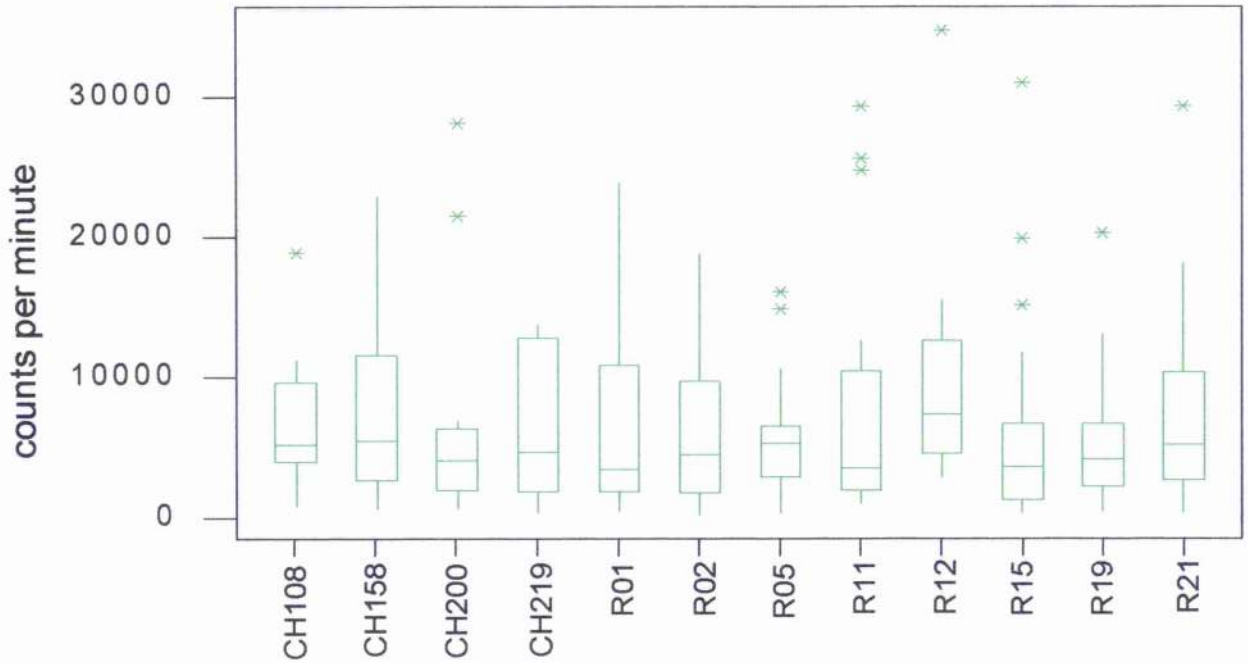


Figure 5.27: Mean PHA-induced *in vitro* PBMC proliferation on day two of culture showing all second generation animals sampled at approximately six months of age (n=316), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire. The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

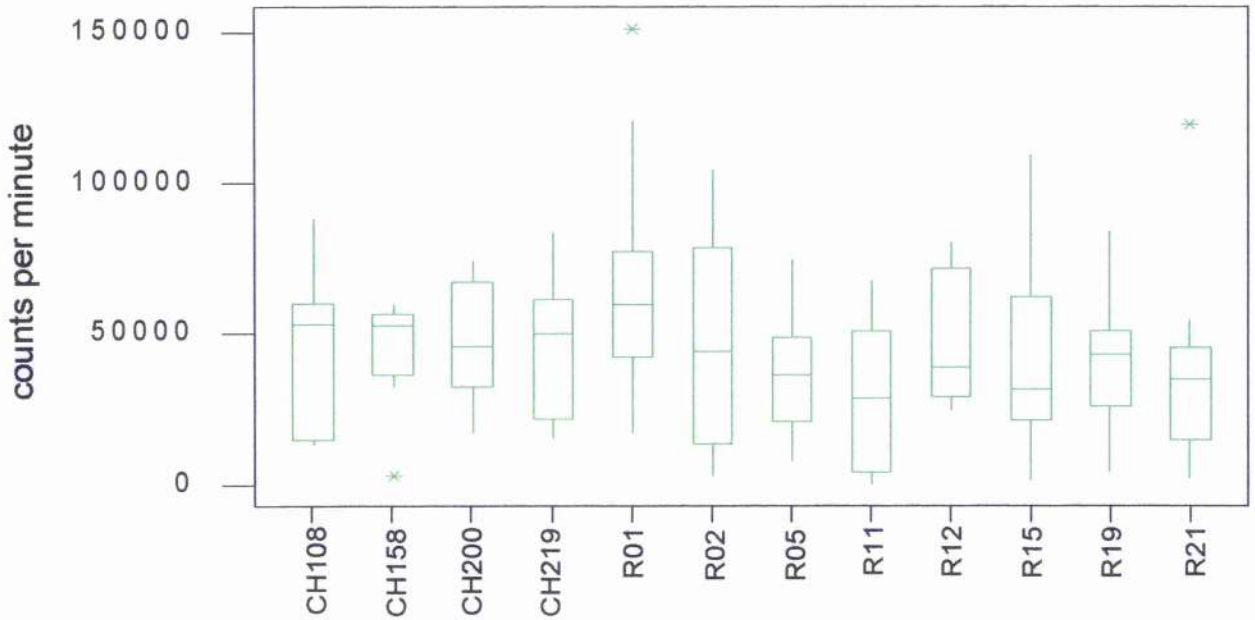


Figure 5.28: Mean PHA-induced *in vitro* PBMC proliferation on day two of culture showing all second generation males sampled at approximately six months of age (n=167), categorised by sire. Peripheral blood mononuclear cell proliferation is expressed as counts per minute (^{3}H thymidine incorporation). R prefix indicates F2 sire, CH prefix indicates Charolais sire. The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

5.3.1.5 Summary of REML analyses

Tables 5.11, 5.12 and 5.13 summarise the significant fixed factors and interactions and, where found to be significant, the random effect of sire, for the control, *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation, respectively. Tables 5.11-5.13 summarise the significant effects following analyses of the whole data set and the significant effects in the males and female subsets, following subsequent analyses.

DATA SET	DAY 2	DAY 3	DAY 4	DAY 5
WHOLE (Males and Females)	sex year of birth	*sex by cross*	*sex by cross*	*sex by cross*
MALES	**SIRE**	none	none	cross
FEMALES	none	cross	cross	cross

Table 5.11: Summary of data output following REML analysis (Genstat, 5th Edition). The table shows significant factors and interactions for Y variate = control *in vitro* PBMC proliferation. All factors shown have a significance level of $p<0.05$. none = no significant factors or interactions; significant interactions are shown in italics and significant random factors are shown in bold and uppercase.

DATA SET	DAY 2	DAY 3	DAY 4	DAY 5
WHOLE (Males and Females)	cross year of birth *sex by control* **SIRE**	*sex by cross* *cross by sample age* *year of birth by sample age*	year of birth sample age	year of birth control
MALES	none	*cross by sample age by control* *cross by year of birth* *year of birth by control* *year of birth by sample age*	year of birth sample age control	none
FEMALES	control **SIRE**	*year of birth by sample age*	year of birth control	year of birth

Table 5.12: Summary of data output following REML analysis (Genstat, 5th Edition). The table shows significant factors and interactions for Y variate = *S. aureus*-induced *in vitro* PBMC proliferation. All factors shown have a significance level of $p<0.05$. none = no significant factors or interactions; significant interactions are shown in italics and significant random factors are shown in bold and uppercase.

DATA SET	DAY 2	DAY 3	DAY 4	DAY 5
WHOLE (Males and Females)	sex year of birth control *sex by sample age* **SIRE**	control	year of birth control	cross sample age control
MALES	cross year of birth **SIRE**	control **SIRE**	year of birth control	cross year of birth sample age
FEMALES	year of birth sample age	sample age	sample age control	sample age control

Table 5.13: Summary of data output following REML analysis (Genstat, 5th Edition). The table shows significant factors and interactions for Y variate = PHA-induced *in vitro* PBMC proliferation. All factors shown have a significance level of $p<0.05$. none = no significant factors or interactions; significant interactions are shown in italics and significant random factors are shown in bold and uppercase.

Chapter 6

Data exploration and analyses

6.1 *In vitro* polymorphonuclear cell phagocytosis

The main statistical analyses were carried out on the data obtained from the experimental animals sampled at approximately six months of age in the 2000 cohort for the *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN. Basic data exploration and statistical analyses which included the assessment of correlation of individual animal responses between time points within a single assay, correlation of individual animal responses at re-sampling (approximately three months after initial sampling) and subsequently, more complex REML analysis was carried out. Only basic descriptive statistics were carried out on the small groups of second generation animals used in the assessment of *in vitro* phagocytosis of FITC-labelled *S. aureus* by isolated PMN and in the assessment of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by PMN in whole blood.

6.2 Data exploration

6.2.1 Distribution of data

A preliminary univariate analysis on approximately 140 animals sampled at approximately six months of age from the 2000 cohort was carried out for

assessment of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN. The percentage total phagocytosis by isolated PMN was assessed at all the time points i.e. 0 hours, 1 hour, 6 hour and 16 hours at 37°C and 1 hour at 4°C (as an indicator of non-specific adherence). The individual gates for 0 beads, one bead, two beads and greater than two beads were also assessed at each of the time points. As described previously, basic parameters such and Mean and Skewness were generated for the data.

Overall, the data was positively skewed for all the gates at each time point, apart from the 0 beads gate, which contained cells that had not undergone phagocytosis and were assumed to be the majority, if not all, of the cells during the early incubation points, which was negatively skewed at 4°C, 0 hours and 1 hour at 37°C (Table 6.1). Positive skewness of the data was high at 4°C for percentage total phagocytosis by isolated PMN, one bead, two beads and greater than two beads gate and the 0 hour and 1 hour incubation at 37°C. By six hours of incubation, the data was normally distributed in the percentage total phagocytosis by isolated PMN (Figure 6.1), 0 bead and two beads gate. By 16 hours of incubation, the data was normally distributed in the percentage total phagocytosis by isolated PMN, one bead and two beads gate. The distribution of the data at 0 hours showing the positive skewness, indicated that most isolated PMN had not phagocytosed any latex beads, and as the incubation progressed, the number of isolated PMN which had phagocytosed FITC-labelled (2.0μM) latex beads increased, and by six hours of incubation, the data was found to be normally distributed.

6.2.2 Box and Cox transformation

The optimal transformation of the data was determined using Box and Cox analysis in Minitab (release 13). Optimal transformations were assessed for the percentage total phagocytosis by isolated PMN at all of the time points at 4°C and 37°C (Table 6.2). Optimal transformations were also assessed for each of the gates i.e. 0 beads, 1 bead, 2 beads and greater than 2 beads at all of the time points at 4°C and 37°C (Table 6.2). Variation was seen in the optimal transformation suggested for the

In vitro phagocytosis of FITC-labelled (2.0μM) latex beads by the isolated PMN	4^0C (1 hour)		0 HOURS		1 HOUR		6 HOURS		16 HOURS	
	Skew	Prob (norm)	Skew	Prob (norm)	Skew	Prob (norm)	Skew	Prob (norm)	Skew	Prob (norm)
% total	2.83	0.0001	**1.82**	**0.0001**	**1.29**	**0.0001**	0.008	0.1340	**-0.48**	**0.0045**
0 bead	-4.35	0.0001	-2.82	0.0001	-1.36	0.0001	-0.06	0.2100	0.479	0.0005
1 bead	2.40	0.0001	1.79	0.0001	1.54	0.0001	1.76	0.0001	-0.21	0.3280
2 beads	2.87	0.0001	7.95	0.0001	1.64	0.0001	0.306	0.7921	-0.14	0.9961
> 2 beads	3.97	0.0001	1.8	0.0001	1.73	0.0001	0.62	0.0001	0.108	0.0014

Table 6.1: Univariate analyses of assessment of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation animals sampled at approximately six months of age from the 2000 cohort was performed using SAS statistical software. Skew = Skewness, Prob (Norm) indicates the significance of the data being significantly different from a normal distribution calculated using the Shapiro-Wilk W test. The skewness for percentage total phagocytosis, 0 bead, 1 bead, 2 beads and greater than 2 beads was shown for 4°C at 1 hour (aqua) and 37°C at 0 hours, 1 hour, 6 hours and 16 hours incubation. Normally distributed data are highlighted in red.

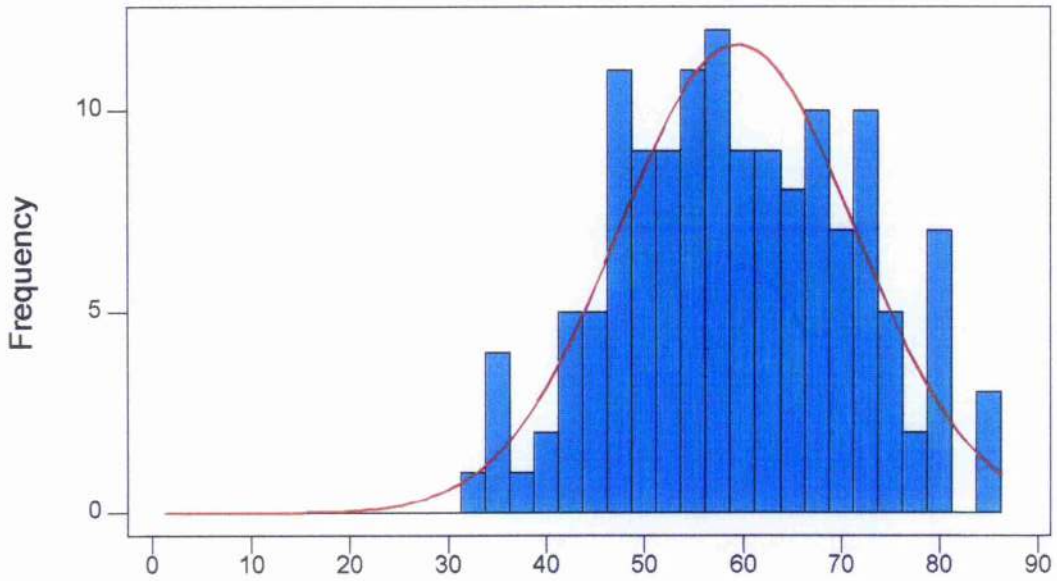


Figure 6.1: Skewness of data obtained from assessment of percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation sampled animals sampled at approximately six months of age from the 2000 cohort. Graphical representation of the percentage total phagocytosis at 6 hours at 37°C. Data is normally distributed (skewness=0.008, p=0.134).

In vitro phagocytosis of FITC-labelled (2.0μM) latex beads by the isolated PMN	4^0C (1 hour)		0 HOURS		1 HOUR		6 HOURS		16 HOURS	
	λ	Trans.	λ	Trans.	λ	Trans.	λ	Trans.	λ	Trans.
% total	0.224	Log_e Y	-0.113	Log_e Y	0.112	Log_e Y	1.011	NT	1.798	Y^2
0 bead	5.000	OB	5.000	OB	3.147	OB	0.786	NT	0.224	Log_e Y
1 bead	0.000	Log_e Y	0.000	Log_e Y	0.225	Log_e Y	0.000	Log_e Y	1.348	NT
2 beads	0.000	Log_e Y	-0.112	Log_e Y	0.112	Log_e Y	1.124	NT	1.124	NT
> 2 beads	0.224	Log_e Y	0.000	Log_e Y	-0.112	Log_e Y	0.224	Log_e Y	0.675	$\sqrt{Y}$

Table 6.2: Box and Cox transformation of assessment of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation animals sampled at approximately six months of age from the 2000 cohort was performed using Minitab (Release 13). λ = mean estimated lambda value. Trans.=optimal recognised transformation for data (Table 2.3). OB= data outwith transformation scale (i.e. extremely skewed). NT= no transformation of the data is required.

various time points and parameters measured (Table 6.2). Although a natural ln transformation was predominate it was decided to not transform the raw data. The raw data was left untransformed due to planned future analysis which would initially focus on the six hour incubation when maximal phagocytosis by the isolated PMN was observed. At the six hour time point no transformation was required for the percentage total phagocytosis by isolated PMN (Figure 6.2). The 0 bead and 2 beads gate, at the six hour time point and the 1 bead and 2 beads gate at the 16 hour time point, also required no transformation of the data (Table 6.2).

6.2.3 Correlations

6.2.3.1 Correlation of individual animal responses between time points

The Pearson's Product Moment Correlation Coefficient in Minitab (Release 13) was used to calculate the correlation of individual animal responses between time points, i.e. the correlation between 0 hours and one hour, one hour and six hours, and six hours and 16 hours incubation at 37°C. This was carried out to assess if individual animals showed any trend in the *in vitro* ability of their isolated PMN to phagocytose FITC-labelled (2.0μM) latex beads. A significant positive correlation was identified between the time points for the assessment of percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by the isolated PMN (Figure 6.3). A significant positive correlation was also identified between the time points for the 0 beads, one bead, two beads and greater than two beads gates. The significant positive correlations increased as the incubation period increased in all the gates except the one bead gate which showed a reduced correlation from 0 hours to 16 hours, the correlations ranged from 0.35 ($p<0.001$) to 0.78 ($p<0.001$) (data not shown). This positive correlation confirmed the validity of the assay and showed that individual animals do not fluctuate in their levels of *in vitro* PMN phagocytosis between time points in a single assay. As only a single time point was assessed at 4°C, no between time points correlations could be calculated.

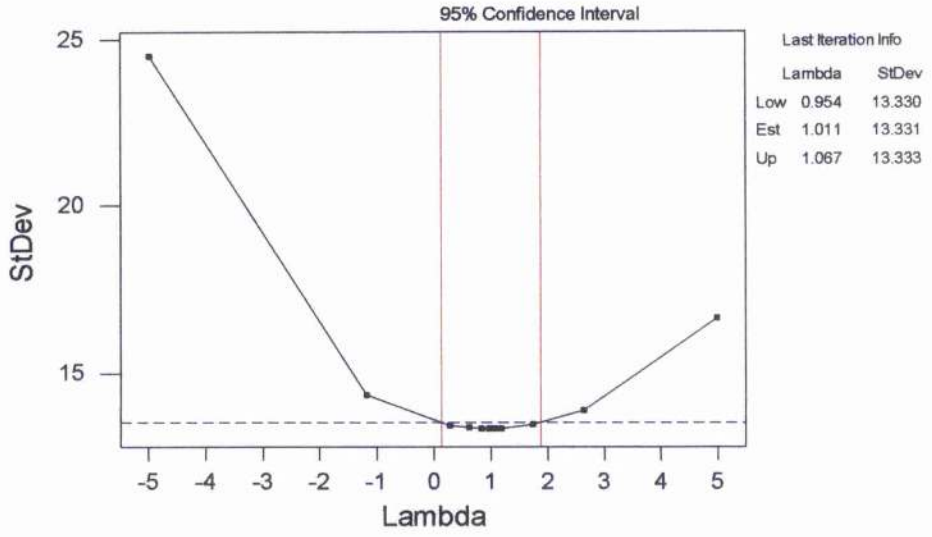


Figure 6.2: Box and Cox transformation of assessment of percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation animals sampled at approximately six months of age from the 2000 cohort.

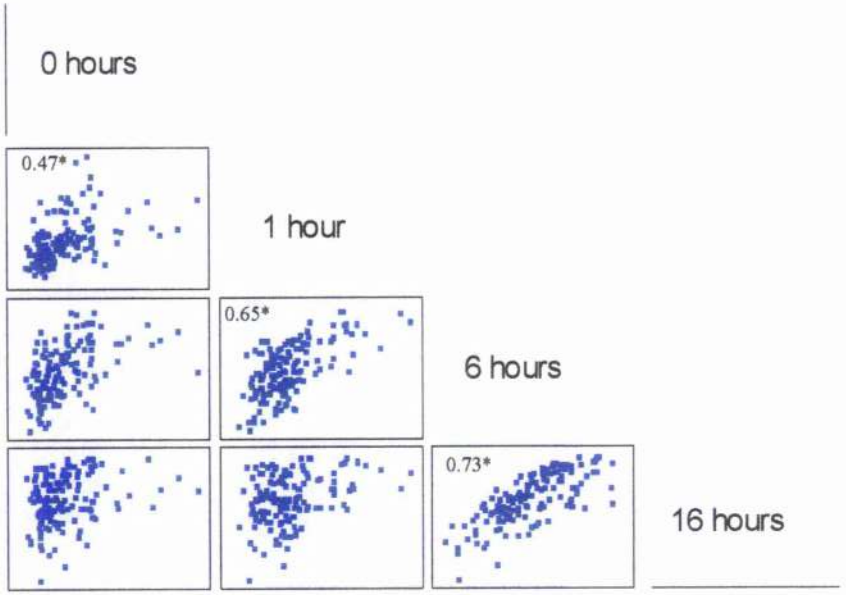


Figure 6.3: Matrix scatter plot showing correlation of individual animal responses between time point for assessment of percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation animals sampled at approximately six months of age from the 2000 cohort. Correlations are shown between incubation time points. * indicates a significant positive correlation ($p<0.05$).

6.2.3.2 Correlation of individual animal responses at re-sampling (approximately three months after initial sampling)

Correlation of individual animal responses at re-sampling (approximately three months after initial sampling) was assessed using the Pearson's Product Moment Correlation Coefficient in Minitab (Release 13). Forty (approximately 30% of the total animals sampled at approximately six months of age) second generation animals were re-sampled approximately three months after the initial sampling of the 2000 cohort. The correlation between initial sampling and repeat sampling was poor for several of the time points (Table 6.3). However, significant positive correlations were identified at some time points. A significant positive correlation was identified at 4°C for the one bead gate. Significant positive correlations were identified at 0 hours for the percentage total phagocytosis by isolated PMN, 1 bead, 2 beads and greater than two beads gates. Significant positive correlations were also identified for the two beads gate at six and sixteen hours. An almost significant correlation was identified for the percentage total phagocytosis by isolated PMN at six hours and the 0 bead gate at 16 hours.

6.2.4 Correlation between *in vitro* peripheral blood mononuclear cell proliferation and polymorphonuclear phagocytosis in the 2000 cohort

The *in vitro* PBMC response to both PHA and *S. aureus* was compared to the isolated PMN phagocytosis of FITC-labelled (2.0μM) latex beads in 140 second generation animals in the 2000 cohort. This was to determine if any correlation existed between the non-specific (PHA-induced) or specific (*S. aureus*-induced) proliferation of PBMC compared with the percentage total phagocytosis by isolated PMN from the same individuals. No significant correlations were identified between the *S. aureus*-induced PMBC proliferation and percentage total phagocytosis by isolated PMN. However, a significant positive correlation was identified between the PHA-induced PBMC proliferation on day two (0.192, $p<0.05$) and day three

In vitro phagocytosis of FITC-labelled (2.0μM) latex beads by the isolated PMN	4^0C (1 hour)		0 HOURS		1 HOUR		6 HOURS		16 HOURS	
	Corr.	*p* value	Corr.	*p* value	Corr.	*p* value	Corr.	*p* value	Corr.	*p* value
% total	-0.066	0.684	0.456	0.003	0.089	0.583	0.276	0.085	0.150	0.357
0 bead	-0.037	0.822	0.133	0.415	0.076	0.641	0.265	0.098	0.308	0.056
1 bead	0.334	0.035	0.574	0.000	0.157	0.332	0.032	0.845	0.053	0.751
2 beads	-0.058	0.723	0.447	0.004	0.124	0.445	0.421	0.007	0.316	0.050
> 2 beads	-0.143	0.378	0.394	0.012	-0.175	0.279	0.030	0.856	0.062	0.708

Table 6.3: Correlation of individual animal responses at re-sampling (approximately three months after initial sampling) assessed using the Pearson's Product Moment Correlation Coefficient in Minitab (Release 13). Forty (approximately 30% of the total animals sampled at approximately six months) second generation animals were re-sampled from the 2000 cohort. Individual animal correlations over time are shown for the percentage total phagocytosis, 0 beads, 1 bead, 2 beads and greater than 2 beads for *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN at 4°C at 1 hour (aqua) and 37°C at 0 hours, 1 hour, 6 hours and 16 hours incubation. Corr=Pearson's Product Moment Correlation Coefficient, *p* value=significance level. Significant correlations are shown in red ($p<0.05$), almost significant correlations are shown in green ($p<0.1$).

(0.168, $p<0.05$) of culture and the percentage total phagocytosis by isolated PMN at six hours incubation (a comparison of the peak days of mitogen-induced proliferation and the peak phagocytosis time by isolated PMN). This significant positive correlation may indicate a link between the non-specific immune responses of these individual animals.

6.3 Data analyses

6.3.1 Linear Mixed Models – Residual Maximum Likelihood

6.3.1.1 Animals and Model

Raw data was used in all REML analyses. All 140 animals from the 2000 cohort were analysed. Initially, the data was assessed as a complete data set analysing all the animals, male and female, together. However, following analyses of the males and females together there was evidence of interactions between sexes, which prompted repeating the analyses on single sex subsets. The percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN was assessed at each time point at 37°C (0 hours, one hour, six hours and 16 hours) and was assessed as the Y variate in the model. The individual bead gates were not assessed by REML analyses. No REML analysis was carried out on the 4°C data, due to the negligible levels of non-specific background adherence measured by flow cytometry.

6.3.1.1.1 Fixed factors used in the model

The fixed factors used in the final model were: the sex of the individual which had two levels, male and female; the cross of the individual, which had three levels, backcross Charolais, backcross Holstein-Friesian or F2 (50:50 Charolais:Holstein-

Friesian); the date the animal was sampled on, which had 14 levels, and the sample age, a covariate, that was the age of the individual animal in days at the point of sampling.

6.3.1.1.2 Random factor used in the model

The sire of the individual animal was assessed as the random factor in the model. There were a total of 12 sires used in the study: eight of the sires were F1 (50:50, Holstein Friesian: Charolais) sires (R01, R02, R05, R11, R12, R15, R18 and R21); and four of the sires were F0 Charolais (CH108, CH158, CH200 CH219).

6.3.1.1.3 Complete data set model

For the complete data set, the percentage total phagocytosis of FITC-labelled (2.0μM) latex beads at six hours incubation was examined. The REML model used was:

Fixed	sex + cross + sample date + sample age
Random	sire

6.3.1.1.4 Subset model

The REML model used for the analyses of the subsets of males and females was:

Fixed	cross + sample date + sample age
Random	sire

The date on which the individual animal was sampled, was open to a wide range of influences including environmental influences such as seasonal variations, housing conditions and the sampling procedures employed in the study which resulted in the animals being sampled in single sex groups. Due to the significant number of factors, which may have influenced the effect of sample date on the levels of

phagocytosis, it was considered appropriate to exclude any significant effect of sample date from the remainder of the analyses.

6.3.1.2 Significant factors affecting polymorphonuclear phagocytosis

On REML analyses of the whole data set, only significant fixed factors were identified. In the whole data set, no significant interactions were identified at any of the time points (Table 6.4). Sex was identified as a significant fixed factor at 0, 1 and 16 hours, with sample age identified as the only other fixed factor at 1 hour incubation ($p<0.05$). The table of effects for sex (Table 6.5) showed that the males had consistently higher levels of PMN phagocytosis than the females, for example, the predicted mean (± S.E.D) for percentage total phagocytosis of FITC (2.0μM) labelled latex beads at one hour incubation was 17.91 ± 5.10% and 30.08 ± 5.10% in the females and males, respectively. The effect of sample age was relatively small, with every increase of 30 days in the age of the individual animal resulting in a increase in the mean (± S.E.) percentage total phagocytosis of 2.87 ± 1.44%.

No significant effects were identified when the males and females were analysed separately at any of the time points. In general, REML analyses requires large sample numbers and when the males and females were analysed separately the sample numbers may have been too small to obtain accurate analysis of the data using this method.

On REML analysis, no significant effect of sire was identified at any of the time points, however, on plotting the data, variation in mean percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN was seen among sires at all of the time points (Figure 6.4). On plotting the data, variation was also seen among sires at individual time points (Figure 6.5).

