Linking phenotype to genotype: a study of antibody responses to bovine respiratory virus vaccines in a crossbred cattle population.

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Abstract

Vaccination is central to prevention of infectious disease in modern livestock production systems. Accurate assessment of the many factors controlling immune responses to vaccination is crucial to maximising their effectiveness. Identification of the genetic component of those immune responses is a prelude to selection for disease resistance. Assignment of those effects at a molecular level should lead to a more complete understanding of the complex processes underlying immunity.

The overall aim of this study was to evaluate the IgG antibody responses induced by vaccination in a juvenile, fully pedigreed bovine population of Holstein-Charolais crossbred animals. Vaccines against bovine respiratory syncytial virus (BRSV), bovine parainfluenza 3 virus (PIV3) and bovine herpesvirus 1 (BHV1) were administered to 463 calves, aged 60 to 167 days old. Antibody responses to field infections against bovine coronavirus (BCV) were also examined. The experimental population comprised second-cross Holstein-Charolais F2, Holstein-backcross and Charolais-backcross animals with a dairy-type calf-rearing scheme employed for females and a beef-type calf-rearing method for males.

All datasets were extensively investigated using Residual Maximum Likelihood (REML) procedures. Across the population, there was a broad spectrum of antibody responses to vaccination. Mean antibody half-lifes were estimated as ~24 days for total BRSV-IgG, ~72 days for BRSV-IgG_2, ~32 days for total PIV3-IgG and ~26 days for total BHV1-IgG. Although PIV3 and BHV1 are both components of a multivalent intranasal vaccine, the BHV1-IgG response was poor while the PIV3-IgG response was substantial. Sex, year-of-birth, cross-breeding, animal age, and levels of pre-existing antibody were significant sources of variation for levels of IgG antibodies against all viruses in this population. Although the effect is confounded by management, female calves had higher levels of BRSV-IgG and PIV3-IgG. Despite year-of-birth effect, including seasonality in climate, environment, wild-type infections and management, it accounted for a lower order of the observed variation than sex. Pre-vaccination, calves from dams with higher percentages of the Holstein breed had higher levels of BRSV-IgG and PIV3-IgG. Dam-age was also positively correlated with pre-vaccination levels of BRSV-IgG, the effect plateauing at about 4 years old. Although calf-age was a significant pre-vaccination determinant, its effect on post-vaccination levels of IgG was much less pronounced. The inhibitory effect of pre-existing BRSV-IgG and PIV3-IgG on vaccine response was analysed using logistic regression and respective relative optical density thresholds of ~26% and ~35% established. Pre-vaccination IgG above these levels meant a positive antibody response was less likely than a negative one.

Pre-vaccination, maternal heritable effects dominated total phenotypic variance but post-vaccination, additive heritable effects became more important. Estimates of heritability (±se) peaked at 0.29 (±0.17) for total BRSV-IgG (Day 35); 0.52 (±0.26) for the deviation in BRSV-IgG levels between Day 0 and Day 35; 0.47 (±0.28; Day 28) and 0.51 (±0.31; Day 42) for total PIV3-IgG in the male and female calves, respectively. Much lower values were recorded for BHV1 and BCV IgG responses.

All calves underwent genotyping and were mapped for 139 microsatellite markers with an average marker interval of 15.8 centiMorgans (cM). Interval analysis for quantitative trait loci (QTL) using the above antibody phenotypes revealed a large number of loci (positions in cM) influencing levels of IgG. Quantitative trait loci on BTA1 (59 cM), BTA2 (108 cM), BTA7 (29 cM), BTA8 (41 cM), BTA10 (55 cM), BTA12 (84 cM), BTA24 (44 cM) and BTA28 (2 cM) proved significant for one or more of the BRSV-, PIV3- and BHV1-IgG antibody traits.

Although the QTL for antibody phenotype identified here are preliminary, they could plausibly be incorporated into breeding programmes to influence antibody response to vaccination. Further, they could act as a basis for further QTL research in this area leading to precise isolation of the relevant genes involved, to a molecular level. However most of all, these results prove that antibody production in cattle is under quantitative genetic control and this influential element should not be disregarded in the future.

Table of Contents

List of tables

List of Figures

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Dedication

To Fiona and baby Euan Conor, and to Mick and Alice.

Declaration

Apart from the assistance acknowledged, I declare that the work described here 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.