TIME POINT (hours)	COMPLETE DATA SET ANALYSES	MALE DATA SET ANALYSES	FEMALE DATA SET ANALYSES
0	sample date sex	*cross . sample date .* sample age *cross . sample date*	sample date
1	sample age sample date sex	*cross . sample date .* *sample age*	sample date
6	sample date	sample date	sample date
16	sample date sex	sample date	sample date

Table 6.4: Data output following REML analysis (Genstat, 5th Edition). Table shows significant final fixed factors and interactions for Y variate = percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN. All factors shown have a significance level of $p<0.05$. Significant interactions are shown in italics.

TIME POINT (hours)	FEMALE	MALE
0	0.000	2.624
1	0.000	12.170
6	0.000	15.730
16	0.000	14.344

Table 6.5: Data output following REML analysis (Genstat, 5th Edition). Table shows table of effects for sex for percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation animals sampled at approximately six months of age from the 2000 cohort.

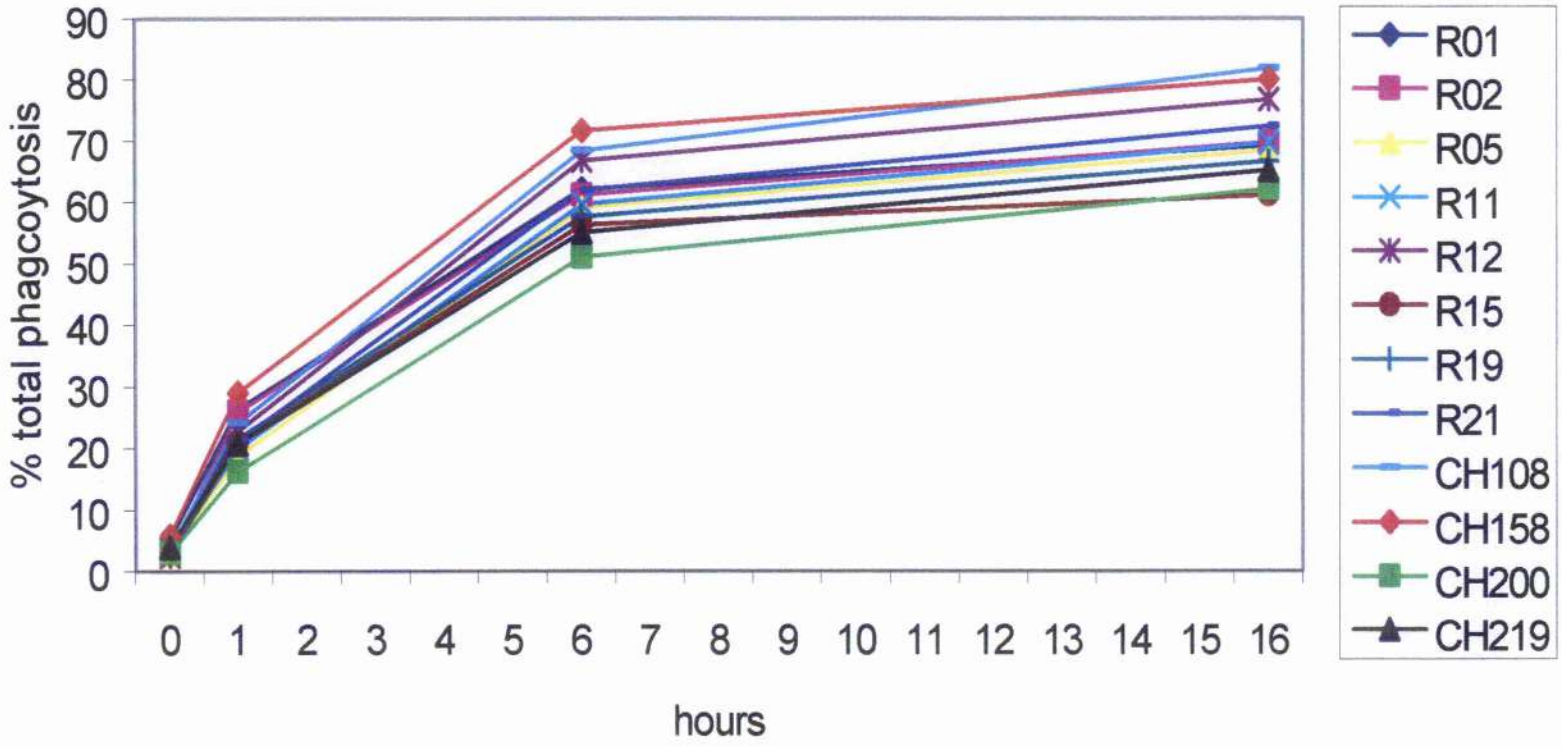


Figure 6.4: Assessment of the percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation animals sampled at approximately six months of age from the 2000 cohort. Graph represents the mean sire variation over time at 37°C.

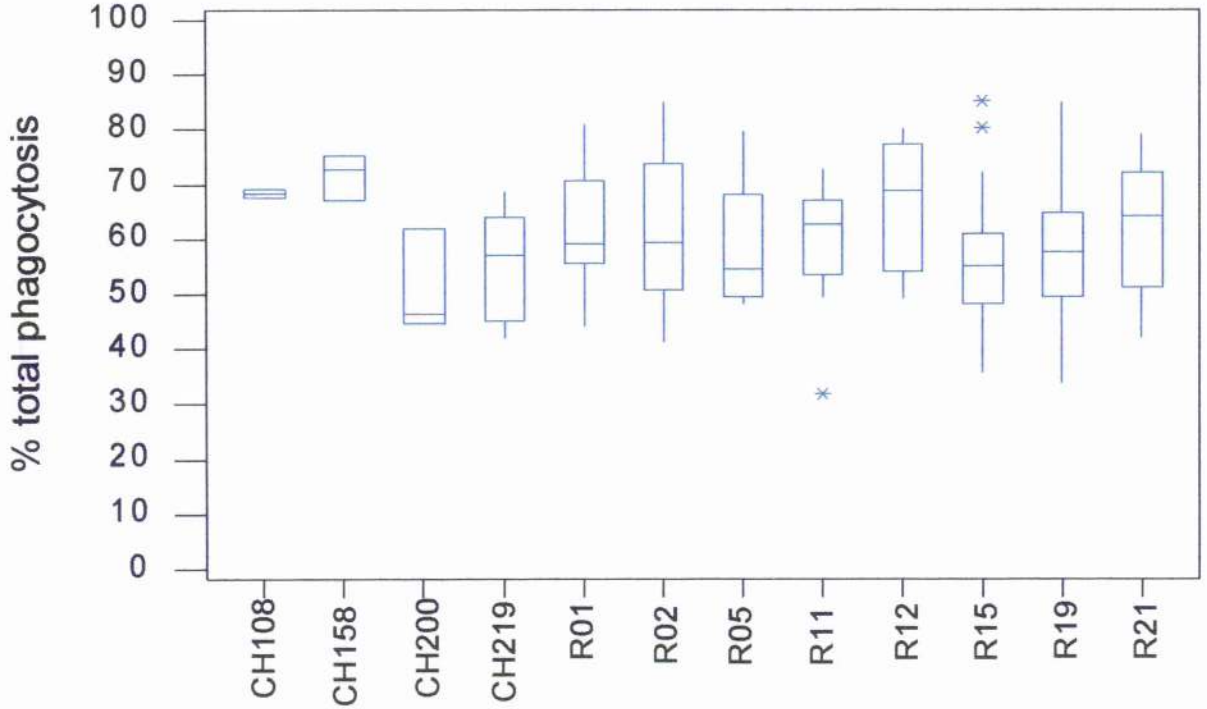


Figure 6.5: Assessment of the percentage total phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN from 140 second generation animals sampled at approximately six months of age from the 2000 cohort. Graph represents sire variation observed at six hours incubation at 37°C. R prefix indicates F2 sire, CH prefix indicates Charolais sire. The plots show the median (line within the box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and the outliers (symbol).

Chapter 7

General discussion

Understanding the processes involved in immunity to infection by pathogenic organisms is critical (Spriggs and Sher, 1999). Improvements in general immune competence should allow enhanced resistance to many pathogens and would, thus, be a desirable selection criterion for livestock (Weigel *et al.*, 1992). Various *in vitro* assays to assess neutrophil function; lymphocyte proliferation, serum Ig, complement and conglutinin concentrations (Kehrli *et al.*, 1991), have been applied to serve as indicators of *in vivo* immune function. The overall aim of this study was to assess the *in vitro* immune function in a genetically defined population of Holstein-Friesian cross Charolais male and female cattle from approximately six months of age. The females were assessed at 12 month intervals for the three years of the study, whereas, the males were sampled at approximately six months of age only, as they were slaughtered at approximately 500kg for assessment of traits such as meat quality and carcass development. The study was designed to measure various peripheral blood mononuclear and polymorphonuclear cellular responses using a number of *in vitro* assays to assess aspects of cellular function induced by both non-specific and specific immunological stimuli.

7.1 *In vitro* peripheral blood mononuclear cell proliferation

An essential aspect of this part of the study was to use the same batch and strain of *S. aureus*, as both strain and batch-to-batch variation have been shown previously to be important in both the kinetics and the magnitude of *S. aureus*-induced proliferation, in addition, in a previous study by Logan (2001), *S. aureus* strain NCTC 13047 had been shown to successfully identify variation among individual animals in their *in vitro* PBMC proliferation when compared to other commercially available preparations of *S. aureus*. The strain of *S. aureus* was confirmed by REFP analysis, and enterotoxin typing was also carried out in order to improve the phenotypic profile of the strain used in the assay. Enterotoxin D was identified as the only enterotoxin produced by *S. aureus* NCTC 13047. Toxin production is one of the essential components that determines the pathogenicity of staphylococci (Anderson, 1986; Kotzin, *et al.*, 1993; Lee, 1996). Enterotoxins A and TSST-1 have been shown to play a role in the stimulation and proliferation of T cells (Yokomizo *et al.*, 1995), whereas, enterotoxin B has been reported by some authors to induce an *in vivo* state of immunosuppression that affects both humoral and cell mediated immunity (Pinto *et al.*, 1978). A single batch of formalin fixed *S. aureus* NCTC 13047, confirmed by REFP analysis, was prepared and optimised for use throughout the three sampling years on all the individuals sampled. The preparation of a single batch of *S. aureus* minimised the levels of assay variability and maximised the assay repeatability.

In vitro PBMC proliferation was assessed in over 300 animals at approximately six months of age over the three years of the study. Non-specific PHA-induced blastogenesis, specific *S. aureus*-induced proliferation and control proliferation of the PBMC was assessed simultaneously. Previous studies have shown variation in *S. aureus*-induced proliferative response among commercial Holstein-Friesian cattle (Fitzpatrick *et al.*, 1999; Logan 2001), measured by ^{3}H-thymidine incorporation in the responding PBMC population, as used routinely by others (Schore, *et al.*, 1981; Concha and Holmberg, 1996; Shafer-Weaver and Sordillo, 1997). This study

expanded these observations in variation in proliferative ability among individual animals into a much larger, genetically defined population, of male and female animals, and investigated the variation in response among these individual animals over three years. Variation in the proliferative ability of these animals in relation to breed or sire was also assessed. Preliminary development of the *S. aureus*-induced PBMC proliferation assay had been carried out in previous studies and PBMC blastogenesis by lectins such as PHA has been described in previously published work (Pearson *et al.*, 1979; Schore, *et al.*, 1981). Results of *in vitro* PBMC proliferation have traditionally been expressed as stimulation indices (SI, PBMC plus antigen, divided by PBMC without antigen), or as Δ counts per minute, (PBMC plus antigen, minus, PBMC without antigen, Lan *et al.*, 1995). In this study, data for both the counts per minute plus antigen (either *S. aureus* or PHA-induced) and the counts per minute minus antigen (control proliferation) were reported, rather than SI or Δ counts per minute.

The *S. aureus*-induced proliferation was shown to peak between day four and day five of culture, as shown previously by Fitzpatrick *et al.* (1999), and Logan (2001). Variation in *S. aureus*-induced *in vitro* PBMC proliferation was seen among individual animals throughout the assay, with a wide range of 62.1-195,107 counts per minute on day five of culture, while control proliferation varied from 42.0-14,812.7 counts per minute, on the same day. Variation in the magnitude of the proliferative response induced by antigenic challenge has been previously reported by others (Fitzpatrick *et al.*, 1995; McCole *et al.*, 1998).

In this study, the non-specific PHA-induced blastogenesis was shown to reach peak proliferation between days two and three of culture, as shown in a previous study (Pearson *et al.*, 1979), which assessed a range of different lectins. The previous study concluded that PHA-induced PBMC proliferation peaked on day three of culture with a mean maximum stimulation ratio of 204.7. Pearson *et al.* (1979) also identified variation among individual animals in their PHA-induced proliferation, with a range of stimulation indices from 82.4 to 394.9 identified in five Holstein-Friesian steers of between six and ten months of age. In the current study, on day three of culture, the PHA-induced proliferation ranged widely from 312.17-169569

counts per minute, while control proliferation ranged from 69.47-16265.6 counts per minute.

In the current study, the assay was further developed by assessing the kinetics of the *in vitro* PBMC proliferation at six time points over a 10 day incubation period. The *in vitro* proliferation was assessed on days two, three, four, five, nine and ten of culture. It was necessary to assess all the different time points, to allow comprehensive assessment of the kinetics of both the non-specific and specific responses of the PBMC. Variation in the kinetics between the specific and non-specific stimuli were confirmed, with the *S. aureus*-induced PBMC proliferation peaking at day four or five, whereas the PHA-induced PBMC proliferation peaked by day two or three of culture, depending on the individual animal. By day nine and ten of culture, very little proliferation was observed with either the stimulated PBMC or the control PBMC, due to the large proportion of dead or dying cells present in the culture at this point in time.

The variability among animals in the intensity of the immune response induced may reflect the efficiency of antigenic processing, association between antigenic peptides and MHC class II, and ultimately presentation to T helper cells (Buus *et al.*, 1987). Individual variability in the intensity of an immune response induced by a specific antigen has been reported in both inbred populations such as mice (Hooper *et al.*, 1987) and in outbred populations, such as cattle (Lutje and Black, 1991). Variation among individual animals was seen in the current study at all of the days assessed, with the largest variation seen around peak proliferation for both *S. aureus*-induced and PHA-induced cultures.

Previous studies by other authors aimed at assessing immune function in cattle have generally been carried out on smaller numbers of female dairy cattle (Detilleux *et al.*, 1995; Weigel *et al.*, 1991). Evaluation of beef cattle has focussed on assessing castrated male calves up to approximately two years of age for traits such as lean tissue growth rate (Kempster *et al.*, 1988) or feed conversion ratios rather than immunity (Arthur *et al.*, 2001). The current study involved sampling a large number of animals, both male and female, at approximately the same age, with known genetic backgrounds, which is unique with regard to the current literature. This was

essential to determine if individual variation among animals was related to the sex, cross, or sire of the animal. In this study, variation in *in vitro* PBMC proliferative ability was identified between males and females, among cross of the animals, i.e. whether they were F2, Holstein-Friesian backcross or Charolais backcross, and among sires.

One aim was to identify if the variation observed among individuals in relation to any of these factors was consistent for *S. aureus*-induced, PHA-induced and control proliferation. The hypotheses to be tested were: i). animals that were high responders to specific stimuli also high responders to non-specific stimuli, ii). animals that were poor responders to specific stimuli were also poor responders to non-specific stimuli, or iii). animals that were high responders to non-specific stimuli were poor responders to specific stimuli. This was assessed in the females from the 1999 cohort due to the availability of additional MHC *DRB* allele RFLP patterns, that had been carried out at the Roslin Institute. The females in the 1999 cohort, 56 in total, were ranked for *S. aureus*-induced and PHA-induced PBMC proliferation and analysed with respect to MHC *DRB 3* allele RFLP patterns. The study indicated that six animals that were ranked in the highest 15 for *S. aureus*-induced proliferation were among the lowest 15 for PHA-induced proliferation and conversely, four animals that were in the highest 15 for PHA-induced proliferation were among the lowest 15 for *S. aureus*-induced proliferation. Only two animals were identified as being in the highest 15 for both *S. aureus*-induced and PHA-induced PBMC proliferation and conversely, and only two animals were identified in the lowest 15 for both *S. aureus*-induced and PHA-induced proliferation. The data suggests that a good specific immune response in an individual does not necessarily mean a good non-specific immune response, and conversely, a poor specific immune response does not necessarily mean that the individual will have a poor non-specific immune response. These observations indicate that the specific and non-specific arms of the immune system may not be controlled in the same way and may reflect functions of different cell types and immune responses. This finding may have implications in the breeding of dairy cattle as resistance to one particular disease, may then lead to susceptibility to another disease. The method of assessment of immune function is, therefore, critical in the identification of genetic markers of resistance or susceptibility, which may be used in future breeding programmes due to the potential

situation which may have resulted in an incorrect marker of immune function being chosen to indicate resistance to a particular pathogen or disease and thus resulting in inadvertently breeding for susceptibility to this disease rather than resistance. In this study many different RFLP patterns were identified in both the high and low groups of animals for *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation. Previous studies in dairy cattle have shown associations of *DRB 16* with both resistance to (Sharif *et al.*, 1998), and susceptibility to (Dietz, *et al.*, 1997a), clinical mastitis. In the current study, only five of the animals possessed *DRB 16*, and of these five animals, three were ranked in the highest 15 for *S. aureus*-induced proliferation and four were ranked in the lowest 15 for PHA-induced proliferation. This data highlights the importance of the assay chosen for assessment of resistance or susceptibility to disease, as the same individuals were ranked in the highest 15 for specific immunity while being in the lowest 15 for non-specific immunity. Choosing the correct method of assessing immune function is therefore an essential aspect on any study. For example, if the current study had examined either *S. aureus*-induced PBMC proliferation or PHA-induced proliferation, rather than both, the possible dichotomy between specific and non-specific immune function highlighted in these animals would not have been identified. Obviously further work in this area would need to be carried out to substantiate this finding.

In addition to the main data set of 300 animals at six months of age, the females were sampled longitudinally at 12 month intervals throughout the three years of the study. A previous study by Ayoub and Yang (1996) involving four female calves, studied changes in peripheral blood lymphocyte subpopulations from two months of age, monthly, until two and a half years of age and again at approximately four years of age. Ayoub and Yang concluded that the proportions of peripheral blood lymphocytes varied from approximately six months of age in the animals, with individual animal variation also occurring. It has been recognised that the numbers and types of cell present in the peripheral blood which are capable of recognising and responding to a pathogenic stimulus will have an impact on the intensity of the subsequent immune response. Previous exposure to a pathogenic stimulus may result in the expression of memory phenotype (CD45RO) $CD4^+$ T cells. These $CD4^+$ memory T cells have been shown to have altered proliferative responses, requiring much lower levels of antigen to induce a proliferative response (Rogers *et al.*, 2000).

When T cells, expressing the memory phenotype, are induced to proliferate by recall antigen, they display rapid proliferative responses when compared to naïve T cells which have been shown to undergo a lag phase prior to induction of a proliferative response (Rogers *et al.*, 2000). This current study attempted to assess if variation existed in the specific and non-specific *in vitro* PBMC proliferative ability in the females in our study over time. There were only 22 females born in the 1998 cohort, which were assessed at 18 and 30 months of age and 57 females born in the 1999 cohort which were assessed at 18 months of age. No obvious trend was identified for either mean (± S.E.M.) *S. aureus*-induced or mean (± S.E.M.) PHA-induced PBMC proliferation when the animals were sampled at 12 month intervals. In the 1998 females there was a reduction in the mean (± S.E.M.) *S. aureus*-induced proliferation between 1998 and 1999 (i.e. 6 and 18 months of age), whereas, with the 1999 cohort there was no significant difference in the mean (± S.E.M.) *S. aureus*-induced proliferation between 1999 and 2000 (i.e. 6 and 18 months of age). The mean (± S.E.M.) PHA-induced proliferation in the 1998 cohort increased significantly between 1998 and 1999 (i.e. 6 and 18 months of age), whereas, in the 1999 cohort there was a significant decrease in the mean (± S.E.M.) PHA-induced proliferation between 1999 and 2000 (i.e. 6 and 18 months of age). The mean (± S.E.M.) PHA-induced proliferation continued to increase in the 1998 cohort between 1999 and 2000 (i.e. 18 and 30 months of age), with a marginal increase observed in the mean (± S.E.M.) *S. aureus*-induced proliferation. Due to the relatively small numbers of females in the 1998 cohort and also that within the scope of this study the 1999 females were followed at 6 and 18 months compared to the 1998 females which were followed at 6, 18 and 30 months of age, any accurate conclusion about variation in specific or non-specific immune function with age, measured by *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation, respectively, was extremely difficult and would require further assessment.

There was also no variation observed in the kinetics of either the *S. aureus*-induced or PHA-induced *in vitro* PBMC proliferation between the sampling years: the peak assay day for *S. aureus*-induced and PHA-induced proliferation remained the same irrespective of the age of the animals sampled.

Univariate statistical analyses of the data was performed on the animals sampled at approximately six months of age to assess factors such as the distribution of the data, the mean, the skewness, optimal transformations, correlation of individual animal responses between days of culture and correlation of individual animal responses at re-sampling (approximately three months after initial sampling). Assessment of the distribution of the 'raw' data is essential, as most statistical procedures require data which fits a normal distribution, unless non-parametric tests such as the Wilcoxin Signed Rank Test (equivalent to a paired t-test) or Spearman's Rank Correlation Coefficient (equivalent to Pearsons Correlation Coefficient) were to be employed.

The *S. aureus*-induced, PHA-induced and control *in vitro* PBMC proliferation were positively skewed on all of the days of culture, with a general increase in the skewness observed for all the parameters with increasing days of *in vitro* culture. The increase in skewness was greatest in the PHA-induced and control proliferation which were approximately five times more skewed on day ten of culture than on day two of culture, whereas, *S. aureus*-induced proliferation showed an approximate two fold increase in skewness between days two and ten of culture. This increase in skewness corresponds with a decrease in the functional viability of the PBMC, thus, as the PBMC proliferation decreased, the skewness increased.

Box and Cox analyses (Box and Cox, 1964) indicated that the overall optimal transformation of the data to provide a more normally distributed data set was a natural logarithmic transformation. Transforming the *S. aureus*-induced, PHA-induced and control proliferation data using the same transformation allowed biological comparison and interpretation of the data for all the parameters following the subsequent REML analyses and was considered appropriate for this study.

Variation in proliferative ability has been shown to be influenced by age, sex, breed, stress, health status and genetic background (Schalm *et al.*, 1976; Kristensen *et al.*, 1982). In order to assess the affect of day to day assay variation compared with 'real' variation between individual animals, the individual animal between day correlation was assessed using the Pearson's Product Moment Correlation Coefficient on each day of culture in the current study. The correlation of individual animal responses between days of culture was high, with significant ($p<0.05$)

positive correlations observed between the days of culture for the *S. aureus*-induced, PHA-induced and control *in vitro* PBMC proliferation, indicating that variation observed among individuals was due to 'real' variation and not between day assay variation.

The correlation of individual animal responses at re-sampling (approximately three months after initial sampling) was lower, than correlation of individual animal responses between days of culture, but still significant ($p<0.05$), with positive correlations observed on days four and five of culture for *S. aureus*-induced and control *in vitro* PBMC proliferation, with an almost significant ($p<0.1$) correlation identified for PHA-induced proliferation on day four of culture. This indicated that, overall, individual animal correlation when animals were re-sampled approximately three months after the initial sample was poor for days two, three, nine and ten of culture, however, their responses at peak proliferation, days four and five of culture, were significantly positively correlated for *S. aureus*-induced, PHA-induced and control proliferation between the initial and repeat sample.

The correlation between individual animals responses at re-sampling (approximately three months after initial sampling) indicated a similarity between the sampling points, i.e. animals that had high levels of *S. aureus*-induced *in vitro* PBMC proliferation on the first sampling point still had high levels of *S. aureus*-induced *in vitro* PBMC proliferation several months after the initial sampling. The significant correlation between individual animals over time on days four and five of culture for the *S. aureus*-induced and control proliferation indicated the validity of use of the assay on these days as an indicator of *in vitro* PBMC proliferative ability and in measuring variation among individuals. Phytohaemagglutinin-induced *in vitro* PBMC proliferation has traditionally shown to have low levels of assay repeatability. In the current study, correlation of individual animal responses at re-sampling (approximately three months after initial sampling) was poorer than for *S. aureus* or control culture, but almost significant on day four of culture indicating that it may serve a useful role as a indicator of non-specific immunity in cattle.

A strong correlation in immune function tests in individual animal responses at re-sampling would not be expected, as the immune systems of these animals have to be

adaptable and are constantly changing, influenced by many factors such as the environment, hormone fluctuations and the exposure to pathogenic organisms. In the current study, as the assay was found to be correlated at day four and five of culture for most parameters, the validity of the assay to assess 'real' variation in immune function among animals rather than just variation in the assay was confirmed, with the proviso that animals studied were sampled over a relatively short period of time.

Residual Maximum Likelihood analysis was performed on a total of 302 animals sampled at approximately six months of age. Preliminary analyses were carried out on the complete data set including males and females. Due to the evidence of interactions between sexes, subsequent analyses were also performed on males and females separately.

Preliminary analyses identified that the control proliferation had a significant effect on both the *S. aureus*-induced and PHA-induced proliferation on all the days of culture and subsequent models created for analyses of the *S. aureus*-induced and PHA-induced data, therefore, included control proliferation as a fixed factor. The control PBMC proliferation was found to have a regression coefficient of between zero and one which meant that the use of delta counts per minute i.e. counts per minute in the presence of antigen minus counts per minute in the absence of antigen, or stimulation indices (SI) i.e. counts per minute in the presence of antigen divided by counts per minute in the absence of antigen, would have been inaccurate. For example, in the current study, the males were found to have consistently higher levels of control PBMC proliferation than the females, and, in the current study, if SI had been used to assess immune function, the calculation of SI would have resulted in a reversal of the current findings, with the male SI being lower than the female SI, whereas, in the current study, in all the parameters assessed, the males were shown to have consistently higher levels of *in vitro* PBMC proliferation than the females.

In the whole data set, sex had a significant effect on control proliferation throughout the assay, with males having consistently higher control proliferation than females. Sex was identified as a significant factor as a fixed effect on day two of culture. On days three, four and five of culture, the sex by cross two-way interaction was found to be significant. The only other factor which was identified as having a significant

effect on the control PBMC proliferation was the year of birth of the individual animal, which showed that the animals born in the 1998 cohort had lower levels of control PBMC proliferation than the animals born in the 1999 or 2000 cohorts. This effect was only significant on day two of culture.

In the males and females the main significant effect on the control PBMC proliferation was the cross of the individual. In the males, the cross was identified as a significant fixed factor on day five of culture, whereas, in the females, the cross was identified as a significant factor on days three, four and five of culture.

In the whole data set, the backcross Charolais animals had higher levels of control PBMC proliferation than the backcross Holstein-Friesian and the F2 animals. When the males and females were analysed separately, the mean control proliferation for each of the crosses was found to differ between the sexes. In the males the F2 animals were found to have higher levels of control proliferation than the backcross Holstein-Friesian or backcross Charolais, whereas, in the females, the backcross Charolais animals were found to have higher levels of mean control proliferation than the backcross Holstein-Friesian of the F2.

The significant cross effect identified in the control proliferation in the females up to and including day five, and the significant random effect of sire identified in the males on day two of culture, may indicate genetic effects on the *in vitro* PBMC proliferation in the absence of either antigenic or mitogenic stimuli which may be important in the early stages of *in vitro* culture.

In the whole data set, the main significant effects on the *S. aureus*-induced PBMC proliferation following analyses were the sex of the individual, the cross, the year of birth, the control and the sample age. As with the control proliferation, the males had consistently higher levels of *S. aureus*-induced *in vitro* PBMC proliferation when compared to the females.

In the whole data set, cross was identified as a significant fixed effect on day two and as a significant two-way interaction on day three. Further assessment of the cross effect in terms of *S. aureus*-induced *in vitro* PBMC proliferation, indicated that at

peak proliferation, the Holstein-Friesian backcross animals had a considerably better responses than the Charolais backcross or the F2 animals. The F2 animals had marginally better levels of *S. aureus*-induced proliferation than the Charolais backcross.

One hypothesis for the ability of the Holstein-Friesian backcross animals to respond to the *S. aureus* most effectively could be due to fact that *S. aureus* is one of the major bovine mastitis pathogens in the UK, causing both clinical and subclinical mastitis. *Staphylococcus aureus* is the predominant cause of subclinical mastitis (Wilson and Richards, 1980), where somatic cell count acts as an indicator of infection. Persistently elevated somatic cell counts indicate chronic infection which is characteristic of *S. aureus* (Holdaway *et al.*, 1996). It is possible, therefore, that Holstein-Friesian cattle that have the ability to mount an effective immune response against *S. aureus* will survive within the genetic population, as animals with poor immune responses against *S. aureus* may have been culled from the herd due to repeated episodes of clinical and subclinical mastitis or persistently elevated somatic cell counts which would be economically detrimental to the dairy farmer. Conversely, one hypothesis to explain the poorer levels of *S. aureus*-induced proliferation in the backcross Charolais animals could be that they have been selected for traits other than resistance to infection with *S. aureus*.

In the whole data set, the year of birth was found to have a significant effect on the *S. aureus*-induced proliferation on all days of culture, i.e. days two, three, four and five, as either a significant fixed factor or a significant interaction on day three of culture. The *S. aureus*-induced proliferation was shown to decrease from 1998 to 2000, this may be due to many factors such as possible deterioration of the antigen in storage, which may have made it less immunogenic. In future studies it may be required that optimisation of the antigen for use should occur prior to each years sampling to test this possibility. Environmental factors, such as increases in herd size from year to year and differing exposure to pathogens, may also potentially influence immune responses.

In the whole data set, the sample age of the individual animals was found to have a significant effect on the *S. aureus*-induced proliferation as a significant two-way or

three-way interaction on day three of culture or as a fixed effect on day four of culture. As the age of the animals increased their levels of *S. aureus*-induced proliferation increased, indicating a possible increase in the specific immune responses of these animals corresponding with the development and maturation of their immune systems.

When the males and females were analysed separately, the main significant effects on the *S. aureus*-induced proliferation, in both the males and females were the year of birth, sample age and control proliferation, with the cross only identified as having a significant effect as part of a significant interaction on day three of culture. The effect of year of birth was similar in both the males and females with both males and females having lower levels of *S. aureus*-induced PBMC proliferation in the 2000 born cohorts than in the 1998 born cohorts. The effect of sample age on the *S. aureus*-induced proliferation was found to differ slightly between the sexes, on day four of culture, the males showed an increase in their levels of *S. aureus*-induced proliferation with increasing age, whereas, in contrast, the females showed a slight decrease in their levels of *S. aureus*-induced proliferation with increasing age.

A significant random effect of sire was identified in the whole data set and in the females on day two of culture only, which may indicate some form of genetic control of early responses of PBMC to *S. aureus*.

The main significant effects on the PHA-induced proliferation when the complete data set was analysed were the sex of the individual, cross, year of birth, control and sample age. As with the control and *S. aureus*-induced proliferation, the male PHA-induced proliferation was markedly higher than the female PHA-induced proliferation.

In the whole data set, the cross of the individual was identified as a significant effect on day five of culture. Further assessment of the cross effect, at peak proliferation indicated that the backcross Charolais animals had greater levels of PHA-induced proliferation than either the Holstein-Friesian backcross or F2 animals. One hypothesis for this variation in PHA-induced proliferation related to breed could be that Charolais animals are traditionally bred for beef production and may be required

to have effective non-specific immune systems to prevent the establishment of generalised infections, including respiratory and enteric disease, which would be detrimental to growth rates and associated production traits.

In the whole data set, the year the animals sampled at approximately six months of age were born, was found to be significant as a fixed effect on days two and four of culture. The PHA-induced proliferation was shown to increase with the year of birth, as the animals sampled at approximately six months of age born in the 1998 cohort had lower levels of proliferation than the animals sampled at approximately six months of age born in the 2000 cohort. This increase in PHA-proliferation may indicate an increase in the levels of non-specific immunity in these animals which may be due to environmental or other non-measured effects.

In the whole data set, in the animals sampled at approximately six months of age, the 'actual' sample age was identified as having a significant effect on the levels of PHA-induced proliferation on days two and five of culture as either a significant two-way interaction or a significant fixed effect. Overall, as the age of the animals increased, the levels of PHA-induced proliferation decreased, indicating a reduction in the levels of non-specific immunity. This reduction in non-specific immunity may be linked to the increase in specific immunity with age as shown by the increase in the levels of *S. aureus*-induced proliferation described earlier.

When the males and females were analysed separately, the main significant effects were cross, control, year of birth and the sample age, in both males and females. The cross was identified as a significant effect in the males only, on days two and five of culture, whereas the year of birth was identified as a significant effect in both the males and females. The effect of year of birth was found to differ between the sexes, with the 1998 females having higher levels of PHA-induced proliferation than the 1999 and 2000 cohort animals, whereas, the 1998 males had the lowest levels of PHA-induced proliferation when compared to the 2000 males, with the 1999 males having the highest levels of proliferation. The sample age was found to have a similar effect on the PHA-induced proliferation in both the males and females, with increasing age in the individual animals resulting in a decrease in the level of PHA-

induced proliferation. This decrease was approximately 10% greater in the females for every 7 day increase in age when compared to the males.

The sire was identified as a significant random factor in the whole data set on day two, and in the males on days two and three of culture, indicating that genetic control may affect the levels of PHA-induced proliferation in individual animals in the early stages of culture.

For all the parameters assessed there are indications that immune function, particularly early in culture may be linked to the sire of the individual animal, with the cross of the individual influencing the immune responses of these animals throughout the assay, thus highlighting a possible genetic link with both specific and non-specific immune function.

7.2 *In vitro* polymorphonuclear cell phagocytosis

Polymorphonuclear cell function is one of the most important host defences against pathogenic challenge (Perticarari *et al.*, 1991). Measuring various aspects of blood polymorphonuclear cell function has been shown to be of increasing importance in medical diagnosis and prognosis (Egger *et al.*, 1997). Neutrophil function in cattle has been assessed in relation to many factors including, neonatal susceptibility to disease (Moiola *et al.*, 1994; Menge *et al.*, 1998), immunosuppression at parturition (Dosogne *et al.*, 1999), reduced PMN function associated with antibiotic administration (Hoeben *et al.*, 1997), and in the selection of dairy cattle in breeding programmes for resistance to disease (Durr *et al.*, 1996; Kelm *et al.*, 1997).

In the past, studies have been performed to quantify phagocytosis (Dunn and Tyrer, 1981; Bjerknes and Bassoe, 1984), distinguish adherent from ingested bacteria, (Fattorossi *et al.*, 1989; Santos *et al.*, 1995) and to monitor the oxidative burst of PMN by flow cytometry (Bass *et al.*, 1983; Salgar *et al.*, 1991). One of the aims of the current study was to develop a method to assess accurately *in vitro* bovine PMN phagocytic ability in an isolated PMN population by flow cytometry. Following development of the assay, another aim of the current study was to assess if variation existed among individual animals in their PMN phagocytic ability, and if so, was the variation associated with the sex, cross, or the sire of the individual animal.

Assessment of a wide range of parameters were carried out during the development and optimisation of the *in vitro* phagocytosis assay by isolated PMN. Non-specific adherence of FITC-labelled particles to the outside of the PMN cells surface, resulting in an overestimation of total phagocytosis has been well documented by others. Quenching this non-specific adherence prior to flow cytometry had been assessed by various methods including the addition of trypan blue (Trinkle *et al.*, 1987), ethidium bromide (Fattorossi *et al.*, 1989), crystal violet (Perticarari *et al.*, 1991), or a commercial lysing agent, Immunolyse™ (White-Owen *et al.*, 1992).

Santos *et al.* (1995) assessed non-specific adherence in isolated human PMN by running a duplicate sample at 4°C, which inhibited phagocytosis by the isolated PMN. Santos *et al.* (1995) used scanning electron microscopy to confirm that adherence and not phagocytosis of the fluorescent (2.0μM) latex particles occurred at 4°C. This method of assessing non-specific adherence was successfully adopted in the current study. Relatively low levels of non-specific adherence of the FITC-labelled latex beads, of approximately 1%, were observed during the optimisation of the assay, which allowed a single assessment of the non-specific adherence at one hour to be used as a background control for all the time points employed. Phagocytosis is facilitated by opsonins such as the immunoglobulins IgG and IgM and complement component C3 (Van Furth and Leijh, 1978). The need for opsonisation has been studied extensively since Wright and Douglas (1903) first described the opsonic effect of serum, reviewed by Horwitz (1982). In contrast, Wood *et al.* (1946) reported that phagocytosis can occur in the absence of opsonins when bacteria are trapped by the PMN against a surface and undergo 'surface phagocytosis'. More recent studies have indicated that the surface charge and hydrophobicity of the bacteria are important factors that determine whether microorganisms need opsonins for the successful uptake by PMN (Van Oss and Gilman, 1972, Stendahl *et al.*, 1979). A study by Vandenbrouke-Graulis *et al.* (1984) examined the interaction between human PMN and *S. aureus* in the presence and absence of opsonins. This study concluded that uptake of unopsonised *S. aureus* proceeded at a slower rate than opsonised bacteria, however, the unopsonised staphylococci induced metabolic activity and degranulation in the PMN to the same extent as opsonised bacteria. A more recent study by Paape *et al.* (1996), identified that in the absence of opsonins, bacteria can adhere to neutrophils by specific binding between mannose-specific lectins on the surface of the bacteria and carbohydrates on the phagocytic cell membrane which is mediated by the integrins CD11a, b, c/CD18 (Gbarah *et al.*, 1991). In this current study, we assessed the phagocytosis of FITC-labelled (2.0μM) latex beads in the presence of heat inactivated autologous serum, heat inactivated foetal calf serum or heat inactivated adult bovine serum. Foetal calf serum resulted in the lowest levels of phagocytosis, with autologous serum having marginally higher levels of phagocytosis than adult bovine serum. Adult bovine serum was chosen for use in the assay, for practical reasons: it was easier to use than

autologous serum which would have required additional stock FITC-labelled (2.0μM) latex beads solutions for each individual, rather than a single solution which could be aliquoted among animals. Assessment of the phagocytosis of different sizes of FITC-labelled beads was also carried out, FITC-labelled latex beads smaller than 2.0μM were not phagocytosed by isolated PMN populations and not detectable visibly by flow cytometry. Assessment of the ratio of PMN to FITC-labelled (2.0μM) latex beads was also carried out to ensure the optimum ratio for maximal phagocytosis. Previous studies have shown that ratios of less than 25 FITC-labelled bacteria to one PMN resulted in lower levels of phagocytosis by PMN in whole bovine blood and a ratio of 25:1 was chosen as optimal (Smits *et al.*, 1997). In the current study a ratio of 50 FITC-labelled latex beads to one PMN was chosen as optimal, as it was shown to have significantly higher levels of phagocytosis than 25:1, and was not significantly different from 100:1. Studies assessing phagocytosis by PMN in whole blood and isolated PMN populations use a range of incubation times to examine phagocytosis (Hasui *et al.*, 1989; Perticarari *et al.*, 1991; Perticarari *et al.*, 1994; Smits *et al.*, 1997). The optimal time point for maximal phagocytosis in the assay conditions developed in the current study, to assess the *in vitro* phagocytosis of FITC-labelled latex beads by isolated PMN, was six hours incubation at 37°C, a lower, but still measurable, level of phagocytosis was seen at one hours incubation. The 16 hours time point was used as an end point for the phagocytosis, as the numbers of viable PMN had decreased due to the relatively short life span of PMN compared to other cells in culture. The percentage total phagocytosis at 16 hours incubation may be an over-estimate of the true levels of phagocytosis due to the possible reduction in the numbers of viable PMN.

Part of the optimisation of the assays used in the assessment of *in vitro* PMN phagocytic function by both isolated PMN and PMN in whole blood involved setting gates within the cell populations. Gates were set on the linear graphs of fluorescence versus total number of cells to correspond with the phagocytosis of zero, one, two, and greater than two FITC-labelled (2.0μM) latex beads by the PMN, as it may be possible that some individual animals may possess PMN populations with limited phagocytic ability, assessed in this study by the phagocytosis of only one latex bead rather than other individuals who may have the capability to phagocytose multiple

beads. In contrast, the gates set in this study may have little or no biological relevance. Larger numbers of animals and possibly alternative methods of assessing the data may be required to answer this hypothesis.

Following successful development and optimisation of the assay to assess *in vitro* PMN phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN, 140 animals, both male and female at approximately six months of age, with a known genetic background, were sampled to determine their *in vitro* isolated PMN phagocytic ability. Variation in *in vitro* PMN phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN was seen among individual animals, between sexes, and among sires at each of the time points. The largest variation among individual animals was seen at six and sixteen hours.

Assessment of the phagocytosis of FITC-labelled *S. aureus* was attempted in a small group of approximately 20 animals. However, considerable difficulty in interpretation of the data was encountered along with extremely high background non-specific fluorescence. It was decided that further work to optimise the assay would be required before accurate results would be obtained and this would be an essential part of any continuation of this aspect of the project. Due to time constraints, development of this method, was outwith the scope of this current study. An essential first step of future work in this area would be to consider labelling the *S. aureus* NCTC 13047 'in-house' rather than using a commercially labelled strain as was used in the current study to try to reduce background non-specific adherence.

Traditionally, assessment of PMN function has been carried out on relatively pure, isolated PMN populations (Roth and Kaeberle, 1981; Kerhli *et al.*, 1989; Durr *et al.*, 1996). Recent studies have shown that the isolation procedure can alter some of the functional capabilities of the PMN (Egger *et al.*, 1997) and subsequently, whole blood assays for assessment of PMN function have now become more favourable. Some of the advantages of whole blood assessment of PMN function are that they require smaller volumes of blood, assess the PMN in their normal environment i.e. in a mixed cell environment, and do not need additional supplementation with sera components associated with opsonisation. Development of an assay for assessment

of *in vitro* PMN phagocytosis of FITC-labelled (2.0μM) latex beads in whole blood was carried out on a small group of approximately 20 animals. Variation in PMN phagocytic ability was seen among individuals with maximal levels of phagocytosis reached by one hour incubation. The background non-specific adherence was also assessed and was found to be marginally higher than the non-specific adherence for the isolated PMN, but the mean was still below 10% throughout the incubation period. For the analysis of phagocytosis by PMN in whole blood an additional gate for the mononuclear cells was set and monitored. No phagocytosis was observed in the mononuclear cell gate throughout the incubation, which confirmed the phagocytosis observed in the whole blood was due to the PMN population. Visually, phagocytosis by the PMN in whole blood was not as clear as the phagocytosis of the isolated PMN in the graphs of side scatter versus forward scatter even although the same number of PMN were counted in the PMN gates in both cases. Overall, the whole blood assay was a rapid technique which required less reagents, less blood, and gave similar results to the phagocytosis observed in isolated PMN populations following flow cytometry. In the current study, the development of the assay to assess PMN phagocytosis in whole blood was undertaken as a future alternative to the method developed which assessed PMN phagocytosis in an isolated PMN population and may form an important aspect of future studies.

Previous studies have assessed the functional ability of blood and milk PMN in dairy cows by assessing the *in vitro* phagocytosis of *E. coli* or *S. aureus*, in the presence of opsonins, and concluded that individual variation in the phagocytic PMN ability varies among animals (Paape and Shultze, 1978; Williams and Bunch, 1981). This observation formed the basis of a study of bulls of different ages performed by Williams *et al.* (1984) to assess if variation in PMN activity could be detected in adult Friesian bulls. If variation was identified among bulls, this could then have the potential to be incorporated into future selective breeding programmes for increased phagocytosis of bacteria by PMN. Williams *et al.* (1984) concluded that individual variation in PMN activity among adult dairy bulls was similar to that reported among dairy cows (Williams and Bunch, 1981).

In the current study, preliminary univariate statistical analyses was carried out on 140 animals from the 2000 cohort sampled at approximately six months of age for the assessment of *in vitro* phagocytosis of FITC-labelled (2.0μM) latex beads by isolated PMN. This data set of 140 male and female animals with known genetic background sampled at a similar age employed in the current study is unique for cattle, as no other literature documenting any similar studies exists. In the current study, the percentage total phagocytosis by isolated PMN was assessed at all the time points at 37°C and 1 hour at 4°C, by univariate procedures in SAS, and subsequently, by more complex REML modelling. The individual gates for 0 beads, one bead, two beads and greater than two beads were assessed for factors such as the optimal transformation of the data, correlation of individual animal responses between time points and the correlation of individual animal response at re-sampling (approximately three months after initial sampling). Overall, the data was positively skewed, except for the 0 beads gate which was negatively skewed at the time points early in the incubation. The negative skewness of the 0 beads gate early in the incubation confirmed the high numbers of cells, generally greater than ninety percent, which had not undergone phagocytosis at the early stages of the incubation. As the isolated PMN reached maximal phagocytosis the data generally reduced in skewness and was found in most of the gates to be normally distributed. Due to the variation in optimal transformation that would have been required at each of the time points for the different gates, and as the data was generally normally distributed at maximal phagocytosis, no transformation of the data was undertaken prior to further statistical analyses.

A study by Guidry *et al.* (1974), assessed within animal repeatability at several time points over a 48 hour period and concluded that within animal repeatability was excellent under identical physiological conditions, however, within animal variation was seen during periods when physiological changes such as parturition were occurring. Other studies indicated considerable between assay variability in PMN function in the same individuals over time (Paape and Miller 1988). However, Paape and Miller (1988) concluded that despite this variation, relevant data regarding individual variation among animals in PMN functional ability could still be obtained if the time scale between sampling remained relatively short. In the current study,

correlation of individual animal responses between time points for the percentage total phagocytosis were high, with significantly positive correlations also observed for the 0 beads, 1 bead, 2 beads and greater than 2 beads gate. This significant correlation of individual animal responses between time points indicated that individuals with relatively high levels of percentage total phagocytosis of FITC-labelled latex beads at one time point remained at a high level within the population at the subsequent time points during the incubation, and conversely, animals with low levels of PMN phagocytosis early in the incubation period, showed consistently low levels of phagocytosis throughout the assay. The correlation of individual animal responses at re-sampling (approximately three months after initial sampling) was low when the animals were sampled approximately three months after the initial sampling took place. The correlation of individual animal responses at re-sampling (approximately three months after initial sampling) were identified for the total phagocytosis at six hours incubation ($p<0.1$), and significant correlations were also found between some individual gates and time points ($p<0.05$), but overall the correlation was low. This variability identified among individuals in their phagocytic ability, which occurred within a relatively short time span, highlights the ever changing immune systems in these individual animals due to many influences both within the animals, such as, fluctuations in hormone levels and external influences such as environmental challenge by pathogens .

One observation which would require further investigation, was the significant positive correlation identified between the non-specific arms of the immune system assessed in the study. A significant positive correlation was identified between PHA-induced PBMC proliferation at peak proliferation and the percentage total phagocytosis at maximal phagocytosis measured. No correlation was observed between the specific arm of the immune response, *S. aureus*-induced PBMC proliferation, and the PMN phagocytosis at any of the time points measured. This apparent dichotomy between the non-specific and specific immune responses indicates that different regions of the genome may control non-specific and specific immune function.

Residual maximum likelihood analyses were primarily carried out on the complete data set, and subsequently on the males and females as separate data sets. Analyses

of the complete data set identified the consistently significant effect of sex as a fixed factor. The significance of sex indicated that males had consistently higher levels of *in vitro* PMN phagocytosis compared to females. When the males and females were analysed separately, no significant factors were identified. Following REML analyses, there was no significant effect of sire identified on the levels of PMN phagocytosis of FITC-labelled latex beads at any of the time points, however, graphical variation was observed among sires at each of the time points with the largest variation observed at the six hour time point. The relatively low animal numbers in terms of REML analyses and the high levels of between day variability of the PMN phagocytosis assay may all contribute to the lack of significance of sire.

7.3 Summary of significant factors affecting immune function

7.3.1 Age

In the current study the age of the individual was found to be significant factor for *in vitro* PBMC specific proliferation and non-specific proliferation in the animals sampled at approximately six months of age. The age of the individual animal was shown to influence both the specific and non-specific 'arms' of the immune system in different ways. An increase in the age of the individual animal was found to result in an increase in specific immune function and a decrease in non-specific immune function, which were assessed in the current study by measuring *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation, respectively.

Studies have been carried out in many species to examine the long-term effects of ageing on immunological function. To date, one of the most universally detectable measures of immunosenescence is the decline in *in vitro* PBMC proliferative response to mitogens in individuals, which has been described in a wide range of species from rodents to humans (Greeley *et al.*, 2001).

In the current study, when the females from the 1998 cohort were sampled over subsequent years at 18 and 30 months of age, variation in the mean *S. aureus*-induced and PHA-induced PBMC proliferation was observed among years. There was no clear pattern in the variation of the PHA-induced proliferation with age in the females: females from the 1998 cohort showed an increase in the PHA-induced proliferation from approximately six months of age to thirty months of age, whereas, the females from the 1999 cohort showed a reduction in the PHA-induced proliferation from approximately six to eighteen months of age. These observed variations could be due to a wide variety of factors including environmental factors or fluctuations in hormone levels. One explanation for the variation in cohort PHA-

induced responses could be variation in individual group numbers as there was only 22 females in the 1998 cohort and approximately 57 in the 1999 cohort. This may result in variation in the reported mean response, as only a relatively small number of animals with extreme PHA-induced responses would be required to skew the mean value in the data, by either increasing or decreasing the mean PHA-induced response. Environmental variation could also play a role in the cohort variation as the year of birth of the animals has been shown to be a consistent significant factor.

Most understanding of cellular and subcellular changes associated with alterations in the immune system related to age has been derived from cross-sectional and longitudinal studies (Greeley *et al.*, 2001). Cross-sectional studies have predominantly been carried out in rodents and humans (Makinodan and Kay, 1980; Miller, 1999) and to a lesser extent in other species including primates (Coe *et al.*, 1992; Bowden *et al.*, 1994), and dogs (Davila *et al.*, 1992; Greeley *et al.*, 1996; Kearns *et al.*, 1999; Strasser *et al.*, 2000). It has been assumed that the immune related changes observed in these cross-sectional studies reflect the changes that occur within individuals over time and have been verified by longitudinal studies which assessed immunological parameters in mammals with a relatively short life span (Boersma *et al.*, 1985; Miller, 1997; Heller *et al.*, 1998). In these studies the data collected spans from between fifty to eighty five percent of the anticipated life span of the subjects. Longitudinal studies in animals with a relatively longer life-span have been limited. Studies examining immune function over an approximate four year period, corresponding to approximately five to ten percent of the anticipated life span, have been carried out in primates (Bowden *et al.*, 1994), humans (Adler and Nagel, 1981; Wikby *et al.*, 1998) and cattle (Ayoub and Yang, 1996). A longitudinal study assessing humans over a ten year span, by Adler and Nagel (1981) found no age-related changes in numbers of leucocytes or lymphocytes. A study in primates by Gould *et al.* (1998), demonstrated a significant decline in the numbers of lymphocytes from infancy to five years of age, while Bowden *et al.* (1994), looking at a broader age span, identified a marginal decline in numbers. A recent study in dogs by Greeley *et al.* (2001) observed that there was an age-related decline in absolute numbers of lymphocytes in both males and females from four to eleven years of age. A study by Ayoub and Yang (1996) assessed changes in peripheral blood cell numbers and changes in peripheral blood cell

proportions in terms of lymphocyte subsets in four calves from birth to four years of age. Ayoub and Yang (1996) showed variation in the absolute counts of peripheral blood lymphocytes among animals, that also altered with age and variation in the peripheral blood lymphocyte subsets among animals, that also altered with age. An earlier study by Hauser *et al.* (1986), assessed variation in neutrophil function in calves from four weeks of age to approximately twelve months of age at four different time points. Hauser *et al.* (1986) identified that younger calves had lower levels of mycloperoxidase-hydgrogen peroxide-halide activity and lower hydrogen peroxidase activity in the youngest group of animals, which indicated decreased degranulation of primary granules in young calves. Hauser *et al.* (1986) also observed that younger animals were capable of higher levels of *S. aureus* phagocytosis than older animals. The study by Greeley *et al.* (2001) assessed a wide variety of parameters and identified a shift in the lymphocyte subsets proportions with age in all individuals, this being most pronounced in females. Greeley *et al.* (2001) identified a decline in the *in vitro* proliferative ability of lymphocytes to the mitogens PHA and Con A over time, a negative trend in the cytotoxicity in the natural killer cell activity, but found no variation in the *in vitro* percentage of PMN able to phagocytose latex beads. One explanation for the lack of variation in the PMN phagocytosis could be that the methodology assessed the levels of phagocytosis following incubation of the PMN on ice for 60 minutes, which may inhibit the phagocytosis completely. Many of the changes observed in the study by Greeley *et al.* (2001) were most significant in the period between four and seven years of age with less variation observed in the seven to eleven year period.

In the current study, the majority of the animals were assessed within a relatively short time span of several months at a relatively young age, even within this short time, a significant effect of sample age was identified, highlighting that immune function in cattle is continually influenced by a variety of factors.

7.3.2 Sex

As the complexity of the immune system has become increasingly clear, it is apparent that it interacts with other systems, particularly the endocrine and nervous systems (Everaus, 1992). A growing body of evidence has accumulated and shown that hormones can modulate the immune system (Cohen and Crnic, 1982). One hormonal complex which exerts profound influences on the immune system is that of the hypothalamic-pituitary-adrenal cortical axis (Gisler, 1974), while significant effects on the immune system are also mediated by the sex steroids (Grossman, 1984).

In the current study, the sex of the individual was identified as a significant factor affecting all aspects of non-specific and specific immunity which were assessed. Variation between the sexes was observed in the *in vitro S. aureus*-induced, PHA-induced and control PBMC proliferation and in the phagocytosis of FITC-labelled latex beads by isolated PMN. In all assessments of immune function carried out in this study, the males were consistently higher responders than the females. Traditionally, sexual dimorphisms in immune function has been identified in many species, with females generally exhibiting enhanced immune responses to antigenic challenge than males. However, males have been shown to have enhanced natural killer cell activity compared to females (Sorachi *et al.*, 1993), and in some cases males have been shown to exhibit greater levels of cell-mediated immunity (Alexander and Stimson, 1988; Brabin and Brabin, 1992). Although social or epidemiological mechanisms may account for some of these gender-associated differences (Bundy, 1988), hormone-influenced immunological mechanisms, many of which have been recreated in laboratory models of disease, would appear to be the primary determining factor. Hormones have been shown to alter immunologic factors and responses, including antigen expression and presentation, cytokine production, and expression of apoptotic factors and cell death (Huber *et al.*, 1999). In mice, the presence of oestrogen receptors on various immune cells has been demonstrated, along with identification of the presence of androgen receptors on T and B cells (Olsen and Kovacs, 1996). Sex steroid hormones have also been shown to influence the immune response in part via the thymus in rodents and the Bursa of

Fabricus in birds through specific androgen and oestrogen binding sites (Sullivan and Wira, 1979; Gaillard and Spinedi, 1998)

Female mice have been shown to have higher titers of immunoglobulins, as well as a higher incidence of autoimmune diseases than males (Grossman, 1984). Additionally, females have been shown to display higher splenocyte blastogenic responses to T and B cell mitogens than males (Krzych *et al.*, 1981). Women have also been shown to have higher immunoglobulin levels than men, and suffer from a higher incidence of autoimmune diseases such as erythematosus (Sakans *et al.*, 1978) and rheumatoid arthritis compared with men (Gaillard and Spinedi, 1998). Helminth infections are generally more severe in male than in female vertebrate hosts (Poulin, 1996) and males of species such as sheep, deer or moorhens often have higher parasite loads than females (Zuk and McKean, 1996; Stear *et al.*, 1995; Eens *et al.*, 2000). However, some studies have identified males with better immunological functional ability than females. A study by Mitchell (1999) assessed antibody and cell-mediated immune response to rubella re-immunisation in humans and identified significantly higher antibody and lymphocyte proliferative responses in males at two and four weeks post-immunisation. Overall, Mitchell (1999) concluded that there were kinetic differences between males and females in the recall response of both B and T cells to the rubella antigen, which suggested hormonal influences on the immune response to rubella virus. Another study, which looked at sex-determined resistance to *Toxoplasma gondii* infection in mice by Roberts *et al.* (1995), identified a dramatic difference in susceptibility and resistance to infection between males and females. The females were identified as being significantly more susceptible to infection than the males with higher and more rapid levels of mortality. The males had higher splenocytic lymphocyte stimulation indices and higher levels of cytokine production throughout the study than females. Roberts *et al.* (1995) observed that females had complete suppression of their splenocytic lymphocyte proliferative ability on day 15 post-infection in conjunction with low levels of cytokines when compared to males. Roberts *et al.* (1995) concluded that there were clear differences in the kinetics and magnitude of T cell responses and in the production of the cytokines TNF α, IFN γ and IL-10 between the sexes. A study by Bilbo and Nelson (2001) assessed the influence of sex steroid hormones in male and female Siberian

hamsters and concluded that sex steroid hormones enhanced immunity in both males and females, however, a sexual dimorphism was evident following a direct mitogen challenge, in which LPS significantly decreased cell-mediated immunity in males but not in females.