Ronan G. O'Neill, April 2006

Chapter 1

General Literature Review

1.1 General Introduction

Infectious disease among domestic animals is currently controlled using many methods including eradication, quarantine, disinfection, sanitation, medication and vaccination (Detilleux *et al.*, 1994). Control is imperfect, and real or perceived disadvantages of the existing systems include: the high cost of replacing culled animals, increasing public concerns about animal welfare, side-effects and withdrawal times of drugs, development of antimicrobial drug resistance, environmental damage due to chemical residues, poor performance in preventing clinical disease, and ever escalating difficulties and costs in the development of new medication (Rauw *et al.*, 1998). A more sustainable approach would be to optimise host immunity, removing or diminishing many of these difficulties and reducing the need for more invasive methods (Lofthouse and Kemp, 2002).

Present levels of disease resistance among farm animals are a product of natural selection/survival and those immune responses which fit with production aims (Visscher *et al.*, 2002). Reduction of disease by improving natural immunity would have cumulative and permanent effects, through successive generations. Disease transmission should drop as more resistant animals persist, resulting in lower pathogenic contamination of the environment (MacKenzie and Bishop, 1999). Furthermore improvements in genetic resistance-based disease status, could lower susceptibility to other pathogens and ultimately would lead to improvement in general health and welfare (Simm *et al.*, 1996). Additionally, an adequate and competent natural resistance should reduce opportunities for pathogens to evolve and overcome the host immune response (Bishop, 2003).

Resistance to infectious disease is likely to be under polygenic control (Wilkie and Mallard, 1999) with the involvement and regulation of both innate and acquired immune systems (Malo and Skamene, 1994). Even for immune mechanisms expressed by a single genetic locus, other loci will nonetheless modulate and regulate the resistance (Adams and Templeton, 1998). Development of disease is confounded by so many additional factors to genetic

resistance, that heritabilities tend to be significant but low (Soller and Andersson, 1998).

Direct selection based on observation of resistance and susceptibility to specific infections is not feasible on a commercial scale (Wilkie and Mallard, 1998). Indirect selection criteria may be used, based on morphological, physiological and immunological characteristics. Surrogate indicator traits such as serum antibody responses, *in vitro* cellular bioassays, and similar correlates of protection could be used to quantify immune reactions which could then be linked to genetic markers and eventually to the relevant immune system genes (Detilleux *et al.*, 1994).

By more closely reflecting protection, this approach may be used effectively to advance beneficial traits without minute knowledge of gene function. Such selection may be achieved by incremental quantitative improvements in resistance, without major changes in mechanisms of overall action (Gavora, 1996). Thus this approach is exposed to less detrimental effects than other "genetic modification" techniques, and lends itself for use in conventional breeding programmes (Shook, 1989).

1.2 The Immune System

Living animals make a very attractive resource to microorganisms, to the extent that resistance to infection is paramount to any animal's survival. Multicellular organisms must be able to eliminate infectious agents capable of causing disease, or inhibit the replication of pathogens, once the host has been invaded (Tizard, 2000). This is a dynamic process with pathogen gene frequencies coevolving and tracking those genes involved in host defences (Dybdahl and Storfer, 2005). To deal with a highly variable range of pathogens, from viruses to nematodes, the immune system has evolved multiple, intricate defence mechanisms (Engelhard, 1994). Mammals possess two highly integrated defence systems, the innate and the acquired immune systems which cooperate and interact extensively, depending on the pathogen and its route of entry (Kaufmann and Kabelitz, 1998). Innate immune responses act as limited but rapid responses to infection, without prior exposure to the pathogen (Parham, 2004), whereas the acquired immune

system relies on direction from the innate immune system, focuses on specific pathogen epitopes, and leads to cellular memory (Bachmann and Kopf, 1999).

Pathogens, themselves, can alter immune function. The likelihood of secondary bacterial pneumonia is greatly increased by the lethal effect of bovine herpesvirus 1 infection on the pulmonary epithelium, directly compromising ciliary activity in the respiratory tract (Babiuk *et al.*, 1988). Bovine viral diarrhoea virus (BVDV) infection produces a lymphopaenia in cattle with severe depletion of B-cell and T-cell populations throughout many lymphoid tissues. In addition, neutrophil functions are depressed and surviving B-cells are poor producers of antibodies (Radositis *et al.*, 2003). Bovine respiratory syncytial virus (BRSV) infection depresses lymphocyte blastogenesis in sheep (Keles *et al.*, 1999).