Although oestrogens and androgens have been reported to be both immunoinhibitory and immunostimulatory, they have been shown to be mainly suppressers of cell-mediated immunity. It may be possible, however, that the oestrogens and androgens suppress different subsets of lymphocytes and it may be plausible to suggest that the oestrogen to androgen ratio may determine whether the hormones will be immunostimulatory or immunoinhibitory (Grossman, 1985).

Other hormonal factors could also influence variation in immune function in the animals in the current study. Growth hormone and prolactin have been shown to have a stimulatory effect on the immune system at many levels (Weigent and Blalock, 1990; Kelley *et al.*, 1992, 1994). A study by Wise and Klindt (1995) assessed changes in thymic weight and endocrine relationships in male and female cattle at four, six, ten, twelve and fourteen months of age. This study observed significant increases in growth hormone and thymosin β4 up to six months of age and increases of prolactin up to ten months of age. Levels of pre-pubertal testosterone increased up to between ten and twelve months of age in both males and females, however, the concentration of testosterone in males was up to a 100-fold greater than that of females. The results of this study by Wise and Klindt (1995) highlight the complexity of the cause and effect relationships that exist between gonadal and thymic function.

In the current study, although the males and females were sampled at approximately the same age variation in hormone levels, both between the sexes, and within the sexes were likely and would have possibly had an effect on immune function. As within the sexes, variation in factors such as body weight and size were observed, which may have indicated variations in hormone levels among animals of a similar age.

7.3.3 Environment

Environmental factors such as season, stress associated factors and housing conditions can all effect immune function. Previous studies in Siberian hamsters looking at the effect of photoperiod on immune cell function in males (Yellon *et al.*, 1999; Drazen *et al.*, 2000), and females (unpublished observation Bilbo and Nelson, 2001), found decreased cell-mediated and humoral immune function during simulated winter conditions in the laboratory. A study by Stefanski (2001) assessed the effect of social and rearing conditions prior to weaning on the proportions post weaning of peripheral blood immune cells in male offspring of Long-Evans rats. This study found that male offspring from females housed in a mixed sex colony had significantly higher numbers of $CD4^+$ and $CD8^+$ T cells than males from females housed in breeding pairs. No significant variation was observed in the numbers of B cells and natural killer cells or in the proliferative response to Con A between the two groups. Stefanski (2001) concluded that the early social environment of these male rats could effect the numbers and proportions of many peripheral blood cell subsets in later life. In the current study, the male and female calves were reared under completely separate management regimes. The females were housed indoors and fed artificial milk replacer almost immediately after birth, whereas, the male calves were allowed to remain with their mothers on pasture until they were weaned before winter housing at approximately six months of age. This would result in exposure to different pathogens e.g. respiratory and enteric viruses would be more common in calves housed indoors, whereas, parasite challenge, especially with nematodes would only occur in calves with access to grazing. These completely different management regimes could potentially have a huge influence on the immune responses of the males and females and may also influence the proportions of the peripheral blood cell subsets. The assessment of the proportions of peripheral blood cell subsets in the male and female calves was outwith the scope of this project, however, due to the significant variation identified between the sexes, such an investigation would be an essential aspect of future work.

Psychological stress may also influence immune function. Overall, the effects of stress are very complex and may suppress or activate various immune cells (Dantzer

and Mormede, 1995). Depending on the immune variables assessed, some immune functions may be enhanced while others are suppressed in animals or human subjects subjected to environmental stressors (Maes *et al.*, 1999). Maes *et al.* (1999) assessed the psychological stress on leucocyte subset distribution in a group of male and female students in relation to examination stress. The study identified an increase in the numbers of neutrophils, monocytes, $CD8^{+}$, $CD2^{+}CD26^{+}$ and $CD2^{+}HLADR^{+}$ T cells and $CD19^{+}$ B cells and significant reductions in the $CD4^{+}/CD8^{+}$ T cell ratio in stressed pupils. Maes *et al.* (1999) concluded that academic stressors are sufficient to promote T cell activation and B cell proliferation and increase the numbers of circulating leucocytes and neutrophils. One explanation for this alteration in immune cells numbers and proportions may be that there is a stress-induced production of pro-inflammatory cytokines such as IL-6, TNF α and IFN γ, which stimulate certain populations of immune cells. Another study in humans by Maes *et al.* (1990) showed variation in immune cell proportions and function in conjunction with an up-regulation of IL-2 receptors in patients diagnosed with major depression. The most consistently observed changes in peripheral cell populations during depression are: a reduced mitogen-induced lymphocyte blastogenesis (Schleifer *et al.*, 1984), an increased $CD4^{+}/CD8^{+}$ ratio (Irwin, 1988) and reduced natural killer cell activity (Irwin *et al.*, 1990).

In livestock the stresses of weaning and transportation on both the physiological and behavioural function of the animal have been well documented. In cattle, the most important disease associated with transportation is shipping fever which lowers the animals' resistance to infection (Grandin, 2000). A study by McKenzie *et al.* (1997) showed that the humoral immune system in calves was affected by both weaning and transportation. Murata (1995) showed that serum collected from animals 48 hours after transportation had an immunosuppressive effect on PMN function, resulting in a decrease in their bactericidal ability. A study by Tarrant *et al.* (1992) also showed an alteration in the immune systems of cattle after long journeys. This author observed an increase in white blood cell count and neutrophil numbers and a reduction in the numbers of lymphocytes and eosinophils.

Physiological stressors, such as nutrient deficiency, can also lead to impaired immune function. Vitamin E is essential for body functions such as growth, reproduction, prevention of various diseases and for integrity of tissues (McDowell *et al.*, 1996). Vitamin E and selenium may play an essential role in the protection of leucocytes and macrophages from toxic compounds during phagocytosis (Badwey and Karnovsky, 1980). In a number of mammalian species, selenium deficiency has been associated with decreased immune system function. Decreased glutathione peroxidase activity in phagocytic cells has been reported in selenium deficient heifers (Boyne and Arthur, 1979). In bovine neutrophils, the bacterial capacity for *Candida albicans* and *S. aureus* is lowered in selenium-deficient cattle (Gyang *et al.*, 1984). Mastitis incidence has also been shown to be related to vitamin E and selenium status of dairy herds (McDowell *et al.*, 1996). Weiss *et al.* (1990) surveyed dairy herds and observed a negative correlation between dietary vitamin E intake and rates of clinical mastitis. Another essential requirement for efficient growth and immune function is chromium. In human nutrition chromium has been recognised as a trace element, required for normal metabolism of carbohydrates, proteins and lipids and as an active component of glucose tolerance factor (Mertz, 1992). Several reports have documented performance and health augmented effects of supplemental dietary organic chromium in domestic animals (Mowat, 1997). Dietary chromium supplementation has been shown to increase the proliferation of PBMC *in vitro* with or without Con A stimulation, in both beef calves (Chang *et al.*, 1994) and periparturient dairy cows (Burton *et al.*, 1993). A study by Chang *et al.* (1996) assessed the effects of chromium on health status and various immune cell functions including blood neutrophil phagocytosis in dairy cows. This study concluded that supplemental chromium had no effect on the neutrophil phagocytic function in cows from six weeks prepartum to six weeks postpartum and no overall benefit on health status and mastitis-related parameters in dairy cows, however, supplemental chromium did have an effect on lymphocyte proliferation which may be associated with the actions of insulin or cortisol.

7.3.4 Breed/Cross

The immune system is affected by both environmental and genetic factors (Nguyen, 1984). Genetic control of the immune system has been studied extensively using inbred species of animals, such as mice, rats and guinea pigs (Newman *et al.*, 1996). Effects mediated through genes and/or cell surface expressed products of the MHC or other genes have been documented using experimental immunisations or infections with different types of pathogen (Benacceraf and Germain, 1978). In mice, strain variation resulting in resistance or susceptibility to disease has been well documented. In autoimmune diseases, the New Zealand Black strain of mice have been shown to be susceptible to spontaneous development of systemic lupus erythematosus (Klein and Horejski, 1997). Strain variation had also been shown in mice in their resistance to parasite challenge, as inbred strains of mice have been shown to differ in their ability to expel intestinal nematodes. This variability was linked to the activity of their Th cell subsets, shown by a consistent predisposition to infection in outbred mice reinfected with *Trichuris trichiura* (Tizard, 2000). Another study in mice by Wakelin *et al.* (1993) looked at resistant and susceptible strains of inbred mice following infection with *Eimeria vermiformis*. Wakelin *et al.* (1993) identified a kinetic difference in their ability to mount protective Th cell subset responses, in particular variation in the cytokine levels and kinetics between the strains. In this study no evidence was found for Th2-mediated interference with the ability of the two strains to release IFN γ.

Breed differences in immune response to different antigens resulting in resistance or susceptibility to disease have been reported for many diseases such as respiratory disease in chickens (Ewbank *et al.*, 1999), caprine arthritis-encephalitis virus-induced arthritis (Ruff and Lazary, 1988) and scrapie in sheep (Millot *et al.*, 1985, 1988). In sheep breeds such as the Cheviot, Shetland and Swaledale, three different sites on the PrP gene have been linked to resistance or susceptibility to scrapie, whereas in Suffolk sheep, only one site on the PrP gene has been identified in association with resistance or susceptibility to scrapie (Hosie, 1997). The same genotypes have been linked to resistance or susceptibility to naturally occurring scrapie in different breeds of sheep. Studies have shown that the AA_{136}, RR_{154},

QQ_{171} genotype has been associated with resistance in Cheviots (Hunter *et al.*, 1996), but also with susceptibility in Suffolks (Westaway *et al.*, 1994).

At present little research has been done to evaluate the immune response to similar antigens among different breeds of cattle. A study by Blecha *et al.* (1984) reported that Brahman cross Angus cattle and pure bred Angus cattle had different immunological responses to shipping stress. Muggli *et al.* (1987) reported that Angus calves had higher mean IgG_1 concentrations at 24-48 hours of age than Hereford or Red Poll calves. Research in dairy cattle (Shultz *et al.*, 1971) demonstrated that Brown Swiss calves developed a greater number of antibody plaque-forming cells to chicken red blood cells than Holstein-Friesian, Ayrshire or Guernsey calves. A recent study by Engle *et al.* (1999) assessed the effect on immune function and response to challenge with infectious bovine rhinotracheitis virus in Angus and Simmental calves. Engle *et al.* (1999) identified higher rectal temperatures, higher total Ig and IgM titres against pig red blood cells and higher cell-mediated immune response to PHA in Angus calves compared to Simmental calves. A study by Losson *et al.* (1999) assessed *Psoroptes ovis* infestation in traditionally resistant Holstein-Friesian and susceptible Belgian White and Blue cattle. Losson *et al.* (1999) assessed various immune parameters including serology and lymphocyte proliferation. This study identified that there was a positive correlation between antibody titre and the extent of the lesions and that the antibody response was associated with a marked *in vitro* lymphocyte proliferation. Losson *et al.* (1999) concluded that there was no significant difference between susceptible and resistant cattle in the immunological parameters assessed in the study and that a combination of immunological and non-immunological factors may be responsible for this previously documented breed related susceptibility or resistance to *P. ovis* infection (Meleney and Fisher, 1979; Fisher and Wright, 1981; Pouplard *et al.*, 1990). Studies in cattle have also identified variation in resistance to trypanosomosis among *Bos indicus* cattle breeds (Mwangi *et al.*, 1998). Studies comparing trypanotolerant breeds such as N'Dama and West African Shorthorn cattle with breeds known to be more susceptible have shown that trypanotolerance is associated with the ability of the host to resist the development of anaemia and control parasitaemia once infected (Dargie *et al.*, 1979; Murray *et al.*, 1979, 1982; Akol *et al.*, 1986; Paling *et al.*, 1991).

In the current study significant differences among breed crosses were identified for *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation on several of the days of culture. These differences indicated that up to and including day four of culture, Holstein Friesian backcross animals had greater *S. aureus*-induced PBMC proliferation than Charolais backcross animals, and that Charolais backcross animals had greater peak PHA-induced PBMC proliferation than Holstein-Friesian backcross animals. One hypothesis that may explain this breed related difference in immune function may be that traditionally, beef and dairy cattle have been selected for different purposes.

In broad terms, the breeding goal of most livestock producers is to increase the profitability of their animals (Simm 1998). Genetic improvement of disease resistance is a relatively slow process, however, genetic improvement may reduce costs incurred in isolation, treatment and culling of diseased animals (Shook, 1989). Replacement of a diseased animal incurs a large cost and is considered as the last resort, therefore, genetic improvement must be cost effective in comparison to other methods if it is to form part of a disease control strategy. Traditionally dairy cattle have been selected for traits such as milk production, milk quality, conformational type traits such as stature, foot angle, teat length and more recently traits associated with disease susceptibility, reproduction, feed intake, live weight and body score. The relatively recent incorporation of these additional conformational and disease associated traits takes into account longevity of the animals rather than just productivity (Simm, 1998). In contrast, beef cattle have traditionally been produced for production traits such as growth rate, carcass composition, calving ease and feed conversion ratio. This variation in traits for selection between beef and dairy cattle may account for the variability in breed cross identified in the current study between Holstein backcross and Charolais backcross animals. In the current study the predominantly Holstein animals have greater specific immune responses as measured by *S. aureus*-induced cell proliferation than the predominantly Charolais animals. It may be hypothesised that the Holstein backcross animals have better specific immunity because they have been traditionally developed to have longer lifespans and have the ability to resist pathogenic challenge by mastitis-causing pathogens including *S. aureus*. In contrast, the Charolais backcross animals may have better

non-specific immunity as they have been traditionally developed to have relatively short life spans with rapid growth rates, which may require alternative immune responses to prevent the establishment of infection, as established infections requiring a specific immune response may be have detrimental effects on factors such as growth rate.

The significance of sire in the current study in both non-specific and specific immunity indicates that immune function is under genetic influence.

7.4 Future

The current study focused on assessing various aspects of immune function in over 300 male and female genetically defined Holstein-Friesian cross Charolais calves from approximately six months of age. The immune system is very complex and involves a wide variety of functional immune responses rather than simply lymphocyte proliferation and neutrophils phagocytosis, thus, additional functional assays would be employed in future projects.

Further development of the *in vitro* PBMC proliferation may involve the assessment of stimulation by other specific bacterial/mastitis pathogens such as *E. coli* or *S. uberis* or the assessment of T-cell epitopes specific for the *S. aureus* NCTC 13047 strain used in the current study. T cell epitopes to foot and mouth disease virus have been previously identified by peptide scanning of cattle immunised with whole-virus vaccine (Collen *et al.*, 1991; van Lierop *et al.*, 1994), with a further study identifying selection of these epitopes linked to MHC class I and Class II haplotype (Glass *et al.*, 1991). The identification of T cell epitopes for the *S. aureus* NCTC 13047 would enhance the specificity of the current assay. Additional development of the assay may also involve measurement of cytokine production induced by both specific and non-specific antigenic stimuli as an alternative to, or in conjunction with, measurement of cell proliferation. Levels of cytokine production could be detected by ELISA, ELISPOT or cytokine messenger RNA could be detected by reverse-transcription PCR (Czerkinsky *et al.*, 1988; Favre *et al.*, 1997). Development of future assays involving cytokines could initially target cytokines traditionally associated with the Th_1, Th_2 paradigm.

Alternatively, assessment of *in vitro* PBMC proliferation could be carried out by flow cytometry to minimise the use of radioactivity. Lymphocyte proliferation has been shown to be measurable by PKH2 fluorescent cell tracking dye, which is incorporated into the membrane of living cells. Proliferating cells may be identified by a reduction in fluorescence intensity relative to cells that have not undergone cell division (Horan *et al.*, 1990; Samlowski *et al.*, 1991). Additionally, the phenotype

and activation marker expression of the PKH2-labelled proliferating lymphocytes could be identified using lymphocyte subset specific monoclonal antibodies and dual-colour flow cytometry (Shapiro, 1998; Quade and Roth, 1999). Further options to develop the *in vitro* PBMC proliferation would be to use a whole blood method for assessment of PBMC proliferation rather than an isolated population, which may give a better indication of the *in vivo* function of these immune cells.

To continue development of the *in vitro* PMN phagocytosis by isolated PMN population, the initial development may include the 'in-house' labelling of *S. aureus* NCTC 13047 for use in the optimisation of the phagocytosis of FITC-labelled *S. aureus* by PMN. Phagocytosis of other bacterial pathogens such as *E. coli* and *S. uberis* could also be assessed. Alternative methods of isolating PMN from blood could be attempted to continue to assess the phagocytosis of isolated PMN populations. A method using biomagnetic beads coated with a monoclonal antibody that recognises an abundant surface antigen on bovine neutrophils has been described by Soltys *et al.* (1999). This type of separation was shown to result in a higher yield and purity of PMN, with normal or even improved PMN function in assays of chemotaxis, phagocytosis, degranulation and respiratory burst. Further development of the whole blood assay for routine use would be an important aspect of future studies. The assessment of oxidative burst in combination with levels of phagocytosis using dual-colour flow cytometry may also be a useful development. This has been described using both isolated PMN populations and PMN from whole blood in many species including humans (Hasui *et al.*, 1989; Perticarari *et al.*, 1994) and cattle (Smits *et al.*, 1997). Hydrogen peroxide is one of the species of reduced oxygen that are produced by PMN when they encounter invading pathogens or other appropriate stimuli and is considered to be essential for intracellular killing (Babior *et al.*, 1975; Rosen and Klebanoff, 1979). A previous study by Smits *et al.* (1997) identified significant differences in the PMN from whole blood samples from ten cows in their ability to phagocytose *S. aureus* and to produce reactive oxygen intermediates.

During the current study the females for the 1999 cohort were ranked for *S. aureus*-induced and PHA-induced *in vitro* PBMC proliferation and the MHC RFLP pattern obtained was studied in order to determine any association with MHC RFLP pattern

and specific or non-specific immunity. This highlighted possible links between specific and non-specific immunity and MHC RFLP pattern. Future work would be required to clarify any such link and further investigation would be essential in order to identify any genetic regions associated with specific and non-specific immunity in cattle. The identification of these regions may have significant implications on future breeding programmes and the identification of quantitative trait loci.

Two main strategies have been described to 'move' from the identification of linked markers to the genes responsible, they are positional cloning and the positional candidate gene approach (Collins, 1995). The positional candidate gene approach has been shown to be successful in the cloning of human disease genes and is likely to be similarly effective in livestock (Clark, 1998). The identification of the ryanidine receptor gene (*RYR1*) as a gene responsible for malignant hyperthermia in pigs confirms that this strategy may be useful in livestock (Fujii *et al.*, 1991). It remains to be seen if similar methods may be useful in designing improved breeding programmes for commercial cattle.

Glossary

1°	primary
2°	secondary
3°	tertiary
±	plus or minus
αβ	alpha beta
β2M	beta 2M
γδ	gamma delta
Ii	invariant chain
^3H	methyl tritiated thymidine
APC	antigen presenting cell
ANOVA	analysis of variance
B cells	B lymphocytes
BCR	B cell receptor
BHI	brain heart infusion
BME	basal media Eagles
BoLa	bovine leukocyte antigen complex
CO_2	carbon dioxide
°C	centigrade (celsius)
cm	centimetre
CD	cluster of differentiation
cpm	counts per minute
Δcpm	delta counts per minute
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetate
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
HBSS	hanks balanced salt solution
HLA	human leukocyte antigen complex
ICAM	intracellular adhesion molecule

IFN	interferon
IL	interleukin
Ig	immunoglobulin
ITEM	index of total economic merit
IU	international units
kDA	kilo Dalton
λ	lambda
l	litre
m	metre
MACE	multiple trait across country evaluation
mAb	monoclonal antibody
mg	milligram
ml	millilitre
mm	millilmetre
MHC	major histocompatibility complex
MNC	mononuclear cell
NK	natural killer
PBMC	peripheral blood mononuclear cell/s
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PIN	profit index number
PLI	profitable life index
PMN	polymorphonuclear cell/s
p	significance value
QTL	quantitative trait loci
REFP	restriction enzyme fragmentation pattern
REML	residual maximum likelihood
RFLP	restriction fragment length polymorphism
RPLA	rapid passive latex agglutination
SCC	somatic cell count
SE	Staphylococcal enterotoxin
S.E.	standard error
S.E.D.	standard error of the difference

S.E.M.	standard error of the mean
SI	stimulation indices
TAP	transporter associated protein
T cell	T lymphocyte
TCR	T cell receptor
TNF	tumour necrosis factor
TSST	toxic shock syndrome toxin
μ	micro
vol/vol	volume per volume
w/vol	weight per volume
WC	workshop cluster
WCCF	white cell counting fluid

NUMBER	DATE OF BIRTH	SEX	SIRE	DAM	SAMPLE DATE
14456	*	M	R02	13468	07/12/1998
14475	28/05/1998	F	R12	13543	30/11/1998
14480	20/06/1998	M	R15	13498	18/01/1999
14501	31/03/1998	M	R19	13743R42	11/01/1999
14502	31/03/1998	M	R15	13709R17	07/12/1998
14504	02/04/1998	F	R19	13706R13	16/11/1998
14505	03/04/1998	M	R11	13705R10	07/12/1998
14507	04/04/1998	F	CH158	13716R26	18/01/1999
14508	06/04/1998	M	R19	13732R43	11/01/1999
14509	09/04/1998	M	CH200	13715R25	11/01/1999
14510	09/04/1998	F	R11	13600	18/01/1999
14511	09/04/1998	M	CH158	13728R38	07/12/1998
14512	09/04/1998	F	CH158	13702R04	16/11/1998
14513	10/04/1998	M	CH200	13720R30	11/01/1999
14514	12/04/1998	F	R01	13735R46	18/01/1999
14515	13/04/1998	F	CH158	13714R24	02/11/1998
14516	18/04/1998	M	CH200	13745R55	11/01/1999
14517	19/04/1998	M	R11	13746R56	23/11/1998
14518	23/04/1998	M	R21	13750R60	23/11/1998
14519	23/04/1998	M	R01	13712R22	23/11/1998
14520	24/04/1998	F	R12	13703R07	16/11/1998
14521	24/04/1998	M	R05	13713R23	14/12/1998
14522	24/04/1998	F	R11	13755R65	16/11/1998
14523	26/04/1998	F	R02	13756R66	30/11/1998
14524	26/04/1998	M	R02	13717R27	14/12/1998
14525	27/04/1998	M	R19	13736R47	09/11/1998
14526	27/04/1998	F	R11	13725R35	18/01/1999
14527	27/04/1998	M	R15	13742R54	11/01/1999
14528	27/04/1998	M	R21	13704R09	11/01/1999
14529	27/04/1998	F	R15	13727R37	16/11/1998
14530	27/04/1998	M	R11	13741R53	11/01/1999
14532	28/04/1998	F	R01	13738R50	16/11/1998
14533	29/04/1998	M	CH108	13744R48	07/12/1998
14534	29/04/1998	M	R19	13724R34	09/11/1998
14535	30/04/1998	F	CH200	13759R69	18/01/1999
14536	01/05/1998	M	R15	13721R31	14/12/1998
14537	01/05/1998	M	R05	13731R41	23/11/1998
14538	02/05/1998	M	R21	13708R16	23/11/1998
14539	03/05/1998	M	R15	13734R45	07/12/1998
14540	03/04/1998	F	R01	13752R62	18/01/1999
14541	03/05/1998	M	R19	13599	09/11/1998
14542	05/05/1998	F	R01	13568	18/01/1999
14543	22/05/1998	M	R05	13747R57	09/11/1998
14544	14/05/1998	M	*	13465	14/12/1998
14545	13/05/1998	F	R21	13733R44	30/11/1998
14546	13/05/1998	F	R11	13701R03	02/11/1998
14547	21/05/1998	F	THEBOYS	13541	02/11/1998

14548	28/05/1998	F	R02	13718R28	02/11/1998
14549	26/05/1998	M	R12	13710R18	14/12/1998
14550	28/05/1998	F	R12	13757R67	30/11/1998
14611	31/05/1998	M	R02	13753R63	14/12/1998
14612	01/06/1998	F	R19	12157	30/11/1998
14613	02/06/1998	M	R05	13726R36	09/11/1998
14614	06/06/1998	M	R05	13737R49	23/11/1998
14615	10/06/1998	F	R05	13729R39	30/11/1998
14616	20/06/1998	M	R02	13758R68	09/11/1998
14617	01/07/1998	M	R19	13557	02/11/1998
14618	03/07/1998	M	R05	11998	18/01/1999
14804	28/03/1999	F	R21	13577	25/10/1999
14805	28/03/1999	M	R19	13839 R86	01/11/1999
14807	30/03/1999	M	R21	13691 R135	01/11/1999
14808	30/03/1999	F	R01	13856 R105	18/10/1999
14809	31/03/1999	M	R02	13692 R137	01/11/1999
14810	31/03/1999	M	CH108	13828 R77	08/11/1999
14811	31/03/1999	F	R02	13847 R96	18/10/1999
14812	01/04/1999	M	R19	13690 R 133	08/11/1999
14813	01/04/1999	M	CH108	13754 R 64	01/11/1999
14814	01/04/1999	F	CH108	13681 R136	25/10/1999
14815	01/04/1999	F	R02	13186	18/10/1999
14816	02/04/1999	F	CH108	13858 R107	18/10/1999
14817	01/04/1999	M	CH219	13840 R89	01/11/1999
14818	01/04/1999	M	CH219	13840 R89	08/11/1999
14819	01/04/1999	F	CH158	13831 R80	18/10/1999
14820	01/04/1999	F	R21	13826 R75	18/10/1999
14821	02/04/1999	M	R01	13735 R46	15/11/1999
14822	02/04/1999	M	R21	13848 R97	08/11/1999
14823	02/04/1999	M	R21	13442	01/11/1999
14824	03/04/1999	M	R21	13844 R93	01/11/1999
14826	05/04/1999	F	CH108	13744 R48	25/10/1999
14827	05/04/1999	M	R12	13836 R87	08/11/1999
14828	05/04/1999	M	R12	13846 R95	22/11/1999
14829	05/04/1999	F	R02	13652	25/10/1999
14830	03/04/1999	M	CH158	13823 R72	08/11/1999
14831	03/04/1999	M	R01	13696 R141	22/11/1999
14832	03/04/1999	M	CH108	13698 R143	08/11/1999
14833	03/04/1999	M	R12	13863 R112	08/11/1999
14834	03/04/1999	F	R05	13731 R41	18/10/1999
14835	04/04/1999	F	R15	13825 R74	18/10/1999
14836	04/04/1999	M	R01	13678 R124	15/11/1999
14837	04/04/1999	M	CH200	13694 R139	15/11/1999
14838	04/04/1999	F	R15	13851 R100	18/10/1999
14839	04/04/1999	M	CH158	13716 R26	01/11/1999
14840	04/04/1999	M	R02	13657	08/11/1999
14841	04/04/1999	F	CH200	13715 R25	18/10/1999
14842	04/04/1999	M	R21	13153	08/11/1999
14843	04/04/1999	F	R15	13709 R17	11/10/1999