Furthermore, imbalances in immunity such as immunodeficiencies and autoimmune diseases stress the importance of the controlled production of the multitude of factors involved in a normally functioning immune system; any departure from this dynamic equilibrium increases susceptibility to disease (Klein and Horejsi, 1997).

Many of the various components of the immune system are peptides and glycoproteins coded for in the genome, although post-transcriptional and post-translational modifications mean the functional proteome greatly exceeds the genome. In the text that follows, where possible reference will be be made to the coding chromosome (*Bos taurus* autosome (BTA)) and the segment (q) or position on that BTA e.g. [BTA.. q..; published reference].

1.3 The innate immune system

Innate immunity acts as a sentinel system at the interface between the host and the environment. The innate immune system recognises pathogenic material via cellular pattern recognition receptors (PRR), typically found on macrophages and dendritic cells (Lazarus *et al.*, 2002). PRR are stimulated by pathogen-associated molecular patterns (PAMP), essentially conserved molecular constituents of microbial pathogens such as lipopolysacchride, peptidoglycan, lipoteichoic acid,

unmethylated DNA (CpG motif) as well as various endogenous molecules from dead or dying cells (Medzhitov and Janeway, Jr., 1997).

It is a key role of the innate immune system to trigger and channel an appropriate response from the acquired immune system. The cumulative action of many genes, structural, physiological and immunological, determine this phenotype, which is responsible for 95% of host defence. Although relative to the diversity of pathogens the number of innate immunity genes is small, they gain functional plasticity by high rates of polymorphism and heterodimerisation (Lazarus *et al.*, 2002). Due to their critical position in initialising and dictating the type of acquired immunity, any genetic variation could have a major impact on all downstream immune respones, including antibody production. Innate immunity also includes physical defences; for example, the lungs have developed innate defences to protect the airways from infection such as the cough reflex, mucociliary clearance, extensive patrolling populations of immune cells and antimicrobial properties of the mucosal surface (Strieter *et al.*, 2003).

1.3.1 Cell-mediated innate immunity

Although many subsets of leukocytes contribute to a complete immune response, the term "cell-mediated immunity" broadly describes any response in which antibody plays a subordinate role. Pluripotent hematopoietic stem cells in the bone marrow differentiate into lymphoid and myeloid stem cells, a further segregating development of the lymphoid stem cells producing the three major populations of mature lymphocytes: T-cells, B-cells and Natural Killer (NK) cells. Myeloid stem cells differentiate into granulocytes and other non-immune system blood cells (Chaplin, 2003). Cell-mediated immunity (CMI) is operated and coordinated primarily by T-cells (Janeway, Jr., 1994) but is not totally dependent on this cell-type (Bancroft *et al.*, 1991). By common convention, leukocyte cell surface molecules are named by assigning them a cluster of differentiation (CD) antigen number, dependent on the reactivity to a panel of monoclonal antibodies. Lymphocytes may have over 100,000 CD and they are extremely useful in determining cellular function.

1.3.1.1 Antigen presenting cells

Antigen processing serves to regulate the amount and type of antigen presented to the immune system allowing the development of a restricted and specific response. Dendritic cells appear to the most potent antigen presenting cells (APC) but B-cells, macrophages, epithelial Langerhans cells, hepatic Kupffer cells and nervous microglial cells are also important APC (Tizard, 2000). Antigen presenting cell involvement is essential for the active induction of helper T-cell function whereas APC interact with other cell types to modulate the homeostatic maintenance of an effective immune system (Glass and Spooner, 1989).

1.3.1.2 Dendritic cells

Dendritic cells (DC) are professional, myeloid-derived APC, distributed in small numbers throughout the body, but concentrated in the secondary lymphoid tissues where they are ideally placed for antigen uptake (Howard *et al.*, 1999). A number of phenotypic subsets have been distinguished with different functions, locations, structure, trafficking properties and levels of maturity (Shortman and Caux, 1997). Dendritic cells are highly efficient processors of antigen and form a widespread surveillance network, the local density of which depends on the likelihood of insult. Dendritic cells may be the only cells able to present antigen to naïve T-cells, thereby initiating the acquired immune response (Banchereau and Steinman, 1998). Furthermore, as part of a secondary immune response, follicular DCs trap circulating antigen-antibody complexes and process them into iccosomes (Tew *et al.*, 1997). These packaged antigen-antibody complexes are then ingested by B-cells, processed, and T-cell help elicited, greatly amplifying the resulting immune reaction (Bajer *et al.*, 2003). Dendritic cells are critical to the initation of many acquired immune responses, acting as an intelligent bridge between the innate and acquired immune systems.

1.3.1.3 Monocytes

Monocytes are physically the largest of all blood cells, but form only 5% of the total leukocyte population (Tizard, 2000). Monocytes leave the systemic circulation by diapedesis and develop into larger, more efficient and specialised phagocytes in the tissues: macrophages (Volkman, 1970).

1.3.1.4 Macrophages

Macrophages are long-lived, mononuclear phagocytes, capable of longer, repeated phagocytic activity than neutrophils (Klein and Horejsi, 1997). They kill ingested pathogens using nitric oxide and release many cytokines including interleukin (IL)-6, IL-10, IL-12 and IL-18, acting as regulators in acquired immune responses (Mosser and Karp, 1999). Pulmonary alveolar macrophages and pulmonary intravascular macrophages are specialist populations of macrophages found in cattle, the latter in intimate association with the endothelial cells of pulmonary capillaries (Ackermann and Brogden, 2000).

1.3.1.5 Neutrophils

Neutrophils have been described as the primary cellular killers of invading micro-organisms (Hasui *et al.*, 1989). Their major importance immunologically derives from their ability to sequester microbes and particles internally. The neutrophil is the major descendant of the myeloid lineage in all species, forming however a much lower proportion of total leukocytes in healthy ruminants (~25%) than other animals (~70%) (Carlson and Kaneko, 1973). Neutrophils play a major role in the clearance of microbial pathogens and repair of tissue injury (Chertov *et al.*, 2000).

A common form of bacterial bovine pneumonia, caused by *Mannheimia haemolytica*, is characterised by a profound influx of neutrophils into lung tissue (Caswell *et al.*, 1998). Neutrophil migration into the lung parenchyma occurs from capillaries, as opposed to post-capillary venules in most other tissues (Albertine *et al.*, 1993). Due to their large size, neutrophils must adopt an elongated shape to pass the lung capillaries (Gebb *et al.*, 1995). When activated, neutrophils become enlarged and inflexible which increases their pulmonary retention (Soethout *et al.*, 2002). Upregulation of the adherence molecules, integrins, is critical for neutrophil migration to the lung in cattle (Wagner and Roth, 2000), with bovine leukocyte adhesion deficiency (BLAD) affected cattle demonstrating atypical pneumonic pathology.

1.3.1.6 Toll-like receptors

Toll-like receptors (TLR) are one of the primary types of PRR expressed on the surface of many cells. Stimulation of TLR initiates the production of reactive oxygen and nitrogen intermediates and induces secretion of many pro-inflammatory cytokines and co-stimulatory molecules (Chaplin, 2003) to further amplify the innate immune response. In cattle, ten TLR have been identified with varying functions (McGuire *et al.*, 2005). Generally, the TLR trigger specific signalling pathways leading to pathogen-specific immune response serving as a crucial link between innate and acquired immunity through the entirety of the host immune response (Table 1.1).

TLR	Ligand	Bovine chromosome	Reference
TLR-1	G+ bact. lipoprotein, interacts with TLR2	BTA6	(McGuire *et al.*, 2005)
TLR-2	G± bact. lipoprotein	BTA17	(White *et al.*, 2003)
TLR-3	viral ssRNA, type 1 IFN	BTA27	(McGuire *et al.*, 2005) (Alexopoulou *et al.*, 2001)
TLR-4	G- bact. Lipopolysacchride hRSV F-protein	BTA8 distal	(White *et al.*, 2003) (Tal *et al.*, 2004)
TLR-5	flagellin	BTA16	(McGuire *et al.*, 2005)
TLR-6	lipoproteins, interacts with TLR2	BTA6	(McGuire *et al.*, 2005)
TLR-7	Imiquimol, resiquimol	X	(McGuire *et al.*, 2005)
TLR-8	sRNA	X	(McGuire *et al.*, 2005)
TLR-9	methylated CpG dinucleotides	BTA22	(McGuire *et al.*, 2005)
TLR-10	asthma in humans	BTA6	(McGuire *et al.*, 2005)