14844	05/04/1999	M	CH158	13679 R127	01/11/1999
14845	05/04/1999	M	CH219	13688 R131	08/11/1999
14846	05/04/1999	F	R02	13827 R76	25/10/1999
14848	06/04/1999	M	R21	13708 R16	08/11/1999
14849	06/04/1999	M	R15	13860 R109	01/11/1999
14850	07/04/1999	M	R11	13755 R65	01/11/1999
14851	07/04/1999	F	CH108	13854 R103	25/10/1999
14852	08/04/1999	F	R05	13857 R106	18/10/1999
14853	08/04/1999	M	R21	13750 R60	01/11/1999
14854	08/04/1999	M	R12	13581	25/10/1999
14855	08/04/1999	M	R15	13583	25/10/1999
14856	08/04/1999	F	R19	13544	25/10/1999
14857	08/04/1999	M	R19	13706 R13	01/11/1999
14858	09/04/1999	M	CH158	13674 R119	08/11/1999
14859	09/04/1999	F	CH200	13720 R30	11/10/1999
14860	10/04/1999	F	R19	13609	18/10/1999
14861	*	*	*	*	18/10/1999
14862	12/04/1999	M	R02	13682 R120	22/11/1999
14863	11/04/1999	F	CH200	13759 R69	18/10/1999
14864	11/04/1999	M	R02	13714 R24	01/11/1999
14865	10/04/1999	M	CH158	13707 R14	22/11/1999
14866	12/04/1999	M	CH108	13845 R94	01/11/1999
14867	12/04/1999	M	CH219	13680 R134	08/11/1999
14868	14/04/1999	M	CH108	13850 R99	01/11/1999
14869	14/04/1999	F	CH200	13855 R104	11/10/1999
14870	14/04/1999	M	R19	13132	25/10/1999
14873	18/04/1999	F	R12	13683 R125	11/10/1999
14874	23/04/1999	F	R19	13499	25/10/1999
14875	23/04/1999	F	R05	13697 R142	11/10/1999
14876	23/04/1999	F	R15	13838 R85	25/10/1999
14877	24/04/1999	F	R15	13064	25/10/1999
14878	24/04/1999	F	R15	13064	25/10/1999
14879	26/04/1999	F	R01	13502	11/10/1999
14880	28/04/1999	F	CH219	13582 R101	25/10/1999
14881	29/04/1999	M	R11	13741 R53	22/11/1999
14882	29/04/1999	F	R05	13722 R32	11/10/1999
14883	30/04/1999	F	R21	13597	25/10/1999
14884	29/04/1999	F	CH158	13835 R84	25/10/1999
14885	29/04/1999	M	R19	13837 R88	15/11/1999
14888	04/05/1999	M	R12	13699 R144	15/11/1999
14889	06/05/1999	M	R05	13721 R31	15/11/1999
14890	06/05/1999	F	R19	13724 R34	25/10/1999
14891	06/05/1999	F	R15	13748 R58	11/10/1999
14892	06/05/1999	M	R19	13865 R114	08/11/1999
14893	06/05/1999	M	CH108	13751 R61	22/11/1999
14894	06/05/1999	M	R21	13733 R44	22/11/1999
14896	08/05/1999	M	CH219	13685 R128	22/11/1999
14897	10/05/1999	F	R11	11445	11/10/1999
14898	18/05/1999	F	R21	13651	11/10/1999

14899	18/05/1999	F	R21	13651	25/10/1999
14900	20/05/1999	F	R12	13447	11/10/1999
14901	22/05/1999	F	R12	13710 R18	11/10/1999
14904	24/05/1999	M	R11	13824 R73	15/11/1999
14905	24/05/1999	F	R15	13532	04/10/1999
14906	24/05/1999	F	R01	13582	22/11/1999
14907	26/05/1999	M	R19	13598	15/11/1999
14908	27/05/1999	M	R19	13743 R42	15/11/1999
14909	27/05/1999	F	CH200	13829 R78	04/10/1999
14910	29/05/1999	F	CH200	13676 R122	04/10/1999
14911	29/05/1999	F	R19	13736 R47	04/10/1999
14912	30/05/1999	M	R11	13746 R56	15/11/1999
14913	01/06/1999	F	R15	13608	04/10/1999
14914	02/06/1999	M	R19	13579	22/11/1999
14915	03/06/1999	F	R15	13593	04/10/1999
14916	04/06/1999	F	CH200	13745 R55	22/11/1999
14917	04/06/1999	F	CH219/R19	13864 R113	22/11/1999
14918	10/06/1999	M	R02	13601	04/10/1999
14919	14/06/1999	M	R12	11900	04/10/1999
14920	15/06/1999	M	R05	13615	22/11/1999
14921	14/06/1999	F	R19	13705 R10	04/10/1999
14922	15/06/1999	F	R19	13726 R36	22/11/1999
14923	16/06/1999	M	R19	13753 R63	15/11/1999
14924	17/06/1999	M	R19	13712 R22	15/11/1999
14925	17/06/1999	M	R19	13675 R121	15/11/1999
14927	19/06/1999	F	R19	13713 R23	04/10/1999
14928	20/06/1999	M	R19	13747 R57	15/11/1999
14929	20/06/1999	M	R01	13633	04/10/1999
14930	22/06/1999	M	R19	13734 R45	15/11/1999
14931	22/06/1999	M	R19	13702 R04	15/11/1999
14932	23/06/1999	F	R01	13752 R62	22/11/1999
14933	23/06/1999	F	R19	13758 R68	04/10/1999
14935	04/07/1999	M	R19	13737 R49	29/11/1999
15001	22/03/2000	F	R21	13848 R97	11/09/2000
15002	24/03/2000	M	R11	13853 R102	06/11/2000
15003	24/03/2000	M	R21	13754 R64	31/10/2000
15004	23/03/2000	M	R11	13840 R89	31/10/2000
15005	24/03/2000	M	R12	13683 R125	13/11/2000
15006	24/03/2000	F	R21	13480	11/09/2000
15007	24/03/2000	M	R21	13733 R44	31/10/2000
15008	26/03/2000	M	R21	13673 R118	06/11/2000
15009	27/03/2000	F	R11	13701 R03	04/09/2000
15010	27/03/2000	F	R11	13755 R65	11/09/2000
15011	27/03/2000	F	R05	13697 R142	11/09/2000
15012	27/03/2000	F	R05	13697 R142	11/09/2000
15013	28/03/2000	M	R21	13863 R112	31/10/2000
15014	28/03/2000	M	R11	13693 R138	06/11/2000
15015	28/03/2000	F	R02	13827 R76	16/10/2000
15016	28/03/2000	F	R05	13647	11/09/2000

15019	29/03/2000	M	R05	13543	11/09/2000
15020	29/03/2000	F	R05	13543	02/10/2000
15021	29/03/2000	F	R05	13565	11/09/2000
15022	29/03/2000	F	R21	13704 R09	13/11/2000
15023	29/03/2000	M	CH158	13831 R80	31/10/2000
15024	29/03/2000	M	13773R08	13757 R67	13/11/2000
15025	29/03/2000	M	R02	13718 R28	13/11/2000
15026	29/03/2000	M	R02	13847 R96	16/10/2000
15027	29/03/2000	F	R02	13756 R66	11/09/2000
15028	30/03/2000	F	R15	13517	16/10/2000
15029	30/03/2000	F	R02	13597	16/10/2000
15030	29/03/2000	F	R21	13844 R93	31/10/2000
15031	29/03/2000	M	R01	13698 R143	13/11/2000
15032	31/03/2000	M	R02	13577	02/10/2000
15033	31/03/2000	F	CH200	13855 R104	02/10/2000
15034	31/03/2000	F	CH108	13689 R132	11/09/2000
15035	31/03/2000	F	R21	13740 R52	31/10/2000
15036	31/03/2000	M	R01	13821 R70	11/09/2000
15037	31/03/2000	F	R05	13684 R126	16/10/2000
15038	31/03/2000	F	R02	13707 R14	06/11/2000
15039	31/03/2000	M	R15	13836 R87	13/11/2000
15040	31/03/2000	M	R21	13826 R75	13/11/2000
15041	01/04/2000	M	R21	13716 R26	02/10/2000
15042	01/04/2000	F	R15	13845 R94	31/10/2000
15043	01/04/2000	M	R01	13622	11/09/2000
15044	02/04/2000	F	R02	13849 R98	31/10/2000
15045	02/04/2000	M	R21	13671 R116	11/09/2000
15046	02/04/2000	F	R19	13837 R88	06/11/2000
15047	02/04/2000	M	R01	13735 R46	06/11/2000
15049	03/04/2000	M	CH219	13833 R82	02/10/2000
15050	24/04/2000	F	R01	13719 R29	06/11/2000
15051	04/04/2000	M	CH158	13674 R119	11/09/2000
15052	04/04/2000	F	R19	13455	11/09/2000
15053	04/04/2000	F	CH219	13835 R84	13/11/2000
15054	05/04/2000	M	R05	13729 R39	06/11/2000
15055	04/04/2000	M	CH200	13720 R30	31/10/2000
15056	06/04/2000	M	R21	13708 R16	06/11/2000
15057	06/04/2000	M	CH158	13728 R38	16/10/2000
15058	07/04/2000	F	R15	13748 R58	06/11/2000
15059	07/04/2000	M	R01	13053	31/10/2000
15060	08/04/2000	M	CH219	13852 R101	11/09/2000
15061	09/04/2000	F	R01	13696 R141	02/10/2000
15063	10/04/2000	F	R01	13052	02/10/2000
15064	16/04/2000	F	R11	13858 R107	06/11/2000
15065	17/04/2000	M	R01	13738 R50	13/11/2000
15066	18/04/2000	M	R15	20210	02/10/2000
15067	18/04/2000	F	R15	20223	02/10/2000
15068	18/04/2000	F	R19	20215	02/10/2000
15069	18/04/2000	F	R05	13722 R32	04/09/2000

15072	24/04/2000	F	CH108	13744 R48	31/10/2000
15073	21/04/2000	M	R01	12281	04/09/2000
15074	21/04/2000	F	R12	11445	04/09/2000
15075	22/04/2000	F	R19	20227	04/09/2000
15076	22/04/2000	F	R19	20218	06/11/2000
15077	23/04/2000	M	R19	20232	31/10/2000
15078	23/04/2000	M	R21	13111	06/11/2000
15079	24/04/2000	M	R15	20229	06/11/2000
15080	24/04/2000	M	CH219	13862 R111	04/09/2000
15081	25/04/2000	F	R02	13677 R123	04/09/2000
15082	24/04/2000	F	R01/R02	13157	13/11/2000
15083	25/04/2000	M	R15	20208	02/10/2000
15085	25/04/2000	F	R15	20219	02/10/2000
15086	25/04/2000	F	CH219	13828 R77	31/10/2000
15087	25/04/2000	M	R19	20213	04/09/2000
15088	25/04/2000	F	R19	14401	02/10/2000
15089	25/04/2000	F	R11	13509	04/09/2000
15090	26/04/2000	F	R19	20234	04/09/2000
15091	26/04/2000	F	R12	13626	31/10/2000
15092	26/04/2000	M	CH200	13745 R55	06/11/2000
15093	27/04/2000	M	R19	20220	04/09/2000
15095	27/04/2000	F	R12	13688 R131	31/10/2000
15096	27/04/2000	M	R01	13856 R105	02/10/2000
15097	02/05/2000	F	R19	13706 R13	04/09/2000
15098	28/04/2000	F	R19	13690 R133	23/10/2000
15099	28/04/2000	M	R15	20238	16/10/2000
15100	28/04/2000	M	R19	13724 R34	04/09/2000
15101	29/04/2000	F	R02	13864 R113	04/09/2000
15103	29/04/2000	F	R19	13681 R136	23/10/2000
15104	29/04/2000	M	R15	20203	04/09/2000
15105	29/04/2000	F	R11	13824 R73	16/10/2000
15106	30/04/2000	M	R15	13709 R17	02/10/2000
15108	01/05/2000	F	R15	13908	13/11/2000
15109	03/05/2000	M	R15	20236	23/10/2000
15110	01/05/2000	M	R19	20217	13/11/2000
15111	03/05/2000	M	R01	13186	04/09/2000
15112	08/05/2000	F	R15	13838 R85	02/10/2000
15113	10/05/2000	F	R19	13468	16/10/2000
15114	12/05/2000	M	R15	13758 R68	16/10/2000
15115	11/05/2000	F	R11	13501	09/10/2000
15117	14/05/2000	F	R11	13741 R53	09/10/2000
15118	16/05/2000	F	R19	13825 R74	09/10/2000
15119	16/05/2000	F	R02	13823 R72	23/10/2000
15120	17/05/2000	M	R05	13749 R59	16/10/2000
15121	18/05/2000	F	R15	14704	16/10/2000
15122	19/05/2000	F	R15	13721 R31	16/10/2000
15123	19/05/2000	F	R05	13726 R36	23/10/2000
15124	19/05/2000	M	R21	13691 R135	09/10/2000
15125	19/05/2000	F	R21	13714 R24	09/10/2000

15126	20/05/2000	F	R01/R19	13739 R51	09/10/2000
15127	21/05/2000	F	R11/R19	13859 R108	23/10/2000
15128	22/05/2000	M	R19	13712 R22	09/10/2000
15129	22/05/2000	F	R01	13635	23/10/2000
15130	22/05/2000	M	R01	13546	23/10/2000
15131	23/05/2000	M	R11/R19	13746 R56	16/10/2000
15132	23/05/2000	M	R15/R19	13866 R115	09/10/2000
15133	25/05/2000	F	R19	13723 R33	23/10/2000
15134	25/05/2000	M	R15/R19	13710 R18	23/10/2000
15135	26/05/2000	M	R01/19	13148	23/10/2000
15136	27/05/2000	M	R19	13725 R35	16/10/2000
15137	28/05/2000	M	R19	13854 R103	23/10/2000
15139	30/05/2000	M	R19	13682 R120	23/10/2000
15140	01/06/2000	M	R15	13685 R128	09/10/2000
15142	04/06/2000	M	R01	13035	09/10/2000
15143	05/06/2000	F	R19	13747 R57	09/10/2000
15144	06/06/2000	M	R15	13656	09/10/2000
15145	06/06/2000	F	R19	13692 R137	23/10/2000
15147	07/06/2000	M	R19	13699 R144	23/10/2000
15148	08/06/2000	F	R19	13857 R106	09/10/2000
15150	11/06/2000	M	R19	13715 R25	09/10/2000
15151	14/06/2000	F	R19	13717 R27	09/10/2000
15152	14/06/2000	M	R19	13860 R109	23/10/2000
15153	19/06/2000	M	R01	13593	09/10/2000
15154	22/06/2000	F	R19	13850 R99	09/10/2000

Appendix A: Basic information about animals used in the study. F1 (50:50 Holstein Friesian:Charolais) sires were coded R01, R02, R05, R11, R12, R15, R19 and R21, and F0 (Charolais) sires were coded CH108, CH158, CH200 and CH219. M=Male and F=Female. * indicates missing data.

RANK	*S. aureus*-induced PBMC proliferation		PHA-induced PBMC proliferation	
	Cow Number	RFLP Pattern	Cow Number	RFLP Pattern
1	14804	3\23	14882	1\10
2	14898	24\22	14859	23/8
3	14846	15/23	14869	24/22/7/28
4	14815	16\26	14921	23\10
5	14852	7\10	14820	22\24
6	14835	23\10	14876	22/23
7	14879	24\22	14900	18\23
8	14897	24\1	14922	11\3
9	14826	22/24/16	14910	28\23
10	14878	23\13	14811	23\24
11	14829	23\10	14804	3\23
12	14910	28\23	14890	10\22
13	14915	16/23	14838	10\8
14	14820	22\24	14933	24\11
15	14819	11\22	14856	24\11
16	14911	7\11	14932	26/24
17	14909	23	14879	24\22
18	14933	24\11	14901	11\23
19	14880	10\12	14873	23\1
20	14875	10\3	14815	16\26
21	14860	10\11	14874	18\10
22	14900	18\23	14899	24\22
23	14883	16/3	14819	11\22
24	14869	24/22/7/28	14875	10\3
25	14814	23\28	14917	7\23
26	14890	10\22	14843	23
27	14874	18\10	14911	7\11
28	14863	23	14852	7\10
29	14851	23	14916	23

30	14838	10\8	14851	23
31	14932	26/24	14841	3\23
32	14873	23\1	14884	12\22
33	14891	23\3	14898	24\22
34	14834	16\7	14905	24\23
35	14856	24\11	14814	23\28
36	14811	23\24	14897	24\1
37	14841	3\23	14808	1\26
38	14927	10\2	14913	28\23
39	14913	28\23	14880	10\12
40	14916	23	14906	3\22
41	14922	11\3	14891	23\3
42	14816	13\23	14829	23\10
43	14882	1\10	14826	22/24/16
44	14901	11\23	14927	10\2
45	14884	12\22	14915	16/23
46	14921	23\10	14835	23\10
47	14861	22\24	14860	10\11
48	14906	3\22	14816	13\23
49	14859	23/8	14909	23
50	14877	23\13	14878	23\13
51	14843	23	14877	23\13
52	14808	1\26	14883	16/3
53	14917	7\23	14861	22\24
54	14876	22/23	14846	15/23
55	14905	24\23	14834	16\7
56	14899	24\22	14863	23

Appendix B: MHC alleles identified by RFLP pattern for females in the 1999 Cohort.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way	year of birth . sample age cross . sample date cross . year of birth	0.046 0.031 0.032	none		year of birth . sample age cross . sample date	0.012 0.021
re-run significant	all lose significance				one remains significant	
Fixed	**sample date** **year of birth** **sex**	**<0.001** **<0.001** **0.002**	**none**		**cross . sample date**	**0.019**
Random	**sire**	**none**	**sire**	**0.05<p>0.02**	**sire**	**none**

Appendix C.1: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = control *in vitro* PBMC proliferation on day two of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way	cross . sample date sex . sample date sex . cross	<0.001 0.034 0.008	none		year of birth . sample age (cross . sample date)	0.034 0.052
re-run significant	all remain significant				both lose signifcance	
Fixed	**sex . cross** **sex . sample date** **cross . sample date**	**0.001** **0.008** **0.003**	**sample date**	**0.003**	**cross** **sample date**	**0.007** **<0.001**
Random	**sire**	**0.1<p>0.05**	**sire**	**0.1<p>0.05**	**sire**	**none**

Appendix C.2: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = control *in vitro* PBMC proliferation on day three of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way re-run significant	cross . sample date sex . cross both remain significant	0.003 0.006	none		none	
Fixed	**cross . sample date** **sex . cross**	**0.013** **<0.001**	**sample date**	**0.005**	**cross** **sample date**	**0.005** **<0.001**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.3: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = control *in vitro* PBMC proliferation on day four of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way	cross . sample date sex . cross	0.020 <0.001	none		none	
re-run significant	both remain significant					
Fixed	**cross . sample date** **sex . cross**	**0.014** **<0.001**	**cross**	**0.045**	**sample date** **cross**	**<0.001** **0.003**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.4: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = control *in vitro* PBMC proliferation on day five of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way re–run significant	none		none		cross . sample date . sample age loses significance	0.049
2 way	none		none		none	
Fixed	**year of birth** **sex**	**0.033** **<0.001**	**sample age**	**0.034**	**sample date** **year of birth**	**<0.001** **0.031**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.5: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = control *in vitro* PBMC proliferation on day nine of culture. Fixed indicates the final significant factors when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way	none		none		None	
Fixed	**sample date** **year of birth** **sex**	**0.008** **0.035** **0.008**	**none**		**sample date** **year of birth**	**0.002** **0.022**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.6: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = control *in vitro* PBMC proliferation on day ten of culture. Fixed indicates the final significant factors when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	sex . year of birth . control	0.007	none		cross . year of birth . control	0.047
re-run significant	loses significance				loses significance	
2 way	sex . control sample date . sample age	0.037 0.017	sample date . sample age cross . sample date (sample date . control)	0.003 0.009 0.057	none	
re-run significant	one remains signifcant		all remain significant			
Fixed	**sex . control** **sample date** **year of birth** **cross**	**0.001** **<0.001** **0.002** **0.011**	**sample date . control** **cross . sample date** **sample date . sample age**	**<0.001** **0.011** **<0.001**	**control** **sample date**	**<0.001** **<0.001**
Random	**sire**	**0.05**	**sire**	**none**	**sire**	**0.02<p>0.01**

Appendix C.7: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = *S. aureus*-induced *in vitro* PBMC proliferation on day two of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor. ( ) = significance level of P<0.1.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	sample date . sample age . control	0.038	cross . sample age .control	0.013	cross . sample date . control	0.016
			cross . sample date . control	0.029		
	year of birth . sample age . control	0.002	cross . sample date . sample age	0.005		
	sex . sample date . sample age	0.034				
	sex . cross . sample date	0.001				
	(cross . sample date . control)	0.053				
re-run significant	2 out of 5 remain significant		all remain significant		remains significant	
2 way	4 are significant as part of significant 3 way		4 are significant as part of significant 3 way		3 are significant as part of significant 3 way	
Fixed	**sex . sample date . sample age**	**0.033**	**cross . sample age . control**	**<0.001**	**cross . sample date . control**	**0.008**
			cross . sample date . control	**0.019**	**sample date . sample age**	**0.023**
	cross . sample date . control	**0.008**	**cross . sample date . sample age**	**0.004**	**year of birth . sample age**	**0.008**
	year of birth . sample age	**0.014**			**cross . sample date**	**0.028**
	cross . sample age	**0.010**	**year of birth . control**	**<0.001**		
	cross . sample date	**0.048**	**year of birth . sample age**	**<0.001**		
	sex .sample date	**0.009**	**cross . sample date**	**0.005**		
	sex . cross	**0.040**	**cross . year of birth**	**0.007**		
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.8: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = *S. aureus*-induced *in vitro* PBMC proliferation on day three of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor. ( ) = significance level of P<0.1.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		cross . sample age . control cross . sample date . sample age	0.037 0.013	none	
re-run significant			both lose significance			
2 way	sex . control (cross . year of birth)	0.013 0.056	sample date . sample age cross . sample date	0.019 <0.001	none	
re-run significant	both lose significance		both lose significance			
Fixed	**control** **sample age** **sample date** **year of birth** **(cross)**	**<0.001** **0.039** **<0.001** **<0.001** **0.056**	**control** **sample age** **sample date** **year of birth**	**<0.001** **0.015** **0.004** **0.003**	**sample date** **year of birth**	**<0.001** **0.011**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.9: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = *S. aureus*-induced *in vitro* PBMC proliferation on day four of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor. ( ) = significance level of P<0.1.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	sex . cross . year of birth	0.049	sample date . sample age . control cross . sample date . sample age	0.036 0.004	none	
re-run significant	loses significance		both remain significant			
2 way	sex. control	0.012	2 are significant as part of significant 3 way		sample date . control	0.043
re-run significant	loses significance				loses significance	
Fixed	**control** **sample date** **year of birth**	**<0.001** **<0.001** **<0.001**	**cross . sample date . sample age** **sample date . sample age . control** **sample date . control** **cross . sample date**	**0.004** **0.022** **0.005** **<0.001**	**sample date** **year of birth**	**<0.001** **0.009**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.10: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = *S. aureus*-induced *in vitro* PBMC proliferation on day five of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	sample date . sample age . control sex . sample age . control cross . sample date . control sex . sample date . control sex . sample date . sample age		sample date . sample age . control	<0.001	sample date . sample age . control cross . sample date . control	<0.001 0.028
re-run significant	4 out of 5 remain significant		remains signficant		one remains significant	
2 way	none		2 are significant as part of significant 3 way		4 are significant as part of significant 3 way	
re-run significant						
Fixed	**sex . sample date . sample age** **cross . sample date . control** **sample date . sample age . control** **sex . sample age . control** **cross . sample date** **sex . sample date**	**<0.001** **0.006** **<0.001** **0.020** **0.004** **0.002**	**sample date . sample age . control** **sample date . control** **cross . sample date** **(sample date . sample age)**	**0.014** **0.030** **<0.001** **0.051**	**sample date . sample age . control** **sample date . control** **sample date . sample age** **year of birth . sample age** **cross . sample date**	**0.005** **0.022** **0.002** **0.024** **0.032**
Random	**sire**	**none**	**sire**	**none**	**sire**	**0.01<p>0.05**

Appendix C.11: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = *S. aureus*-induced *in vitro* PBMC proliferation on day nine of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor. ( ) = significance level of P<0.1.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	sample date . sample age . control	0.041	sample date . sample age . control	0.041	sample date . sample age . control	0.018
	cross . sample date . control	0.045			(cross . sample date . control)	0.053
	sex . sample date . control	0.049				
	sex . year of birth . control	0.032			(cross . sample date . sample age)	0.059
	sex . sample date . sample age	0.027				
re-run significant	all lose significance		loses significance		all lose significance	
2 way	sample date . sample age	0.045	sample date . control	0.004	none	
	(sex . sample age)	0.055	year of birth . sample age	0.029		
	(cross . year of birth)	0.059	cross . sample date	0.035		
re-run significant	2 out of 3 remain significant		2 out of 3 remain significant			
Fixed	**sex . sample age**	**0.045**	**sample date . control**	**<0.001**	**sample date**	**0.016**
	sample date . sample age	**0.005**	**cross . sample date**	**0.001**		
	sample date	**<0.001**				
	year of birth	**0.037**				
	sex	**0.015**				
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.12: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = *S. aureus*-induced *in vitro* PBMC proliferation on day ten of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor. ( ) = significance level of P<0.1.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way	sex . control sex . sample age	0.031 0.047	none		sample date . control	0.030
re-run significant	one remains significant				loses significance	
Fixed	**sex . sample age** **control** **sample date** **year of birth** **sex**	**0.006** **0.025** **<0.001** **<0.001** **<0.001**	**control** **sample date** **year of birth** **cross**	**0.055** **<0.001** **<0.001** **0.027**	**sample age** **sample date** **year of birth**	**0.034** **0.012** **0.035**
Random	**sire**	**0.01<p>0.001**	**sire**	**0.01<p>0.001**	**sire**	**none**

Appendix C.13: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = PHA-induced *in vitro* PBMC proliferation on day two of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way re-run significant	sex . sample age sample date . sample age one remains significant	0.008 0.018	sample date . sample age remains significant	0.039	none	
Fixed	**sample date . sample age** **control** **sample date**	**0.005** **<0.01** **<0.001**	**sample date . sample age** **control** **sample date**	**<0.001** **<0.001** **0.002**	**sample age** **sample date**	**0.010** **<0.001**
Random	**sire**	**none**	**sire**	**0.02<p>0.01**	**sire**	**none**

Appendix C.14: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = PHA-induced *in vitro* PBMC proliferation on day three of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way re-run significant	none		year of birth . sample age . control cross . sample age . control cross . sample date . control all lose significance	0.048 0.049 0.036	none	
2 way re-run significant	none		(cross . control) loses significance	0.055	none	
Fixed	**control** **sample age** **sample date** **year of birth** **(cross)**	**<0.001** **0.007** **<0.001** **<0.001** **0.056**	**control** **sample date** **year of birth**	**0.013** **0.020** **0.001**	**control** **sample age** **sample date**	**<0.001** **<0.001** **<0.001**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.15: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = PHA-induced *in vitro* PBMC proliferation on day four of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor. ( ) = significance level of P<0.1.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	none		none		none	
2 way	sex . control	0.045	cross . control	0.022	none	
re-run significant	loses significance		loses significance			
Fixed	**control** **sample age** **sample date** **cross**	**<0.001** **0.001** **0.007** **0.032**	**sample age** **sample date** **year of birth** **cross**	**0.017** **0.004** **0.007** **0.037**	**control** **sample age** **sample date**	**<0.001** **<0.001** **<0.001**
Random	**sire**	**0.10<p>0.05**	**sire**	**none**	**sire**	**none**

Appendix C.16: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = PHA-induced *in vitro* PBMC proliferation on day five of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	cross . sample date . sample age	0.048	cross . sample age . control	0.040	cross . sample date . sample age	0.005
	cross . year of birth . sample age	<0.001			cross . year of birth . sample age	<0.001
	sex . cross . sample age	0.015				
	sex . cross . sample date	0.038				
re-run significant	all lose significance		loses significance		both lose significance	
2 way	sample age . control	0.029	sample date . control	0.032	none	
			sample date . sample age	0.002		
			cross . sample date	0.010		
re-run significant	loses significance		all remain significant			
Fixed	**sample date**	**<0.001**	**cross . sample date**	**0.004**	**sample date**	**<0.001**
	year of birth	**<0.001**	**sample date . sample age**	**0.002**	**year of birth**	**0.005**
	sex	**0.040**	**sample date . control**	**0.006**		
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.17: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = PHA-induced *in vitro* PBMC proliferation on day nine of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

INTERACTION	COMPLETE DATA SET ANALYSES	P VALUE	MALE DATA SET ANALYSES	P VALUE	FEMALE DATA SET ANALYSES	P VALUE
3 way	cross . sample date . control	0.022	cross . sample age . control	0.048	sample date . sample age . control year of birth . sample age . control cross . sample date . control cross . sample date . sample age	<0.001 <0.001 <0.001 <0.001
re-run significant	loses significance		loses significance		3 out of 4 remain significant	
2 way	sample age . control sex . cross	0.040 0.010	none		4 are significant as part of significant 3 way	
re-run significant	both lose significance					
Fixed	**sample date** **year of birth** **sex**	**<0.001** **<0.001** **0.007**	**control** **sample date** **year of birth**	**0.044** **<0.001** **0.018**	**sample date . sample age . control** **cross . sample date . control** **cross . sample date . sample age** **year of birth . control** **year of birth . sample age** **cross . sample date** **cross . year of birth**	**<0.001** **<0.001** **0.004** **<0.001** **<0.001** **<0.001** **<0.001**
Random	**sire**	**none**	**sire**	**none**	**sire**	**none**

Appendix C.18: Data output following REML analysis (Genstat, 5th Edition). Table shows step-wise significant factors and interactions for Y variate = PHA-induced *in vitro* PBMC proliferation on day ten of culture. Fixed indicates the final significant factors and significant interactions when sire is a random factor.

References

Adler, W. H., Nagel, J. E. 1981. Studies of immune function in a human population. In Immunobiological Aspects of Ageing. 295-311.

Akol, G. W., Authie, E., Pinder, M., Moloo, S. K., Roelants, G. E., and Murray, M. 1986. Susceptibility and immune responses of zebu and taurine cattle of West Africa to infection with *Trypanosoma congolense* transmitted by *Glossina morsitans centralis*. Veterinary Immunology and Immunopathology, 11, 361-373.

Alexander, J. and Stimson, W. H. 1988. Sex hormones and the course of parasitic infection. Parasitology, 134, 204-210.

Allen, R. C., Stjernholm, R. C., and Steele, R. H. 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochemical and Biophysical Research Communications, 47, 679-684.

Amer, P. R., Simm, G., Keane, M. G., Diskin, M. G., and Wickham, B. W. 2001. Breeding objectives for beef cattle in Ireland. Livestock Production Science, 67, 223-239.

Anderson, J. C. 1986. Pathogenesis of Bacterial Infections in Animals. Library of Congress Catologing in Publication Data.

Andersson, L., Lunden, A., Sigurdardottir, S., Davies, C. J., and Rask, L. 1988. Linkage relationships in the bovine MHC region - high recombination frequency between Class-II subregions. Immunogenetics, 27, 273-280.

Arthur, P. F., Renand, G., and Krauss, D. 2001. Genetic and phenotypic relationships among different measures of growth and feed efficiency in young Charolais bulls. Livestock Production Science, 68, 131-139.

Ashwell, M. S., Da, Y., Vanraden, P. M., Rexroad, C. E., and Miller, R. H. 1998. Detection of putative loci affecting conformational type traits in an elite population of united states Holsteins using microsatellite markers. Journal of Dairy Science, 81, 1120-1125.

Avoncroft Sire Information, 2001. Charolais breeding pamphlet.

Ayoub, I. A. and Yang, T. J. 1996. Age-dependent changes in peripheral blood lymphocyte subpopulations in cattle: A longitudinal study. Developmental and Comparative Immunology, 20, 353-363.

Babior, B. M., Curnutte, J. T., and Kipnes, R. S. 1975. Biological defense mechanisms. Evidence for the participation of superoxide in bacterial killing by xanthine oxidase. Journal of Laboratory Clinical Medicine, 85, 235-244.

Bachmann, M. F. and Kopf, M. 1999. The role of B cells in acute and chronic infections. Current Opinion in Immunology, 11, 332-339.

Badwey, J. A. and Karnovsky, M. L. 1980. Active oxygen species and the functions of phagocytic leukocytes. Annual Reviews of Biochemistry, 49, 695-726.

Bainton, D. F., Ullyot, J. L., and Farquhar, M. G. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Journal Experimental Medicine, 134, 907-934.

Baker, P. E. and Knoblock, K. F. 1979. Monoclonal cytolytic T-cell lines. Journal of Experimental Medicine, 149, 273-278.

Baldwin, C. L., Teale, A. J., Naessens, J. G., Goddeeris, B. M., Machugh, N. D., and Morrison, W. I. 1986. Characterization of a subset of bovine T-lymphocytes that express BOT4 by monoclonal-antibodies and function - similarity to lymphocytes defined by human T4 and murine L3T4. Journal of Immunology, 136, 4385-4391.

Banchereau, J. and Steinman, R. M. 1998. Dendritic cells and the control of immunity. Nature, 392, 245-252.

Barnstable, C. J., Jones, E. A., and Crumpton, M. J. 1978. Isolation, structure and genetics of *HLA-A*, *-B*, *-C* and -DRw (Ia) antigens. British Medical Bulletin, 34, 241-246.

Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C., and Thomas, M. 1983. Flow cytometric studies of oxidative product formation by neutrophils - a graded response to membrane stimulation. Journal of Immunology, 130, 1910-1917.

Batra, T. R., Lee, A. J., Gavora, J. S., and Stear, M. J. 1989. Class-1 alleles of the bovine major histocompatibility system and their association with economic traits. Journal of Dairy Science, 72, 2115-2124.

Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A., and Schall, T. J. 1997. A new class of membrane-bound chemokine with a CX(3)C motif. Nature, 385, 640-644.

Bembridge, G. P., Machugh, N. D., McKeever, D., Awino, E., Sopp, P., Collins, R. A., Gelder, K. I., and Howard, C. J. 1995. CD45RO expression on bovine T cells: Relation to biological function. Immunology, 86, 537-544.

Benacerraf, B. and Germain, R. N. 1978. The immune response genes of the major histocompatibility complex. Immunology Reviews, 38, 70-119.

Berek, C. 1992. The development of B-cells and the B-cell repertoire in the microenvironment of the germinal centre. Immunological Reviews, 126, 5-19.

Beverley, P. C. L., Merkenschlager, M., and Terry, L. 1988. Phenotypic diversity of the CD45 antigen and its relationship to function. Immunology, 3-5.

Bigby, M., Markowitz, J. S., Bleicher, P. A., Grusby, M. J., Simha, S., Siebrecht, M., Wagner, M., Nagleranderson, C., and Glimcher, L. H. 1993. Most gamma-delta-

T-cells develop normally in the absence of MHC Class-II molecules. Journal of Immunology, 151, 4465-4475.

Bilbo, S. D. and Nelson, R. J. 2001. Sex steroid hormones enhance immune function in male and female Siberian hamsters. American Journal of Physiology, Regulatory Integrative and Comparative Physiology, 280, 207-213.

Billiau, A. 1996. Interferon-gamma: Biology and role in pathogenesis. Advances in Immunology, 62, 61-130.

Biozzi, G., Mouton, D., Heumann, A. M., Bouthillier, Y., Stiffel, C., and Mevel, J. C. 1979. Genetic analysis of antibody responsiveness to sheep erythrocytes in crosses between lines of mice selected for high or low antibody synthesis. Immunology, 36, 427-438.

Bjerknes, R. and Bassoe, C. F. 1983. Human-leukocyte phagocytosis of zymosan particles measured by flow-cytometry. Acta Pathologica Microbiologica et Immunologica Scandinavica Section C-Immunology, 91, 341-348.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. 1987. Structure of the human Class-I histocompatibility antigen, HLA- A2. Nature, 329, 506-512.

Blecha, F., Boyles, S. L., and Riley, J. G. 1984. Shipping suppresses lymphocyte blastogenic responses in Angus and Brahman X Angus feeder calves. Journal of Animal Science, 59, 576-583.

Blowey, R.W. 1990. A veterinary book for dairy farmers, Second Edition, Farming Press Limited.

Boersma, W. J., Steinmeier, F. A., and Haaijman, J. J. 1985. Age-related changes in the relative numbers of Thy-1- and Lyt-2-bearing peripheral blood lymphocytes in mice: a longitudinal approach. Cellular Immunology, 93, 417-430.

Boettcher, P. J., Dekkers, J. C. M., and Kolstad, B. W. 1998. Development of an udder health index for sire selection based on somatic cell score, udder conformation, and milking speed. Journal of Dairy Science, 81, 1157-1168.

Bowden, D. M., Short, R., Williams, D. D., and Clark, E. A. 1994. Immunologic markers in a longitudinal study of aging in pigtailed macaques (*Macaca nemestrina*). Journal of Gerontology, 49, 93-103.

Box, G. E. P. and Cox, D. R. 1964. An analysis of transformations. Journal of the Royal Statistical Society.Series B, 2, 211-252.

Boyne, R. and Arthur, J. R. 1979. Alterations of neutrophil function in selenium-deficient cattle. Journal of Comparative Pathology, 89, 151-158.

Brabin, L. and Brabin, B. J. 1992. Parasitic infections in women and their consequences. Advances in Parasitology, 31, 1-81.

Briles, R. W., McGibbon, W. H., and Irwin, M. R. 1950. Multiple alleles affecting cellular antigens in the chicken. Genetics, 35, 633-652.

Briles, W. E., Stone, H. A., and Cole, R. K. 1977. Marek's disease: effects of B histocompatibility alloalleles in resistant and susceptible chicken lines. Science, 195, 193-195.

Brotherstone, S. and Hill, W. G. 1991. Dairy-herd life in relation to linear type traits and production .1. Phenotypic and genetic analyses in pedigree type classified herds. Animal Production, 53, 279-287.

Brown, W. C., Rice-Ficht, A. C., and Estes, D. M. 1998. Bovine Type I and Type 2 responses. Veterinary Immunology and Immunopathology, 63, 45-55.

Bundy, D. A. 1988. Population ecology of intestinal helminth infections in human communities. Philosophical transactions of the Royal Society of London. Series B: Biological Sciences, 321, 405-420.

Burton, J. L., Mallard, B. A., and Mowat, D. N. 1993. Effects of supplemental chromium on immune responses of periparturient and early lactation dairy cows. Journal of Animal Science, 71, 1532-1539.

Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell, 67, 1033-1036.

Butcher, E. C. and Picker, L. J. 1996. Lymphocyte homing and homeostasis. Science, 272, 60-66.

Buus, S., Sette, A., Colon, S. M., Miles, C., and Grey, H. M. 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. Science, 235, 1353-1358.

Cabrera, W. H., Ibanez, O. M., Oliveira, S. L., Sant'Anna, O. A., Siqueira, M., Mouton, D., and Biozzi, G. 1982. Evidence for distinct polygenic regulation of antibody responses to some unrelated antigens in lines of mice selected for high or low antibody responses to somatic antigen of Salmonella. Immunogenetics, 16, 583-592.

Carlson, G. P. and Kaneko, J. J. 1973. Isolation of leukocytes from bovine peripheral blood. Proceedings of the Society of Experimental Biology and Medicine, 142, 853-856.

Carter, L. L. and Swain, S. L. 1997. Single cell analyses of cytokine production. Current Opinion in Immunology, 9, 177-182.

Chan, A. C., Desai, D. M., and Weiss, A. 1994. The role of protein-tyrosine kinases and protein-tyrosine phosphatases in T-cell antigen receptor signal-transduction. Annual Review of Immunology, 12, 555-592.

Chang, X., Mallard, B. A., and Mowat, D. N. 1996. Effects of chromium on health status, blood neutrophil phagocytosis and in vitro lymphocyte blastogenesis of dairy cows. Veterinary Immunology and Immunopathology, 52, 37-52.

Claman, H. N., Chaperon, E. A., and Triplett, R. F. 1966. Thymus-marrow cell combination. Synergisms in antibody production. Proceedings of the Society of Experimental Biology, 122, 1167-1171.

Clark, A. G. 1998. Mutation-selection balance with multiple alleles. Genetics, 102-103, 41-47.

Clark, A. J. 1998. Animal Breeding Technology for the 21st Century. Harwood Academic Publishers.

Clark, F. A. and Klebanoff, S. J. 1978. Chronic granulomatous disease: studies of a family with impaired neutrophil chemotactic, metabolic and bactericidal function. American Journal of Medicine, 65, 941-948.

Clevers, H., Machugh, N. D., Bensaid, A., Dunlap, S., Baldwin, C. L., Kaushal, A., Iams, K., Howard, C. J., and Morrison, W. I. 1990. Identification of a bovine surface-antigen uniquely expressed on CD4- CD8- T-cell receptor gamma-delta positive T-lymphocytes. European Journal of Immunology, 20, 809-817.

Coe, C. L., Lubach, G. R., Schneider, M. L., Dierschke, D. J., and Ershler, W. B. 1992. Early rearing conditions alter immune-responses in the developing infant primate. Pediatrics, 90, 505-509.

Cohen, J., Crnic, L. S. 1982. Immunopharmacology and the regulation of leukocyte function. (ed D. R. Webb), p61.

Collen, T., Dimarchi, R., and Doel, T. R. 1991. A T cell epitope in VP1 of foot-and-mouth disease virus is immunodominant for vaccinated cattle. Journal of Immunology, 146, 749-755.

Collins, F. S. 1995. Positional cloning moves from perditional to traditional. Nature Genetics, 9, 347-350.0

Concha, C., Hu, S., and Holmberg, O. 1996. The proliferative responses of cow stripping milk and blood lymphocytes to pokeweed mitogen and ginseng *in vitro*. Veterinary Research, 27, 107-115.

Constant, S. L. and Bottomly, K. 1997. Induction of TH1 and TH2 CD4+ T cell responses: The alternative approaches. Annual Review of Immunology, 15, 297-322.

Cooper, N. R. 1985. The classical complement pathway - activation and regulation of the 1st complement component. Advances in Immunology, 37, 151-216.

Correa, I., Bix, M., Liao, N. S., Zijlstra, M., Jaenisch, R., and Raulet, D. 1992. Most gamma delta T-cells develop normally in beta-2-microglobulin deficient mice. Proceeding of the National Academy of Sciences of the United States of America, 89, 653-657.

Cox, E., Mast, J., MacHugh, N., Schwenger, B., and Goddeeris, B. M. 1997. Expression of beta 2 integrins on blood leukocytes of cows with or without bovine leukocyte adhesion deficiency. Veterinary Immunology and Immunopathology, 58, 249-263.

Czerkinsky, C., Moldoveanu, Z., Mestecky, J., Nilsson, L. A., and Ouchterlony, O. 1988. A novel two colour ELISPOT assay. I. Simultaneous detection of distinct types of antibody-secreting cells. Journal of Immunological Methods, 115, 31-37.

Dantzer, R., Mormede, P. 1995. Psychoneuroimmunology of stress. In Stress, the immune system and psychiatry p 48-83.

Dargie, J. D., Murray, P. K., Murray, M., Grimshaw, W. R., and McIntyre, W. I. 1979. Bovine trypanosomiasis: the red cell kinetics of ndama and Zebu cattle infected with *Trypanosoma congolense*. Parasitology, 78, 271-286.

Davila, D. R., Guilmette, R. A., Bice, D. E., Muggenburg, B. A., Swafford, D. S., and Haley, P. J. 1992. Long-term consequences of 239PuO2 exposure in dogs:

persistent T lymphocyte dysfunction. International Journal of Radiation Biology, 61, 123-133.

De Libero, G. 1997. Sentinel function of broadly reactive human gamma delta T cells. Immunology Today, 18, 22-26.

Detilleux, J. C., Kehrli, M. E., Freeman, A. E., Fox, L. K., and Kelley, D. H. 1995. Mastitis of periparturient holstein cattle - a phenotypic and genetic-study. Journal of Dairy Science, 78, 2285-2293.

Dice, L. R. 1945. Measures of the amount of ecological association between species. Ecology, 26, 297-302.

Dietz, A. B., Detilleux, J. C., Freeman, A. E., Kelley, D. H., Stabel, J. R., and Kehrli, M. E. 1997 (a). Genetic association of bovine lymphocyte antigen *DRB3* alleles with immunological traits of Holstein cattle. Journal of Dairy Science, 80, 400-405.

Dietz, A. B., Cohen, N. D., Timms, L., and Kehrli, M. E. 1997 (b). Bovine lymphocyte antigen class II alleles as risk factors for high somatic cell counts in milk of lactating dairy cows. Journal of Dairy Science, 80, 406-412.

Dinarello, C. A. 1996. Biologic basis for interleukin-1 in disease. Blood, 87, 2095-2147.

Dosogne, H., Burvenich, C., Freeman, A. E., Kehrli, M. E., Detilleux, J. C., Sulon, J., Beckers, J. F., and Hoeben, D. 1999. Pregnancy-associated glycoprotein and decreased polymorphonuclear leukocyte function in early post-partum dairy cows. Veterinary Immunology and Immunopathology, 67, 47-54.

Drazen, D. L., Kriegsfeld, L. J., Schneider, J. E., and Nelson, R. J. 2000. Leptin, but not immune function, is linked to reproductive responsiveness to photoperiod. American journal of physiology. Regulatory, integrative and comparative physiology., 278, 1401-1407.

Duncan, J. R., Wilkie, B. N., and Hiestand, F. 1972. The serum and secretory immunoglobulins of cattle: characterisation and quantitation. Journal of Immunology, 108, 965-976.

Dunn, P. A. and Tyrer, H. W. 1981. Quantitation of neutrophil phagocytosis, using fluorescent latex beads. Correlation of microscopy and flow cytometry. Journal of Laboratory Clinical Medicine, 98, 374-381.

Durr, J. W., Monardes, H. G., and Turner, J. D. 1996. Correlations of neutrophil phagocytosis for proven bulls with traits of economic importance of their daughters. Journal of Dairy Science, 79, 133-139.

Edfors Lilja, I., Wattrang, E., Marklund, L., Moller, M., AnderssonEklund, L., Andersson, L., and Fossum, C. 1998. Mapping quantitative trait loci for immune capacity in the pig. Journal of Immunology, 161, 829-835.

Edfors Lilja, I., Johansonn, M., Wattrang, E., Johansson, A., Andersson, L., and Fossum, C. 2001. Regions on porcine chromosomes 1 and 8 influence the number of peripheral blood cells and haemoglobin levels. Proceedings of the Sixth International Veterinary Immunology Symposium, Sweden, 222.

Eens, M., Van Duyse, E., Berghman, L., and Pinxten, R. 2000. Shield characteristics are testosterone-dependent in both male and female moorhens. Hormonal Behaviour, 37, 126-134.

Egger, G., Kukovetz, E. M., Hayn, M., and Fabjan, J. S. 1997. Changes in the polymorphonuclear leukocyte function of blood samples induced by storage time, temperature and agitation. Journal of Immunological Methods, 206, 61-71.

Eigler, A., Sinha, B., Hartmann, G., and Endres, S. 1997. Taming TNF: strategies to restrain this proinflammatory cytokine. Immunology Today, 18, 487-492.

Ellis, J. A., Baldwin, C. L., Machugh, N. D., Bensaid, A., Teale, A. J., Goddeeris, B. M., and Morrison, W. I. 1986. Characterization by a monoclonal-antibody and functional- analysis of a subset of bovine T-lymphocytes that express BOT8, a molecule analogous to human CD8. Immunology, 58, 351-358.

Engelhard, V. H. 1994. How cells process antigens. Scientific American, 271, 54-61.

Engle, T. E., Spears, J. W., Brown, T. T., and Lloyd, K. E. 1999. Effect of breed (Angus vs Simmental) on immune function and response to a disease challenge in stressed steers and preweaned calves. Journal of Animal Science, 77, 516-521.

Estes, D. M., Hirano, A., Heussler, V. T., Dobbelaere, D. A. E., and Brown, W. C. 1995. Expression and biological-activities of bovine interleukin-4 - effects of recombinant bovine interleukin-4 on T-cell proliferation and B-cell differentiation and proliferation *in- vitro*. Cellular Immunology, 163, 268-279.

Everaus, H. 1992. Hormones and immune responsiveness in chronic lymphocytic leukemia. Leukocyte Lymphoma, 8, 483-489.

Ewbank, R., Kim-Madslien, F., Hart, C. B. 1999. Management and Welfare of farm animals. The UFAW farm handbook. Halstons and Co. UK Limited.

Exley, M., Wileman, T., Mueller, B., and Terhorst, C. 1995. Evidence for multivalent structure of T-cell antigen receptor complex. Molecular Immunology, 32, 829-839.

Fattorossi, A., Nisini, R., Pizzolo, J. G., and Damelio, R. 1989. New, simple flow-cytometry technique to discriminate between internalized and membrane-bound particles in phagocytosis. Cytometry, 10, 320-325.

Favre, N., Bordmann, G., and Rudin, W. 1997. Comparison of cytokine measurements using ELISA, ELISPOT and semi-quantitative RT-PCR. Journal of Immunological Methods, 204, 57-66.

Fisher, W. F. and Wright, F. C. 1981. Effects of the sheep scab mite on cumulative weight gains in cattle. Journal of Economic Entomology, 74, 234-237.

Fitzpatrick, J. L. Antigen uptake, presentation and immune responses in the bovine mammary gland. 1992. University of Bristol. Ph.D.

Fitzpatrick, J. L., Williams, N. A., Bailey, M., Bland, P. W., and Stokes, C. R. 1995. Presentation of soluble and bacterial-antigens by milk-derived cells to unprimed bovine T-cells *in-vitro*. Veterinary Immunology and Immunopathology, 46, 251-266.

Fitzpatrick, J. L., Logan, K. E., Young, F. J., Stear, M. J., Platt, D. J., and McGuirk, B. J. 1999. Breeding cattle for mastitis resistance. Proceedings of the British Mastitis Conference, Stoneleigh, UK, p 46-53.

Foreman, K. E., Vaporciyan, A. A., Bonish, B. K., Jones, M. L., Johnson, K. J., Glovsky, M. M., Eddy, S. M., and Ward, P. A. 1994. C5a-induced expression of P-selectin in endothelial-cells. Journal of Clinical Investigation, 94, 1147-1155.

Fries, R., Eggen, A., and Womack, J. E. 1993. The bovine genome map. Mammalian Genome, 4, 405-428.

Fujii, J., Otsu, K., Zorzato, F., de Leon, S., Khanna, V. K., Weiler, J. E., O'Brien, P. J., and MacLennan, D. H. 1991. Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. Science, 253, 448-451.

Furuya, T., Joe, B., Salstrom, J. L., Hashiramoto, A., Dobbins, D. E., Wilder, R. L., and Remmers, E. F. 2001. Polymorphisms of the tumor necrosis factor alpha locus among autoimmune disease susceptible and resistant inbred rat strains. Genes and Immunity, 2, 229-232.

Gaillard, R. C. and Spinedi, E. 1998. Sex- and stress-steroids interactions and the immune system: evidence for a neuroendocrine-immunological sexual dimorphism. Domestic Animal Endocrinology, 15, 345-352.

Gavora, J. S. and Spencer, J. L. 1983. Breeding for immune responsiveness and disease resistance. Animal Blood Groups and Biochemical Genetics, 14, 159-180.

Gbarah, A., Gahmberg, C. G., Ofek, I., Jacobi, U., and Sharon, N. 1991. Identification of the leukocyte adhesion molecules CD11 and CD18 as receptors for type 1-fimbriated (mannose-specific) *Escherichia-coli*. Infection and Immunity, 59, 4524-4530.

Gelhaus, A., Schnittger, L., Mehlitz, D., Horstmann, R. D., and Meyer, C. G. 1995. sequence and PCR-RFLP analysis of 14 novel *BoLA-DRB3* alleles. Animal Genetics, 26, 147-153.

Gellin, J., Brown, S., Graves, J. A. M., Rothschild, M., Schook, L., Womack, J., and Yerle, M. 2000. Comparative gene mapping workshop: progress in agriculturally important animals. Mammalian Genome, 11, 140-144.

Gennaro, R., Dewald, B., Horisberger, U., Gubler, H. U., and Baggiolini, M. 1983. A novel type of cytoplasmic granule in bovine neutrophils. Journal of Cell Biology, 96, 1651-1661.

Georges, M., Dietz, A. B., Mishra, A., Nielsen, D., Sargeant, L. S., Sorensen, A., Steele, M. R., Zhao, X. Y., Leipold, H., Womack, J. E., and Lathrop, M. 1993. Microsatellite mapping of the gene causing weaver disease in cattle will allow the study of an associated quantitative trait locus. Proceedings of the National Academy of Sciences of the United States of America, 90, 1058-1062.

Gerardi, A. S. 1996. Bovine leucocyte adhesion deficiency: A review of a modern disease and its implications. Research in Veterinary Science, 61, 183-186.

Germain, R. N. 1994. MHC-dependent antigen-processing and peptide presentation - providing ligands for T-lymphocyte activation. Cell, 76, 287-299.

Gillis, S., Scheid, M., and Watson, J. 1980. Biochemical and biologic characterization of lymphocyte regulatory molecules. III. The isolation and phenotypic characterization of Interleukin-2 producing T cell lymphomas. Journal of Immunology, 125, 2570-2578.

Gisler, R. H. 1974. Stress and the hormonal regulation of the immune response in mice. Psychotherapy and Psychosomatics, 23, 197-208.

Glas, R., Franksson, L., Une, C., Eloranta, M. L., Ohlen, C., Orn, A., and Karre, K. 2000. Recruitment and activation of natural killer (NK) cells *in vivo* determined by the target cell phenotype: An adaptive component of NK cell-mediated responses. Journal of Experimental Medicine, 191, 129-138.

Glass, E. J. and Spooner, R. L. 1989. Requirement for MHC class-II positive accessory cells in an antigen specific bovine T-cell response. Research in Veterinary Science, 46, 196-201.

Glass, E. J., Oliver, R. A., Collen, T., Doel, T. R., Dimarchi, R., and Spooner, R. L. 1991. MHC Class II restricted recognition of FMDV peptides by bovine T cells. Immunology, 74, 594-599.

Godson, D. L., Baca-Estrada, M. E., Babiuk, C. A. 1997. Application of bovine cytokines. In Cytokines in Veterinary Medicine (eds V. E. C. J. Schijns & M. C. Horzinek).

Gotze, D., Rossler, R., and Thierfelder, S. 1977. Other mammalian histocompatibility systems. Skin graft rejection and graft-versus-host reaction across I-subregion differences: are there many more than three H loci within the H-2 complex? Transplantation Proceedings, 9, 547-550.

Gould, K. G., Akinbami, M. A., and Mann, D. R. 1998. Effect of neonatal treatment with a gonadotropin releasing hormone antagonist on developmental changes in circulating lymphocyte subsets: a longitudinal study in male rhesus monkeys. Developmental and Comparative Immunology, 22, 457-467.

Grandin, T. 2000. Livestock Handling and Transport. Library of Congress Cataloging-in-Publication Data.

Greeley, E. H., Kealy, R. D., Ballam, J. M., Lawler, D. F., and Segre, M. 1996. The influence of age on the canine immune system. Veterinary Immunology and Immunopathology, 55, 1-10.

Greeley, E. H., Ballam, J. M., Harrison, J. M., Kealy, R. D., Lawler, D. F., and Segre, M. 2001. The influence of age and gender on the immune system: a longitudinal study in Labrador Retriever dogs. Veterinary Immunology and Immunopathology, 82, 57-71.

Griffin, F. M., Jr., Griffin, J. A., Leider, J. E., and Silverstein, S. C. 1975. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. Journal of Experimental Medicine, 142, 1263-1282.

Griffin, F. M., Jr., Griffin, J. A., and Silverstein, S. C. 1976. Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. Journal of Experimental Medicine, 144, 788-809.