Figure 1.1 Toll-like receptors (TLR) identified in cattle. Natural ligands, genomic position and related references included. G± bact. (Gram positive / negative bacteria), sRNA (single stranded DNA), ssRNA (double stranded DNA), CpG (cytosine guanine DNA motifs)

1.3.2.1 Cytokines – innate immunity

Cytokines are proteins, active at picomolar concentrations, which control the complex interactions between cells of the immune system (Lunney, 1998). They have transient expression and tend to be limited to local action. There is a good deal of redundancy, ambiguity and pleiotropism in the action of most cytokines. Cytokine production is stimulated by a multitude of mechanisms: antigen or antigen-MHC aggregates acting via T-cell or B-cell receptors, antigen-antibody complexes acting through *Fc* receptors, superantigens acting through T-cell receptors or directly by T-cell interaction with microbial constituents such as lipopolysaccharides (Slifka and Whitton, 2000). Many cytokines such as interleukins (IL): IL-1 and IL-6; interferons (IFN): IFN-α and IFN-β and tumour necrosis factor alpha (TNF-α) are produced by and act on multiple cell types but some such as IL-2, IL-3, IL-4 and IL-5 are secreted by specialised subsets of cells (Carter and Swain, 1997).

Cytokines have a central role in orchestrating the normal maturation of the immune system and regulating defence against infectious disease (Fresno *et al.*, 1997). A series of pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, TNF-α and the type I interferons are synthesized *de novo* following bacterial or viral infection (Lunney, 1998). The broad effect of this is to stimulate the phagocytic cells (monocytes, macrophages, neutrophils and endothelial cells) to attach to or react against pathogens, and to attract other immune cells to the infection site. Some virus constituents can mimic mammalian cytokines, allowing them to suppress and evade the immune response (Kluczyk *et al.*, 2002).

1.3.2.2 Interferon

Interferons (IFN) are major factors in the successful clearance of viral and intracellular infections. There are 3 main types of IFN: IFN-α, IFN-β and IFN-γ, although the latter is unrelated to the former two and will discussed later. These molecules are generated by fibroblasts, T-cells, NK cells and macrophages and have wide ranging effects on many target cells (Boehm *et al.*, 1997). Interferon-α

and IFN-β [both BTA8; q15; (Adkison *et al.*, 1988)] activate macrophages and directly influence virus-infected cells to suppress viral growth.

1.3.2.3 Interleukin-1

Interleukin-1 (IL-1) is produced predominantly by mononuclear phagocytes following induction by bacteria, bacterial products and other soluble factors (Yu *et al.*, 1998). It is potently pro-inflammatory and critical to the mobilisation of the effector cells of the innate immune response (Murtaugh and Foss, 2002). Interleukin-1 [BTA11; (Yoo *et al.*, 1994)] induces pyrexia, initiates the acute phase response, activates many cell types including hepatocytes, osteoclasts and synovial cells, stimulates selectin expression on endothelial cells and is a co-factor for the proliferation and activation of lymphocytes (Dinarello, 1996).

1.3.2.4 Interleukin-12

Interleukin-12 (IL-12) is produced by many types of immune cells, including monocytes, macrophages, dendritic cells and B-cells. Interleukin-12 [BTA1 q34-q36; (Schmidt *et al.*, 2000)] enhances cell-mediated cytotoxicity and mitogenicity and has a pivotal role in directing the immune response to a Th1-type (Collins *et al.*, 1998). Two mediators p35 [BTA1; (Zarlenga *et al.*, 1995)] and p40 [BTA7 q23-q24; (Zarlenga *et al.*, 1995)] control the bioactivity of IL-12. Interleukin-12 also suppresses IL-4 synthesis, driving the immune reaction away from antibody production (Trinchieri, 2003).

1.3.2.5 Tumour Necrosis Factor–alpha

Tumour Necrosis Factor–alpha is expressed early in pneumonia (Mehrad *et al.*, 1999) and is involved in the recruitment and direction of phagocytic cells. Depletion of TNF-α [BTA23 q22; (Agaba *et al.*, 1996)] impairs the clearance of micro-organisms from the lungs (Mehrad *et al.*, 1999) while TNF-α also upregulates genes critical to the innate immune response.

1.3.2.6 Other mediators

There are many other cytokines, lymphokines, interferons and other chemical mediators forming a complex network of factors controlled by regulation of receptor expression, specific binding proteins and antagonistic effects from other cytokines. A host of mediators are involved in each immune response, allowing

it to adapt and evolve to best cope with attack from diverse pathogens (Table 1.1). The precise cytokine pathways which direct resistance to disease remain as yet unclear in larger vertebrates. Excessive production of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα have been associated with the pathogenic effects of Theileriosis and variability in their production linked to disease resistance (Glass *et al.*, 2005).

Mediator	Type	Chromosome	Reference
IL-2	Th1	BTA17 q22-q23;	(Chowdhary *et al.*, 1994)
IL-3	Treg	BTA7	(Mwangi *et al.*, 1995)
IL-6	Th1 (innate)	BTA4	(Barendse *et al.*, 1997)
IL-7	Treg	BTA14	(Barendse *et al.*, 1997)
IL-8	Th1 (innate)	BTA6	(Modi *et al.*, 1990)
IL-10	Th2 (adaptive)	BTA16 q12	(Beever *et al.*, 1997)
IL-13	Th2 (adaptive)	BTA7	(Buitkamp *et al.*, 1999)
IL-15	Treg	BTA17 q13-q14	(Sonstegard *et al.*, 2000)
IL-18	Th1 (innate)	BTA15 q13-q14	(Sonstegard *et al.*, 2000)
TGF-α	Treg	BTA5	(Kappes *et al.*, 1997)
TGF-β	Th2 (adaptive)	BTA18 q24-q25	(Sonstegard *et al.*, 2000)
LTA	Th1 (innate)	BTA23 q22	(Cludts *et al.*, 1993)
CSF -1, -2, -3	Treg	BTA3, 7, 19	(Zhang *et al.*, 1992)
PAX-8	tumours ?	BTA11	(Lopez-Corrales *et al.*, 1999)
PAX-6	tumours ?	BTA15 q25-q27	(Kuiper *et al.*, 2005)

Figure 1.1 Chemical mediators which act as signalling mechanism for mammalian immune system. Position on bovine genome and related references included. BTA (*Bos taurus* autosome). Type (Th1, Th2 and Treg (T regulatory)). Interleukin (IL), tumour growth factor (TGF), lymphotoxin alpha (LTA), colony stimulating factors (CSF), paired box protein (PAX)

1.3.2.7 Acute phase proteins

The acute phase proteins (APPs) are soluble factors released early in the innate immune response with a wide range of pathophysiological effects including pyrexia, leukocytosis, alterations in levels of hormones and serum trace elements and muscle protein depletion (Eckersall, 2000b). Stimulated by cytokines such as

IL-1, IL-6 and TNF-α, the APPs include C-reactive protein, serum amyloid A (SAA), haptoglobin (HP), fibrinogen, ceruloplasmin and α_1-anti protease (Eckersall, 2000a). In cattle, two hepatically derived APPs, SAA and HP, are the most important, the former mapping to BTA29 while the latter is coded for on BTA18 (Barendse *et al.*, 1997). Serum amyloid A is involved in leukocyte activation, chemotaxis and phagocytosis while HP acts as a haemoglobin scavenger (Eckersall, 2000a).

1.3.2.8 Complement

The complement system is a very powerful cascade of at least 30 serum proteins (Manderson *et al.*, 2001) and is a sequential, self-catalysing system that binds specific proteins to the surface of invading pathogens (Klein and Horejsi, 1997). Complement is involved in viral infections in many ways (Lachmann and Davies, 1997). Following virus invasion, complement proteins may directly coat virus particles, preventing further cellular infection or leading to virus lysis (Carroll, 1998). Complement can bind and lyse virus-infected cells (Sissons and Oldstone, 1980) and the specific anti-viral antibody response is enhanced by complement components (Fearon and Carter, 1995). A heritable deficiency of the bovine complement receptor, CR-3, has been described in association with BLAD; (Cox *et al.*, 1997)) and results in impaired phagocyte microbiocidal activity (Nagahata *et al.*, 1996). Other soluble mediators released at this phase include mannose-binding protein, serum amyloid protein and the alternative complement pathway proteins (Tizard, 2000). In Norwegian Red cattle, heritability for haemolytic complement activity was high, estimated at 0.75 (Lie *et al.*, 1983).