Grossman, C. J. 1984. Regulation of the immune system by sex steroids. Endocrine Reviews, 5, 435-455.

Grossman, C. J. 1985. Interactions between the gonadal steroids and the immune system. Science, 227, 257-261.

Gruen, J. R. and Weissman, S. M. 2001. Human MHC class III and IV genes and disease associations. Frontiers in Bioscience, 6, D960-D972.

Guidry, A. J., Paape, M. J., and Miller, R. H. 1974. *In vitro* procedure for measuring phagocytosis of blood neutrophils. American Journal of Veterinary Research, 35, 705-709.

Gyang, E. O., Stevens, J. B., Olson, W. G., Tsitsamis, S. D., and Usenik, E. A. 1984. Effects of selenium-vitamin E injection on bovine polymorphonucleated leukocytes phagocytosis and killing of *Staphylococcus aureus*. American Journal of Veterinary Research, 45, 175-177.

Harding, C. V., Song, R., Griffin, J., France, J., Wick, M. J., Pfeifer, J. D., and Geuze, H. J. 1995. Processing of bacterial-antigens for presentation to class-I and class-II MHC-restricted T-lymphocytes. Infectious Agents and Disease-Reviews Issues and Commentary, 4, 1-12.

Hardy, D. A., Bell, J. I., Long, E. O., Lindsten, T., and McDevitt, H. O. 1986. Mapping of the class-II region of the human major histocompatibility complex by pulsed-field gel-electrophoresis. Nature, 323, 453-455.

Hasui, M., Hirabayashi, Y., and Kobayashi, Y. 1989. Simultaneous measurement by flow-cytometry of phagocytosis and hydrogen-peroxide production of neutrophils in whole-blood. Journal of Immunological Methods, 117, 53-58.

Hauser, M. A., Koob, M. D., and Roth, J. A. 1986. Variation of neutrophil function with age in calves. American Journal of Veterinary Research, 152-153.

Hazel, L. N. 1943. The genetic basis for construction of selection indexes. Genetics, 28, 476-490.

Hein, W. R. and Mackay, C. R. 1991. Prominence of gamma-delta T-cells in the ruminant immune-system. Immunology Today, 12, 30-34.

Hein, W. R., Dudler, L., Marston, W. L., Hopkins, J., Dutia, B. M., Keech, K., Brandon, M. R., and Mackay, C. R. 1991. Summary of workshop findings for leukocyte antigens of sheep. Veterinary Immunology and Immunopathology, 27, 28-30.

Heller, D. A., Ahern, F. M., Stout, J. T., and McClearn, G. E. 1998. Mortality and biomarkers of aging in heterogeneous stock (HS) mice. Journal of Gerontology. A Biology of Scientific Medical Science, 53, 217-230.

Heyen, D. W., Weller, J. I., Ron, M., Band, M., Beever, J. E., Feldmesser, E., Da, Y., Wiggans, G. R., Vanraden, P. M., and Lewin, H. A. 1999. A genome scan for QTL influencing milk production and health traits in dairy cattle. Physiological Genomics, 1, 165-175.

Hines, H. C., Allaire, F. R., and Michalak, M. M. 1986. Association of bovine lymphocyte antigens with milk fat percentage differences. Journal of Dairy Science, 69, 3148-3150.

Hirabayashi, Y., Taniuchi, S., and Kobayashi, Y. 1985. A quantitative assay of oxidative-metabolism by neutrophils in whole- blood using flow-cytometry. Journal of Immunological Methods, 82, 253-259.

Hochrein, H., Shortman, K., Vremec, D., Scott, B., Hertzog, P., and O'Keeffe, M. 2001. Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. Journal of Immunology, 166, 5448-5455.

Hoeben, D., Dosogne, H., Heyneman, R., and Burvenich, C. 1997. Effect of antibiotics on the phagocytotic and respiratory burst activity of bovine granulocytes. European Journal of Pharmacology, 332, 289-297.

Hohenboken, W. D. 1987. Behavioral genetics. Veterinary Clinics of North American Food Animal Practice, 3, 217-229.

Holdaway, R. J., Holmes, C. W., and Steffert, I. J. 1996. A comparison of indirect methods for diagnosis of subclinical intramammary infection in lactating dairy cows .3. Prediction of the incidence of infections and the critical threshold value for somatic cell count from the herd's bulk milk somatic cell count. Australian Journal of Dairy Technology, 51, 79-82.

Honkanen-buzalski, T., Katila, T., and Sandholm, M. 1981. Milk antitrypsin activity during clinical and experimental bovine mastitis. Acta Veterinaria Scandinavica, 22, 360-368.

Hooper, D. C., Chantry, D. H., and Billington, W. D. 1987. Murine pregnancy-associated modulations in lymphocyte- reactivity to mitogens - identification of the cell-populations affected. Journal of Reproductive Immunology, 11, 273-286.

Horan, P. K., Melnicoff, M. J., Jensen, B. D., and Slezak, S. E. 1990. Fluorescent cell labeling for *in vivo* and *in vitro* cell tracking. Methods Cellular Biology, 33, 469-490.

Horwitz, M. A. 1982. Phagocytosis of microorganisms. Reviews of Infectious Diseases, 104-121.

Hosie, B. D. Scrapie susceptibility testing of texel sheep. 1997. SAC Veterinary Services, Edinburgh.

Howard, C. J., Sopp, P., Parsons, K. R., and FINCH, J. 1989. *In vivo* depletion of BOT4 (CD4) and of non-T4/T8 lymphocyte subsets in cattle with monoclonal-antibodies. European Journal of Immunology, 19, 757-764.

Howard, C. J. and Morrison, W. I. 1991. Leukocyte antigens in cattle, sheep and goats. Veterinary Immunology and Immunopathology, 27, 1-20.

Howard, C. J., Brooke, G. P., Werling, D., Sopp, P., Hope, J. C., Parsons, K. R., and Collins, R. A. 1999. Dendritic cells in cattle: phenotype and function. Veterinary Immunology and Immunopathology, 72, 119-124.

Huber, H. and Fudenberg, H. H. 1970. The interaction of monocytes and macrophages with immunoglobulins and complement. Serology and Haematology, 3, 160-175.

Huber, S. A., Kupperman, J., and Newell, M. K. 1999. Estradiol prevents and testosterone promotes Fas-dependent apoptosis in CD4+ Th2 cells by altering Bcl 2 expression. Lupus, 8, 384-387.

Hunter, N., Foster, J. D., Goldmann, W., Stear, M. J., Hope, J., and Bostock, C. 1996. Natural scrapie in a closed flock of Cheviot sheep occurs only in specific *PrP* genotypes. Archives of Virology, 141, 809-824.

Hurley, D. J., Kensinger, M. H., Mastro, A. M., and Wilson, R. A. 1990. An evaluation of the mononuclear-cells derived from bovine mammary- gland dry secretions using leukocyte antigen specific monoclonal- antibodies, light-scattering properties and nonspecific esterase staining. Veterinary Immunology and Immunopathology, 25, 177-193.

Hynes, R. O. 1992. Integrins - versatility, modulation, and signalling in cell- adhesion. Cell, 69, 11-25.

Irwin, M., Patterson, T., Smith, T. L., Caldwell, C., Brown, S. A., Gillin, J. C., and Grant, I. 1990. Reduction of immune function in life stress and depression. Biology and Psychiatry, 27, 22-30.

Irwin, M. R. and Hauger, R. L. 1988. Adaptation to chronic stress. Temporal pattern of immune and neuroendocrine correlates. Neuropsychopharmacology, 1, 239-242.

Janeway, Travers 1994. Immunobiology. The immune system in health and disease. Balckwell Scientific Publications Oxford.

Jutila, M. A., Wilson, E., and Kurk, S. 1997. Characterization of an adhesion molecule that mediates leukocyte rolling on 24 h cytokine- or lipopolysaccharide- stimulated

bovine endothelial cells under flow conditions. Journal of Experimental Medicine, 186, 1701-1711.

Kaufmann, S. H. E. and Kabelitz, D. 1998. Introduction: The immune response to infectious agents. Methods in Microbiology, Vol 25, 25, 1-19.

Kearns, R. J., Hayek, M. G., Turek, J. J., Meydani, M., Burr, J. R., Greene, R. J., Marshall, C. A., Adams, S. M., Borgert, R. C., and Reinhart, G. A. 1999. Effect of age, breed and dietary omega-6 (n-6): omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs. Veterinary Immunology and Immunopathology, 69, 165-183.

Kehrli, M. E. and Goff, J. P. 1989. Periparturient hypocalcemia in cows - effects on peripheral-blood neutrophil and lymphocyte function. Journal of Dairy Science, 72, 1188-1196.

Kehrli, M. E., Nonnecke, B. J., and Roth, J. A. 1989. Alterations in bovine neutrophil function during the periparturient period. American Journal of Veterinary Research, 207-214.

Kehrli, M. E., Weigel, K. A., Freeman, A. E., Thurston, J. R., and Kelley, D. H. 1991. Bovine sire effects on daughters *in vitro* blood neutrophil functions, lymphocyte blastogenesis, serum complement and conglutinin levels. Veterinary Immunology and Immunopathology, 27, 303-319.

Kelley, K. W., Arkins, S., and Li, Y. M. 1992. Growth hormone, prolactin, and insulin-like growth factors: new jobs for old players. Brain Behaviour and Immunology, 6, 317-326.

Kelley, K. W., Arkins, S., Li, Y. M. 1994. Role of growth hormone and insulin-like growth factor I in immunoregulation. In Growth Hormone II. Basic and Clinical Aspects (eds B. B. Bercu & R. F. Walker), pp. 87-103.

Kelm, S. C., Detilleux, J. C., Freeman, A. E., Kehrli, M. E., Dietz, A. B., Fox, L. K., Butler, J. E., Kasckovics, I., and Kelley, D. H. 1997. Genetic association between parameters of innate immunity and measures of mastitis in periparturient Holstein cattle. Journal of Dairy Science, 80, 1767-1775.

Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and Zlotnik, A. 1994. Lymphotactin - a cytokine that represents a new class of chemokine. Science, 266, 1395-1399.

Kelso, A. 1995. Th1 and Th2 subsets - paradigms lost. Immunology Today, 16, 374-379.

Kempster, A. J., Cook, G. L., and Southgate, J. R. 1988. Evaluation of British Friesian, Canadian Holstein and beef breed x British Friesian steers slaughtered over a commercial range of fatness from 16-month and 24-month beef-production systems .2. Carcass characteristics, and rate and efficiency of lean gain. Animal Production, 46, 365-378.

Kim, C. H. and Broxmeyer, H. E. 1999. Chemokines: signal lamps for trafficking of T and B cells for development and effector function. Journal of Leukocyte Biology, 65, 6-15.

Kishimoto, T. 1989. The biology of interleukin-6. Blood, 74, 1-10.

Klein, J., Marusic, M., and Nagy, Z. A. 1982. The seven rules of MHC restriction. Transplantation Proceedings, 14, 581-583.

Klein, J. and Figueroa, F. 1986. Evolution of the major histocompatibility complex. Critical Reviews of Immunology, 6, 295-386.

Klein, J. 1986. Antigen-major histocompatibility complex-T cell receptors: inquiries into the immunological menage a trois. Immunology Research, 5, 173-190.

Klein, J., Horejsi, V. 1997. Immunology. Oxford Cambridge Blackwell Science.

Kong, Y. Y., Kishihara, K., Yoshida, H., Mak, T. W., and Nomoto, K. 1995. Generation of T-cells with differential responses to alloantigens in CD45 exon 6-deficient mice. Journal of Immunology, 154, 5725-5735.

Kotzin, B. L., Leung, D. Y. M., Kappler, J., and Marrack, P. 1993. Superantigens and their potential role in human-disease. Advances in Immunology, 54, 99-166.

Kristensen, F., Kristensen, B., and Lazary, S. 1982. The lymphocyte stimulation test in veterinary immunology. Veterinary Immunology and Immunopathology, 3, 203-277.

Kristensson, K., Kristensen, L., Borrebaeck, C. A. K., and Carlsson, R. 1994. Activation of human CD4(+)45RA(+) T-cells using B-cells as accessory cells. Immunology Letters, 39, 223-229.

Krzych, U., Strausser, H. R., Bressler, J. P., and Goldstein, A. L. 1981. Effects of sex hormones on some T and B cell functions, evidenced by differential immune expression between male and female mice and cyclic pattern of immune responsiveness during the estrous cycle in female mice. American Journal of Reproductive Immunology, 1, 73-77.

Lachmann, P. J. and Hughes-Jones, N. C. 1984. Initiation of complement activation. Springer Seminars in Immunopathology, 7, 143-162.

Lan, H. Y., Nikolicpaterson, D. J., Mu, W., and Atkins, R. C. 1995. Local macrophage proliferation in the progression of glomerular and tubulointerstitial injury in rat anti-gbm glomerulonephritis. Kidney International, 48, 753-760.

Lawrence, M. B. and Springer, T. A. 1991. Leukocytes roll on a selectin at physiological flow-rates - distinction from and prerequisite for adhesion through integrins. Cell, 65, 859-873.

Ledwidge, S. A., Mallard, B. A., Gibson, J. P., Jansen, G. B., and Jiang, Z. H. 2001. Multi-primer target PCR for rapid identification of bovine *DRB3* alleles. Animal Genetics, 32, 219-221.

Lee, C. S., Wooding, F. B. P., and Kemp, P. 1980. Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretions, colostrum, and milk from normal cows. Journal of Dairy Research, 47, 39-50.

Lee, J. C. 1996. The prospects for developing a vaccine against *Staphylococcus aureus*. Trends in Microbiology, 4, 162-166.

Lennard, A. C. 1995. Interleukin-1 receptor antagonist. Critical Reviews in Immunology, 15, 77-105.

Lewin, H. A., Davis, W. C., and Bernoco, D. 1985. Monoclonal-antibodies that distinguish bovine T-lymphocytes and B-lymphocytes. Veterinary Immunology and Immunopathology, 9, 87-102.

Lewin, H. A., Russell, G. C., and Glass, E. J. 1999. Comparative organization and function of the major histocompatibility complex of domesticated cattle. Immunological Reviews, 167, 145-158.

Lewin, R. 1996. Genes chart the spread of farming. New Scientist, 151, 18-18.

Lindberg, P. G. and Andersson, L. 1988. Close association between DNA polymorphism of bovine major histocompatibility complex class-I genes and serological *BoLA-A* specificities. Animal Genetics, 19, 245-255.

Lobb, R. R., Chirosso, G., Leone, D. R., Rosa, M. D., Bixler, S., Newman, B. M., Luhowskyj, S., Benjamin, C. D., Dougas, I. G., Goelz, S. E., Hession, C., and Chow, E. P. 1991. Expression and functional-characterization of a soluble form of endothelial-leukocyte adhesion molecule-1. Journal of Immunology, 147, 124-129.

Logan, K. E. Immunological responses of Holstein-Friesian cattle to *Staphylococcus aureus in vitro*. 2001. University of Glasgow. Ph.D.

Losson, B. J., Lonneux, J. F., and Lekimme, M. 1999. The pathology of *Psoroptes ovis* infestation in cattle with a special emphasis on breed difference. Veterinary Parasitology, 83, 219-229.

Lotz, M. 1995. Interleukin-6: a comprehensive review. Cancer Treatment Research, 80, 209-233.

Lukacs, K., Roth, J. A., and Kaeberle, M. L. 1985. Activation of neutrophils by antigen-induced lymphokine, with emphasis on antibody-independent cyto-toxicity. Journal of Leukocyte Biology, 38, 557-572.

Lund, T. and Jensen, J. 1996. Genetic and phenotypic parameters for clinical mastitis, somatic cell production deviance and protein yield in dairy cattle. Proceedings of the 47th Annual Meeting of the European Association of Animal Producers, 4.

Lunden, A., Sigurdardottir, S., Edfors Lilja, I., Danell, B., Rendel, J., and Andersson, L. 1990. The relationship between bovine major histocompatibility complex class-II polymorphism and disease studied by use of bull breeding values. Animal Genetics, 21, 221-232.

Lunden, A., Sigurdardottir, S., and Andersson, L. 1991. Restriction fragment length polymorphism of a bovine T-cell receptor beta gene. Animal Genetics, 22, 497-501.

Lutje, V. and Black, S. J. 1991. Cellular interactions regulating the *in vitro* response of bovine lymphocytes to ovalbumin. Veterinary Immunology and Immunopathology, 28, 275-288.

Lutje, V. and Black, S. J. 1992. Analysis of pokeweed mitogen-induced *in vitro* proliferative and antibody-responses of bovine lymphocytes. Research in Veterinary Science, 52, 236-242.

Mackay, C. 1988. Sheep leukocyte molecules - a review of their distribution, structure and possible function. Veterinary Immunology and Immunopathology, 19, 1-20.

Mackay, C. R., Kimpton, W. G., Brandon, M. R., and Cahill, R. N. P. 1988. Lymphocyte subsets show marked differences in their distribution between blood and the afferent and efferent lymph of peripheral lymph-nodes. Journal of Experimental Medicine, 167, 1755-1765.

Mackay, C. R. and Hein, W. R. 1990. Analysis of gamma-delta T-cells in ruminants reveals further heterogeneity in gamma-delta T-cell features and function among species. Research in Immunology, 141, 611-614.

Mackay, C. R., Marston, W. L., Dudler, L., Spertini, O., Tedder, T. F., and Hein, W. R. 1992. Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T-cells. European Journal of Immunology, 22, 887-895.

MacKenzie, A. M., Drennan, M., Rowan, T. G., Dixon, J. B., and Carter, S. D. 1997. Effect of transportation and weaning on the humeral immune response of calves. Research in Veterinary Science, 3, 230.

Maes, M., Bosmans, E., Suy, E., Vandervorst, C., De Jonckheere, C., and Raus, J. 1990. Immune disturbances during major depression: upregulated expression of interleukin-2 receptors. Neuropsychobiology, 24, 115-120.

Maes, M., Van Bockstaele, D. R., Gastel, A., Song, C., Schotte, C., Neels, H., DeMeester, I., Scharpe, S., and Janca, A. 1999. The effects of psychological stress on leukocyte subset distribution in humans: evidence of immune activation. Neuropsychobiology, 39, 1-9.

Makinodan, T. and Kay, M. M. 1980. Age influence on the immune system. Advances in Immunology, 29, 287-330.

Mallard, B. A., Leslie, K. E., Dekkers, J. C. M., Hedge, R., Bauman, M., and Stear, M. J. 1995. Differences in bovine lymphocyte antigen associations between immune responsiveness and risk of disease following intramammary infection with *Staphylococcus aureus*. Journal of Dairy Science, 78, 1937-1944.

Manderson, A. P., Pickering, M. C., Botto, M., Walport, M. J., and Parish, C. R. 2001. Continual low-level activation of the classical complement pathway. Journal of Experimental Medicine, 194, 747-756.

Markert, M. L., Hummell, D. S., Rosenblatt, H. M., Schiff, S. E., Harville, T. O., Williams, L. W., Schiff, R. I., and Buckley, R. H. 1998. Complete DiGeorge syndrome: Persistence of profound immunodeficiency. Journal of Pediatrics, 132, 15-21.

Masinovsky, B., Urdal, D., and Gallatin, W. M. 1990. IL-4 acts synergistically with IL-1-beta to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell-adhesion molecule-1. Journal of Immunology, 145, 2886-2895.

Mason, D. and Powrie, F. 1990. Memory CD4+ T-cells in man form 2 distinct subpopulations, defined by their expression of isoforms of the leukocyte common antigen, CD45. Immunology, 70, 427-433.

McCole, D. F., Doherty, M. L., Torgerson, P. R., and Baird, A. W. 1998. Local immune responses in colon from cattle infected with *Fasciola hepatica*. International Journal for Parasitology, 28, 1733-1737.

McDevitt, H. O. 2000. Discovering the role of the major histocompatibility complex in the immune response. Annual Review of Immunology, 18, 1-19.

McDowell, L. R., Williams, S. N., Hidiroglou, N., Njeru, C. A., Hill, G. M., Ochoa, L., and Wilkinson, N. S. 1996. Vitamin E supplementation for the ruminant. Animal Food Science Technology, 60, 273-296.

McEver, R. P. 1994. Selectins. Current Opinion in Immunology, 6, 75-84.

McKellar, Q. A. 1996. How antimicrobials work in mastitis. Proceedings of the British Mastitis Conference, Stoneleigh, UK.

Meerschaert, J. and Furie, M. B. 1995. The adhesion molecules used by monocytes for migration across endothelium include CD11A/CD18, CD11B/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. Journal of Immunology, 154, 4099-4112.

Mejdell, C. M., Lie, O., Solbu, H., Arnet, E. F., and Spooner, R. L. 1994. Association of major histocompatibility complex antigens (*BoLA- A*) with AI bull progeny test-results for mastitis, ketosis and fertility in Norwegian cattle. Animal Genetics, 25, 99-104.

Meleney, W. P. and Fisher, W. F. 1979. The effect of exposure to mites, *Psoroptes ovis* (Acarina: Psoroptidae), on calves of varying susceptibility to common scabies. Proceedings of the Annual Meeting of the U.S. Animal Health Association, 340-346.

Menge, C., Neufeld, B., Hirt, W., Schmeer, N., Bauerfeind, R., Baljer, G., and Wieler, L. H. 1998. Compensation of preliminary blood phagocyte immaturity in the newborn calf. Veterinary Immunology and Immunopathology, 62, 309-321.

Mertz, W. 1992. Chromium. History and nutritional importance. Biology of Trace Element Research, 32, 3-8.

Meyer, A., Parng, C. L., Hansal, S. A., Osborne, B. A., and Goldsby, R. A. 1997. Immunoglobulin gene diversification in cattle. International Reviews of Immunology, 15, 165-183.

Miller, R. A. 1997. Age-related changes in T cell surface markers: a longitudinal analysis in genetically heterogeneous mice. Mechanisms of Ageing and Development, 96, 181-196.

Miller, R. A. 1999. Ageing and immune function. In Fundamental Immunology pp. 947-966.

Millot, P., Chatelain, J., and Cathala, F. 1985. Sheep major histocompatibility complex OLA: gene frequencies in two French breeds with scrapie. Evidence for a linkage between OLA and resistance or susceptibility to the disease. Immunogenetics, 21, 117-123.

Millot, P., Chatelain, J., Dautheville, C., Salmon, D., and Cathala, F. 1988. Sheep major histocompatibility (*OLA*) complex: linkage between a scrapie susceptibility/resistance locus and the *OLA* complex in Ile-de-France sheep progenies. Immunogenetics, 27, 1-11.

Mitchell, L. A. 1999. Sex differences in antibody and cell-mediated immune response to rubella re-immunisation. Journal of Medical Microbiology, 48, 1075-1080.

Moiola, F., Spycher, M., Wyderwalther, M., and Zwahlen, R. D. 1994. Comparative *in-vitro* phagocytosis and F-actin polymerization of bovine neonatal neutrophils. Journal of Veterinary Medicine Series A-Zentralblatt fur Veterinarmedizin Reihe A-Physiology Pathology Clinical Medicine, 202-214.

Montero-Julian, F. A. 2001. The soluble IL-6 receptors: Serum levels and biological function. Cellular and Molecular Biology, 47, 583-597.

Morafo, V., Rath, S., Thomas, M. L., and Durdik, J. M. 1999. Induction of a germinal centre phenotype in B cells *in vitro* by a Th2 cell line. Cellular Immunology, 198, 77-86.

Mosmann, T. R., Cherwinski.H.W., Bond, M. W., Giedlin, M. A., and Coffman, R. L. 1986. Two types of murine helper T-cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology, 136, 2348-2357.

Mosser, D. M. and Karp, C. L. 1999. Receptor mediated subversion of macrophage cytokine production by intracellular pathogens. Current Opinion in Immunology, 11, 406-411.

Mowat, D. N. 1997. Organic chromium in animal nutrition. Chromium Series

Mrode, R. A., Swanson, G. J. T., and Lindberg, C. M. 2000. Genetic correlations of somatic cell count and conformation traits with herd life in dairy breeds, with an application to national genetic evaluations for herd life in the United Kingdom. Livestock Production Science, 65, 119-130.

Muggli, N. E., Hohenboken, W. D., Cundiff, L. V., and Mattson, D. E. 1987. Inheritance and interaction of immune traits in beef calves. Journal of Animal Science, 64, 385-393.

Murata, H. 1995. Suppression of bovine neutrophil function by sera from calves transported from over a long distance by road. Animal Science and Technology (Japan), 66, 976-978.

Murray, M., Clifford, D. J., and McIntyre, W. I. 1979. Diagnosis of African trypanosomiasis in the bovine. Transactions of the Royal Society of Tropical Medicine and Hygene, 73, 120-121.

Murray, M., Morrison, W. I., and Whitelaw, D. D. 1982. Host susceptibility to African trypanosomiasis: trypanotolerance. Advances in Parasitology, 21, 1-68.

Mwangi, E. K., Stevenson, P., Gettinby, G., Reid, S. W., and Murray, M. 1998. Susceptibility to trypanosomosis of three Bos indicus cattle breeds in areas of differing tsetse fly challenge. Veterinary Parasitology, 79, 1-17.

Nadesalingam, J., Plante, Y., and Gibson, J. P. 2001. Detection of QTL for milk production on Chromosomes 1 and 6 of Holstein cattle. Mammalian Genome, 12, 27-31.

Nagahata, H., Nochi, H., Sanada, Y., Tamoto, K., Noda, H., and Kociba, G. J. 1994. Analysis of mononuclear cell functions in Holstein cattle with leukocyte adhesion deficiency. American Journal of Veterinary Research, 55, 1101-1106.

Nagahata, H., Higuchi, H., Nochi, H., Tamoto, K., Araiso, T., Noda, H., and Kociba, G. J. 1996. Biosynthesis of B-2-integrin, intracellular calcium signalling and functional responses of normal and CD18-deficient bovine neutrophils. Research in Veterinary Science, 61, 95-101.

Nepom, G. T. and Erlich, H. 1991. MHC Class-II molecules and autoimmunity. Annual Review of Immunology, 9, 493-525.

Newman, M. J., Truax, R. E., French, D. D., Dietrich, M. A., Franke, D., and Stear, M. J. 1996. Evidence for genetic control of vaccine-induced antibody responses in cattle. Veterinary Immunology and Immunopathology, 50, 43-54.

Newman, S. L. and Johnston, R. B., Jr. 1979. Role of binding through C3b and IgG in polymorphonuclear neutrophil function: studies with trypsin-generated C3b. Journal of Immunology, 123, 1839-1846.

Nguyen, T. C. 1984. The immune response in sheep: analysis of age, sex and genetic effects on the quantitative antibody response to chicken red blood cells. Veterinary Immunology and Immunopathology, 5, 237-245.

Oddgeirsson, O., Simpson, S. P., Morgan, A. L., Ross, D. S., and Spooner, R. L. 1988. Relationship between the bovine major histocompatibility complex (*BoLA*), erythrocyte markers and susceptibility to mastitis in Icelandic cattle. Animal Genetics, 19, 11-16.

Olsen, N. J. and Kovacs, W. J. 1996. Gonadal steroids and immunity. Endocrine Reviews, 17, 369-384.

Outteridge, P. M. and Lee, C. S. 1988. The defence mechanisms of the mammary gland of domestic ruminants. Progress in Veterinary Microbiology and Immunology, 4, 165-169.

Paape, M. J., Guidry, A. J., Kirk, S. T., and Bolt, D. J. 1975. Measurement of phagocytosis of ^{32}P - labelled *Staphylococcus aureus* by bovine leukocytes: Lysostaphin digestion and inhibitory effect of cream. American Journal of Veterinary Research, 36, 1737-1743.

Paape, M. J. and Wergin, W. P. 1977. The leukocyte as a defense mechanism. Journal of American Veterinary Medical Association, 170, 1214-1223.

Paape, M. J., Pearson, R. E., and Schultze, W. D. 1978. Variation among cows in the ability of milk to support phagocytosis and in the ability of polymorphonuclear leukocytes to phagocytose *Staphylococcus aureus*. American Journal of Veterinary Research, 39, 1907-1910.

Paape, M. J. and Miller, R. H. 1988. Sources of variation introduced into a phagocytosis assay as a result of the isolation of neutrophils from bovine blood. American Journal of Veterinary Research, 1210-1213.

Paape, M. J., Miller, R. H., and ZIV, G. 1991. Pharmacological enhancement or suppression of phagocytosis by bovine neutrophils. American Journal of Veterinary Research, 52, 363-366.

Paape, M. J., Lilius, E. M., Wiitanen, P. A., Kontio, M. P., and Miller, R. H. 1996. Intramammary defense against infections induced by *Escherichia coli* in cows. American Journal of Veterinary Research, 57, 477-482.

Paling, R. W., Moloo, S. K., Scott, J. R., Gettinby, G., McOdimba, F. A., and Murray, M. 1991. Susceptibility of N'Dama and Boran cattle to sequential challenges with tsetse-transmitted clones of *Trypanosoma congolense*. Parasite Immunology, 13, 427-445.

Pangburn, M. K. and Muller-Eberhard, H. J. 1980. Relation of putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. Journal of Experimental Medicine, 152, 1102-1114.

Pastoret, P. P., Griebel, P., Bazin, H., Govaerts, A. 1998. Handbook of Vertebrate Immunology. Academic Press.

Pearson, T. W., Roelants, G. E., Lundin, L. B., and Mayor-Withey, K. S. 1979. The bovine lymphoid system: binding and stimulation of peripheral blood lymphocytes by lectins. Journal of Immunological Methods, 26, 271-282.

Perticarari, S., Presani, G., Mangiarotti, M. A., and Banfi, E. 1991. Simultaneous flow cytometric method to measure phagocytosis and oxidative products by neutrophils. Cytometry, 12, 687-693.

Perticarari, S., Presani, G., and Banfi, E. 1994. A new flow cytometric assay for the evaluation of phagocytosis and the oxidative burst in whole-blood. Journal of Immunological Methods, 170, 117-124.

Picker, L. J. 1994. Control of lymphocyte homing. Current Opinion in Immunology, 6, 394-406.

Pinto, M., Torten, M., and Birnbaum, S. C. 1978. Suppression of the *in vivo* humoral and cellular immune response by Staphylococcal enterotoxin B (SEB). Transplantation, 25, 320-323.

Platt, D. J. and Sullivan, L. Molecular Epidemiology: Molmatch and Beyond. International Labmate XVI Issue VII . 1992.

Platt, D. J., Candlish, D., Brown, A., Gillies, E., and Edwards, G. 1995. Strategic application of molecular methods in outbreak investigation. Medical Microbiology, 41, 271.

Plikaytis, B. D., Carlone, G. M., Edmonds, P., and Mayer, L. W. 1986. Robust estimation of standard curves for protein molecular-weight and linear-duplex DNA base-pair number after gel-electrophoresis. Analytical Biochemistry, 152, 346-364.

Ploegh, H. L., ORR, H. T., and Strominger, J. L. 1981. Major histocompatibility antigens - the human (*HLA-A, HLA-B, HLA-C*) and murine (*H-2K, H-2D*) class I molecules. Cell, 24, 287-299.

Poulin, R. 1996. Helminth growth in vertebrate hosts: does host sex matter? International Journal of Parasitology, 26, 1311-1315.

Pouplard, L., Losson, B., Detry, M., and Hollanders, W. 1990. Les gales bovines. Ann.med.vet, 134, 531-539.

Prescott, L. M., Harley, J. P., Klein, D. A. 1990. Microbiology. William C. Brown.

Quade, M. J. and Roth, J. A. 1999. Dual-color flow cytometric analysis of phenotype, activation marker expression, and proliferation of mitogen-stimulated bovine lymphocyte subsets. Veterinary Immunology and Immunopathology, 67, 33-45.

Rabinovitch, M. 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. Experimental Cell Research, 46, 19-28.

Radostits, O. M., Blood, D. C., Gay, C. C. 1994. Veterinary Medicine: A textbook of the diseases of cattle, sheep, pigs, goats and horses. Balliere Tindall.

Reinherz, E. L. and Schlossman, S. F. 1981. Derivation of human T-cell leukemias. Cancer Research, 41, 4767-4770.

Reiter, B. 1978. Review of the progress of dairy science: antimicrobial systems in milk. Journal of Dairy Research, 45, 131-147.

Roberts, C. W., Cruickshank, S. M., and Alexander, J. 1995. Sex-determined resistance to *Toxoplasma gondii* is associated with temporal differences in cytokine production. Infection and Immunity, 63, 2549-2555.

Rogers, G. W., Hargrove, G. L., Lawlor, T. J., and Ebersole, J. L. 1991. Correlations among linear type traits and somatic-cell counts. Journal of Dairy Science, 74, 1087-1091.

Rogers, P. R., Dubey, C., and Swain, S. L. 2000. Qualitative changes accompany memory T cell generation: Faster, more effective responses at lower doses of antigen. Journal of Immunology, 164, 2338-2346.

Roitt, I., Brostoff, J., Male, D. 1993. Immunology. Mosby-Year book Europe Limited.

Rollins, B. J. 1997. Chemokines. Blood, 90, 909-928.

Rosen, H. and Klebanoff, S. J. 1979. Bactericidal activity of a superoxide anion-generating system. A model for the polymorphonuclear leukocyte. Journal of Experimental Medicine, 149, 27-39.

Roth, J. A. and Kaeberle, M. L. 1981. Isolation of neutrophils and eosinophils from the peripheral-blood of cattle and comparison of their functional activities. Journal of Immunological Methods, 45, 153-164.

Roth, M. D. 1994. Interleukin 2 induces the expression of CD45RO and the memory phenotype by CD45RA+ peripheral blood lymphocytes. Journal of Experimental Medicine, 179, 857-864.

Ruff, G. and Lazary, S. 1988. Evidence for linkage between the caprine leucocyte antigen (*CLA*) system and susceptibility to CAE virus-induced arthritis in goats. Immunogenetics, 28, 303-309.

Russell, G. C., Fraser, D. C., Craigmile, S., Oliver, R. A., Dutia, B. M., and Glass, E. J. 2000. Sequence and transfection of *BoLA-DRB3* cDNAs. Animal Genetics, 31, 219-222.

Ruuls, S. R. and Sedgwick, J. D. 1999. Unlinking tumor necrosis factor biology from the major histocompatibility complex: Lessons from human genetics and animal models. American Journal of Human Genetics, 65, 294-301.

Saalmuller, A. 1998. Antigen-specific immune response of porcine T lymphocytes to various pathogens. Revue Scientifique et Technique de l Office International des Epizooties, 17, 71-83.

Sandholm, M. and Mattila, T. 1986. Biochemical aspects of bovine mastitis. Israel Journal of Veterinary Medicine, 42, 4, 405-415.

Sakans, T., Steinberg, A. D., and Green, I. 1978. Studies on immune functions of patients with systemic lupus erythematosus. I. Dysfunction of suppressor T cell activity related to impaired generation of, rather than response to, suppressor cells. Arthritis Rheumatology, 21, 657-667.

Salgar, S. K., Paape, M. J., Alstonmills, B., and Miller, R. H. 1991. Flow cytometric study of oxidative burst activity in bovine neutrophils. American Journal of Veterinary Research, 1201-1207.

Salgar, S. K., Paape, M. J., Alstonmills, B., and Peters, R. R. 1994. Modulation of bovine neutrophil functions by monoclonal-antibodies. American Journal of Veterinary Research, 227-233.

Samlowski, W. E., Robertson, B. A., Draper, B. K., Prystas, E., and McGregor, J. R. 1991. Effects of supravital fluorochromes used to analyze the *in vivo* homing of murine lymphocytes on cellular function. Journal of Immunological Methods, 144, 101-115.

Santos, J. L., Montes, M. J., Gutierrez, F., and Ruiz, C. 1995. Evaluation of phagocytic capacity with a modified flow-cytometry technique. Immunology Letters, 45, 1-4.

Sawhney, S. M. S., Taylor, D. W., and Russell, G. C. 2001. Polymorphism of bovine major histocompatibility complex (MHC) class I genes revealed by polymerase chain reaction (PCR) and restriction enzyme analysis. Animal Genetics, 32, 27-31.

Schalm, O. W., Lasmanis, J., and Jain, N. C. 1976. Conversion of chronic Staphylococcal mastitis to acute gangrenous mastitis after neutropenia in blood and bone marrow produced by an equine anti-bovine leukocyte serum. American Journal of Veterinary Research, 37, 885-890.

Schleifer, S. J., Keller, S. E., Meyerson, A. T., Raskin, M. J., Davis, K. L., and Stein, M. 1984. Lymphocyte function in major depressive disorder. Archives of Genetics and Psychiatry, 41, 484-486.

Schore, C. E., Osburn, B. I., Jasper, D. E., and Tyler, D. E. 1981. B-lymphocytes and T-lymphocytes in the bovine mammary gland rosette. Veterinary Immunology and Immunopathology, 2, 561-569.

Schukken, Y. H., Mallard, B. A., Dekkers, J. C., Leslie, K. E., and Stear, M. J. 1994. Genetic impact on the risk of intramammary infection following *Staphylococcus aureus* challenge. Journal of Dairy Science, 77, 639-647.

Schultz, R. D., Dunne, H. W., and Heist, C. E. 1971. Breed differences in the immune response of newborn calves. American Journal of Veterinary Research, 32, 1337-1341.

Scribner, D. J. and Fahrney, D. 1976. Neutrophil receptors for IgG and complement: their roles in the attachment and ingestion phases of phagocytosis. Journal of Immunology, 116, 892-897.

ShaferWeaver, K. A. and Sordillo, L. M. 1997. Bovine CD8(+) suppressor lymphocytes alter immune responsiveness during the postpartum period. Veterinary Immunology and Immunopathology, 56, 53-64.

Shapiro, H. M. 1998. Practical Flow Cytometry. Wiley-Liss.

Sharif, S., Mallard, B. A., Wilkie, B. N., Sargeant, J. M., Scott, H. M., Dekkers, J. C. M., and Leslie, K. E. 1998. Associations of the bovine major histocompatibility complex *DRB3* (*BoLA-DRB3*) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Animal Genetics, 29, 185-193.

Shook, G. E. 1989. Selection for disease resistance. Journal of Dairy Science, 72, 1349-1362.

Shortman, K. and Caux, C. 1997. Dendritic cell development: Multiple pathways to nature's adjuvants. Stem Cells, 15, 409-419.

Shuster, D. E., Kehrli, M. E., Ackermann, M. R., and Gilbert, R. O. 1992. identification and prevalence of a genetic-defect that causes leukocyte adhesion deficiency in Holstein cattle. Proceedings of the National Academy of Sciences of the United States of America, 89, 9225-9229.

Simm, G. 1998. Genetic improvement of cattle and sheep. Farming Press Miller Freeman UK Limited.

Slanetz, L. W., Bartley, C. H. and Allen, F. E. 1963. Vaccination of dairy cattle against Staphylococcal mastitis. American Journal of Veterinary Research, 24, 102, 923-931.

Slifka, M. K. and Whitton, J. L. 2000. Antigen-specific regulation of T cell-mediated cytokine production. Immunity, 12, 451-457.

Smith, G. M., Laster, D. B., and Gregory, K. E. 1976. Characterization of biological types of cattle I. Dystocia and preweaning growth. Journal of Animal Science, 43, 27-36.

Smits, E., Burvenich, C., and Heyneman, R. 1997. Simultaneous flow cytometric measurement of phagocytotic and oxidative burst activity of polymorphonuclear leukocytes in whole bovine blood. Veterinary Immunology and Immunopathology, 56, 259-269.

Smits, E., Burvenich, C., Guidry, A. J., and Roets, E. 1998. *In vitro* expression of adhesion receptors and diapedesis by polymorphonuclear neutrophils during experimentally induced *Streptococcus uberis* mastitis. Infection and Immunity, 66, 2529-2534.

Smits, E., Burvenich, C., Guidry, A. J., and Massart-Leen, A. 2000. Adhesion receptor CD11b/CD18 contributes to neutrophil diapedesis across the bovine blood-milk barrier. Veterinary Immunology and Immunopathology, 73, 255-265.

Snell, G. D. 1958. Histocompatibility genes in the mouse. II. Production and analysis of isogenic resistant lines. Journal of the national cancer institute, 21, 843-877.

Snustad, D. P., Simmons, M. J. 2000. Principle of Genetics. Wiley Incorporated, Library of Congress Cataloging-in-Publication Data.

Soltys, J., Swain, S. D., Sipes, K. M., Nelson, L. K., Hanson, A. J., Kantele, J. M., Jutila, M. A., and Quinn, M. T. 1999. Isolation of bovine neutrophils with biomagnetic beads: comparison with standard Percoll density gradient isolation methods. Journal of Immunological Methods, 226, 71-84.

Sorachi, K., Kumagai, S., Sugita, M., Yodoi, J., and Imura, H. 1993. Enhancing effect of 17 beta-estradiol on human NK cell activity. Immunology Letters, 36, 31-35.

Spooner, R. L. and Morgan, A. L. G. 1981. Analysis of *BoLA* W6 evidence for multiple subgroups. Tissue Antigens, 18, 178-188.

Spriggs, M. K. and Sher, A. 1999. New lessons for immunology at the host-pathogen interface. Current Opinion in Immunology, 11, 363-364.

Springer, T. A. 1990. Adhesion receptors of the immune-system. Nature, 346, 425-434.

Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration - the multistep paradigm. Cell, 76, 301-314.

Stadler, B. M., Kristensen, F., and de Weck, A. L. 1980. Thymocyte activation by cytokines: direct assessment of G0-G1 transition by flow-cytometry. Cellular Immunology, 55, 436-443.

Stear, M. J., Dimmock, C. K., Newman, M. J., and Nicholas, F. W. 1988. BoLA antigens are associated with increased frequency of persistent lymphocytosis in bovine leukemia-virus infected cattle and with increased incidence of antibodies to bovine leukemia-virus. Animal Genetics, 19, 151-158.

Stear, M. J., Mallard, B. A., Newman, M. J., and Wilkie, B. N. 1989. The current status of major histocompatibility system definition in cattle, goats, horses, pigs and sheep. International Journal of Animal Science, 4, 32-44.

Stear, M. J., Bishop, S. C., Doligalska, M., Duncan, J. L., Holmes, P. H., Irvine, J., McCririe, L., McKellar, Q. A., Sinski, E., and Murray, M. 1995. Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*. Parasite Immunology, 17, 643-652.

Stefanski, V. 2001. Social rearing conditions before weaning influence numbers and proportions of blood immune cells in laboratory rats. Developmental Psychobiology, 39, 46-52.

Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. Annual Review of Immunology, 9, 271-296.

Stendahl, O., Normann, B., and Edebo, L. 1979. Influence of O and K antigens on the surface properties of *Escherichia coli* in relation to phagocytosis. Acta Pathologica Microbiologica Scandinavica [B], 87, 85-91.

Stern, L. J. and Wiley, D. C. 1994. Antigenic peptide binding by class-I and class-II histocompatibility proteins. Structure, 2, 245-251.

Strasser, A., Teltscher, A., May, B., Sanders, C., and Niedermuller, H. 2000. Age-associated changes in the immune system of German shepherd dogs. Journal of Veterinary Medicine Series A Physiology and Pathology of Clinical Medicine, 47, 181-192.

Sullivan, D. A. and Wira, C. R. 1979. Sex hormone and glucocorticoid receptors in the Bursa of Fabricius of immature chicks. Journal of Immunology, 122, 2617-2623.

Sutra, L. and Poutrel, B. 1993. Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. Journal of Medical Microbiology, 40 79-89.

Tanaka, Y., Morita, C. T., Tanaka, Y., Nieves, E., Brenner, M. B., and Bloom, B. R. 1995. Natural and synthetic nonpeptide antigens recognized by human gamma-delta T-cells. Nature, 375, 155-158.

Tarrant, P. V., Kenny, F. J., Harrington, D., and Murphy, M. 1992. Long distance transportation of steers to slaughter:effect of stocking density on physiology, behaviour and carcass quality. Livestock Production Science, 30, 223-238.

Taylor, B. C., Choi, K. Y., Scibienski, R. J., Moore, P. F., and Stott, J. L. 1993. differential expression of bovine MHC Class-II antigens identified by monoclonal-antibodies. Journal of Leukocyte Biology, 53, 479-489.

Terry, L. A., Brown, M. H., and Beverley, P. C. L. 1988. The monoclonal-antibody, UCHL1, recognizes a 180,000 MW component of the human leukocyte-common antigen, CD45. Immunology, 64, 331-336.

Thomas, Y., Sosman, J., Rogozinski, L., Irigoyen, O., Kung, P. C., Goldstein, G., and Chess, L. 1981. Functional analysis of human T cell subsets defined by monoclonal

antibodies. III. Regulation of helper factor production by T cell subsets. Journal of Immunology, 126, 1948-1951.

Tizard, I. R. 2000. Veterinary immunology: an introduction. W.B. Saunders.

Todd, J. A., Bell, J. I., and McDevitt, H. O. 1987. HLA-DQ-Beta gene contributes to susceptibility and resistance to insulin-dependent diabetes-mellitus. Nature, 329, 599-604.

Trinchieri, G. 1989. Biology of natural-killer cells. Advances in Immunology, 47, 187-376.

Trinkle, L. S., Wellhausen, S. R., and Mcleish, K. R. 1987. A simultaneous flow cytometric measurement of neutrophil phagocytosis and oxidative burst in whole-blood. Diagnostic and Clinical Immunology, 5, 62-68.

Trnka, Z. and Cahill, R. N. 1980. Aspects of the immune response in single lymph nodes. Monographs in Allergy, 16, 245-259.

Tsukasaki, K., Miller, C. W., Kubota, T., Takeuchi, S., Fujimoto, T., Ikeda, S., Tomonaga, M., and Koeffler, H. P. 2001. Tumor necrosis factor alpha polymorphism associated with increased susceptibility to development of adult T-cell leukemia/lymphoma in human T-lymphotropic virus type 1 carriers. Cancer Research, 61, 3770-3774.

Unanue, E. R. and Allen, P. M. 1987. Antigen processing. Journal of Cellular Biochemistry, 210-210.

Vaiman, M., Chardon, P., and Rothschild, M. F. 1998. Porcine major histocompatibility complex. Revue Scientifique et Technique de l Office International des Epizooties, 17, 95-107.

Vandenbroucke-graulis, C. M. J. E., Thijssen, H. M. W. M., and Verhoef, J. 1984. Interaction between human polymorphonuclear leukocytes and *Staphylococcus-aureus* in the presence and absence of opsonins. Immunology, 52, 427-435.

Van Eijk, M. J. T., Russ, I., Beever, J. E., and Lewin, H. A. 1992. Polymorphism in exon-2 of the bovine lymphocyte antigen (BoLA)- *DYA* gene. Animal Genetics, 23, 476-476.

Van Eijk, M. J. T., Stewarthaynes, J. A., Beever, J. E., Fernando, R. L., and Lewin, H. A. 1992. Development of persistent lymphocytosis in cattle is closely associated with *DRB2*. Immunogenetics, 37, 64-68.

Van Eijk, M. J. T., Stewarthaynes, J. A., and Lewin, H. A. 1992. Extensive polymorphism of the BoLA-*DRB3* gene distinguished by PCR-RFLP. Animal Genetics, 23, 483-496.

Van Furth, R. 1970. Origin and kinetics of monocytes and macrophages. Seminars in Haematology, 7, 125-141.

Van Furth, R., Diesselhoff-Den Dulk, M. C., and Mattie, H. 1973. Quantitative study on the production and kinetics of mononuclear phagocytes during an acute inflammatory reaction. Journal of Experimental Medicine, 138, 1314-1330.

Van Furth, R., van Zwet, T., Leijh, P. C. J. 1978. In Handbook of Experimental Immunology

Van Lierop, M. J., van Noort, J. M., Wagenaar, J. P., Rutten, V. P., Langeveld, J., Meloen, R. H., and Hensen, E. J. 1994. T cell-stimulatory fragments of foot-and-mouth disease virus released by mild treatment with cathepsin Diagnostic Journal of Genetics and Virology, 75, 2937-2946.

Van Oss, C. J. and Gillman, C. F. 1972. Phagocytosis as a surface phenomenon. II. Contact angles and phagocytosis of encapsulated bacteria before and after opsonisation

by specific antiserum and complement. Journal of the Reticuloendothelial Society, 12, 497-508.

Van Neerven, J., Coligan, J. E., and Koning, F. 1990. Structural comparison of alpha-beta and gamma-delta T-cell receptor-CD3 complexes reveals identical subunit interactions but distinct cross-linking patterns of T-cell receptor chains. European Journal of Immunology, 20, 2105-2111.

Van Snick, J. 1990. Interleukin-6 - an overview. Annual Review of Immunology, 8, 253-278.

Viuff, B., Ostergard, H., Aasted, B., and Kristensen, B. 1991. One-dimensional isoelectric-focusing and immunoblotting of bovine major histocompatibility complex (BoLA) class-I molecules and correlation with class-I serology. Animal Genetics, 22, 147-154.

Vogler, G. P., McClearn, G. E., Snieder, H., Boomsma, D. I., Palmer, R., deKnijff, P., and Slagboom, P. E. 1997. Genetics and behavioral medicine: Risk factors for cardiovascular disease. Behavioral Medicine, 22, 141-149.

Volkman, A. 1970. The origin and fate of the monocyte. Ser.Haematol., 3, 62-92.

Von Boehmer, H. and Kisielow, P. 1990. Self-nonself discrimination by T cells. Science, 248, 1369-1373.

Wakelin, D., Rose, M. E., Hesketh, P., Else, K. J., and Grencis, R. K. 1993. Immunity to coccidiosis: genetic influences on lymphocyte and cytokine responses to infection with *Eimeria vermiformis* in inbred mice. Parasite Immunology, 15, 11-19.

Washington, E. A., Kimpton, W. G., and Cahill, R. N. P. 1992. Changes in the distribution of alpha-beta and gamma-delta T- cells in blood and in lymph-nodes from fetal and postnatal lambs. Developmental and Comparative Immunology, 16, 493-501.

Webster, J. 1993. Understanding the dairy cow. Second Edition, Blackwell Scientific Publications.

Weigel, K. A., Freeman, A. E., Kehrli, M. E., Stear, M. J., and Kelley, D. H. 1990. Association of Class-I bovine lymphocyte antigen complex alleles with health and production traits in dairy-cattle. Journal of Dairy Science, 73, 2538-2546.

Weigel, K. A., Kehrli, M. E., Freeman, A. E., Thurston, J. R., Stear, M. J., and Kelley, D. H. 1991. Association of Class-I bovine lymphocyte antigen complex alleles with *in vitro* blood neutrophil functions, lymphocyte blastogenesis, serum complement and conglutinin levels in dairy cattle. Veterinary Immunology and Immunopathology, 27, 321-335.

Weigel, K. A., Freeman, A. E., Kehrli, M. E., Thurston, J. R., and Kelley, D. H. 1992. Relationship of *in vitro* immune function with health and production in Holstein cattle. Journal of Dairy Science, 75, 1672-1679.

Weigent, D. A. and Blalock, J. E. 1990. Immunoreactive growth hormone-releasing hormone in rat leukocytes. Journal of Neuroimmunology., 29, 1-13.

Weiss, W. P., Hogan, J. S., Smith, K. L., and Hoblet, K. H. 1990. Relationships among selenium, vitamin E, and mammary gland health in commercial dairy herds. Journal of Dairy Science, 73, 381-390.

Westaway, D., Zuliani, V., Cooper, C. M., Da Costa, M., Neuman, S., Jenny, A. L., Detwiler, L., and Prusiner, S. B. 1994. Homozygosity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie. Genes and Development, 8, 959-969.

Whitcomen, C., Alexander, J. W., Sramkoski, R. M., and Babcock, G. F. 1992. Rapid whole-blood microassay using flow-cytometry for measuring neutrophil phagocytosis. Journal of Clinical Microbiology, 30, 2071-2076.

Whittard, J. D. and Akiyama, S. K. 2001. Activation of beta (1) integrins induces cell-cell adhesion. Experimental Cell Research, 263, 65-76.

Wikby, A., Maxson, P., Olsson, J., Johansson, B., and Ferguson, F. G. 1998. Changes in CD8 and CD4 lymphocyte subsets, T cell proliferation responses and non-survival in the very old: the Swedish longitudinal OCTO-immune study. Mechanisms of Ageing and Development, 102, 187-198.

Williams, M. R. and Bunch, K. J. 1981. Variation among cows in the ability of their blood polymorphonuclear leucocytes to kill *Escherichia coli* and *Staphylococcus aureus*. Research in Veterinary Science, 30, 298-302.

Williams, M. R., Hibbitt, K. G., Field, T. R., and Bunch, K. J. 1984. Further-studies on the variation among cows, bulls and calves in the ability of their blood polymorphonuclear leukocytes to kill *Staphylococcus-aureus*. British Veterinary Journal, 307-313.

Wilson, C. D. and Richards, M. S. 1980. A survey of mastitis in the British dairy herd. Veterinary Record, 106, 431-435.

Winkler, G. C. 1988. Pulmonary intravascular macrophages in domestic-animal species - review of structural and functional-properties. American Journal of Anatomy, 181, 217-234.

Wise, T. and Klindt, J. 1995. Thymic weight changes and endocrine relationships during maturation in cattle: effects of age, sex, and castration. Growth Development and Ageing, 59, 139-148.

Wood, W. B., Smith, M. R., and Watson, B. 1946. Studies on the mechanism of recovery in pneumococcal pneumonia. IV. The mechanism of phagocytosis in the absence of antibody. Journal of Experimental Medicine, 84, 387-395.

Wright, A. E. and Douglas, S. R. 1903. An experimental investigation of the role of the blood fluids in connection with phagocytosis. Proceeding of the Royal Society, 357-363.

Wyatt, C. R., Madruga, C., Cluff, C., Parish, S., Hamilton, M. J., Goff, W., and Davis, W. C. 1994. Differential distribution of gamma-delta-T-cell receptor lymphocyte subpopulations in blood and spleen of young and adult cattle. Veterinary Immunology and Immunopathology, 40, 187-199.

Xu, A. L., Van Eijk, M. J. T., Park, C., and Lewin, H. A. 1993. Polymorphism in *BOLA-DRB3* Exon-2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia-virus. Journal of Immunology, 151, 6977-6985.

Yellon, S. M., Fagouga, O. R., and Nehlsen-Cannarella, S. L. 1999. Influence of photoperiod on immune cell functions in the male Siberian hamster. American Journal of Physiology, 276, 97-102.

Yokomizo, Y., Mori, Y., Shimoji, Y., Shimizu, S., Sentsui, H., Kodama, M., and Igarashi, H. 1995. Proliferative response and cytokine production of bovine peripheral-blood mononuclear-cells induced by the superantigens staphylococcal enterotoxins and toxic shock syndrome toxin-1. Journal of Veterinary Medical Science, 299-305.

Young, F. J., Platt, D. J., Logue, D., Ternent, H., and Fitzpatrick, J. L. 2001. Bovine *Staphylcoccus aureus* mastitis: strain recognition and dynamic of infection. Journal of Dairy Research, 68, 377-387.

Young, F. J. Infection and inflammation in bovine *Staphylcoccus aureus* mastitis. 1997 University of Glasgow, M.Sc. (Vet. Sci.).

Yu, P. W., Schuler, L. A., Rejman, J. J., Chen, H. T., Gosink, K., Kehrli, M., Mattocks, L., Nonnecke, B., and Czuprynski, C. J. 1998. Regulation of bovine interleukin-1 receptors. Veterinary Immunology and Immunopathology, 63, 21-25.

Zhang, Q., Boichard, D., Hoeschele, I., Ernst, C., Eggen, N, A., Murkve, B., Pfister-Genskow, M., Witte, L. A., Grignola, F. E., Uimari, P., Thaller, G., and Bishop, M. D. 1998. Mapping quantitative trait loci for milk production and health of dairy cattle in a large outbred pedigree. Genetics, 149, 1959-1973.

Zlotnik, A. and Yoshie, O. 2000. Chemokines: A new classification system and their role in immunity. Immunity, 12, 121-127.

Zuk, M. and McKean, K. A. 1996. Sex differences in parasite infections: patterns and processes. International Journal of Parasitology, 26, 1009-1023.