Non-specific innate immunity is expressed constitively and actively from genes regulating phagocyte uptake and killing, genes encoding cytokines, their receptors and the complement proteins, specific genes such as those coding for Natural Resistance Associated Macrophage Protein (NRAMP) active against intracellular pathogens and others (Rumyantsev, 1998). Almost every aspect of acquired immunity is triggered and governed by signals from the innate immune response, the progression of the former entirely dependent on direction from the latter. General innate immune responses often progress into specific acquired

immune response. These two branches of the immune system, although fundamentally different in their mechanisms, act together, their synergy being essential for a fully effective immune response. Each has strengths and weaknesses, a balance of both being desirable to optimise immune function when selecting for disease resistance (Campos and Godson, 2003).

1.4 The acquired immune system

Acquired immunity involves the induction of cellular and humoral responses directed against a specific antigen alongside the development of cellular memory, thereby permitting a more rapid secondary response on re-exposure to the antigen (Roitt, 2001). Acquired cell-mediated immunity is generally focussed on intracellular pathogens whereas acquired humoral immunity is typically directed against extracellular pathogens (Janeway, Jr., 1994). Unlike the "hard-wired" innate immunity, the acquired immune system relies on immense populations of clonal receptors, generated via somatic expansion during an individual's development, to mediate antigenic recognition (Lazarus *et al.*, 2002).

1.4.1 Cell-mediated acquired immunity

1.4.1.1 Lymphocytes

Lymphocytes play a central role in orchestrating the acquired immune response (Figure 1.1) and are essential in the specific recognition of pathogens (Roitt, 2001). Two of the three major populations of lymphocytes: T lymphocytes (T-cells) and B lymphocytes (B-cells) originate from lymphoid stem cells. T-cells undergo final maturation in the thymus (Klein and Horejsi, 1997) while in mammals B-cells develop in several peripheral lymphoid sites (Meyer *et al.*, 1997). The ileal Peyer's patch is the site of primary B-cell development in ruminants (Meyer *et al.*, 1997).

A third significant type of lymphocyte, the NK cell, recognises virus-infected or tumour cell targets through the use of a sophisticated system of stimulatory and inhibitory cell surface receptors (Moretta *et al.*, 2002). Once activated, NK cells develop into lymphocyte-activated killer (LAK) cells. In cattle, NK cells are

inhibited by MHC class I molecules and are regulated by bovine homologues of the human KIR and mouse Ly49 genes [BTA18; (Storset *et al.*, 2003)].

1.4.1.2 T-cells

T lymphocytes express the T-cell receptor (TCR), a transmembrane heterodimeric surface protein. T-cells exist in several functionally significant subtypes and subsets, acting as the principal managers and coordinators of cell-mediated, antibody and memory immune responses (Howard *et al.*, 2004).

To correctly identify foreign antigen or infected cells, T-cells must discriminate between self-components and associated non-self components such as microbial or other exogenous structures (Germain, 1994). T-cells receive these signals via the TCR, engaged by the antigen bound to a Major Histocompatibility Complex (MHC) molecule on the interacting cell. Antigen presenting cells are particularly involved in the presentation of processed peptide fragments bound with surface bound MHC to T-cells but also provide the co-stimulatory signals obligatory for full T-cell activation (Mellman and Steinman, 2001). Adhesion molecules, APC-derived cytokines, bacterial products such as endotoxins and vaccine adjuvants can all operate as co-stimulatory signals to T-cells undergoing activation (Schwartz, 2003). When fully activated, T-cells differentiate and expand into infinite clonal populations each expressing a unique TCR (Tanaka *et al.*, 1995), in close association with a surface bound complex of transmembrane polypeptides, termed CD3 [BTA 15 (Li *et al.*, 1992)].

A recent expansion of the self-nonself discrimination paradigm is the "Danger model" that proposes that distressed or damaged cells of normal bodily tissues activate local APC which then proceed to alert the immune system proper (Matzinger, 2001).

T-cells may be subdivided into functional subsets according to the dominant surface markers expressed, two major categories being αβ and γδ T-cells. Levels of γδ T-cells peak at birth and gradually decline with age in cattle and sheep (Washington *et al.*, 1992), so that γδ T-cells are believed to provide an early general purpose CMI defence in ruminant neonates, alongside colostral antibody. Ovine γδ T-cells respond more rapidly to mitogens *in vivo* (Evans *et al.*, 1994)

and it has been suggested that this cell type provide a contingency protection until αβ T-cell-dependent mechanisms have fully developed (Ramsburg *et al.*, 2003).

1.4.1.3 αβ T-cells

The αβ T-cells exist as two main subpopulations, each with defined repertoires of effector functions, distinguished by their exclusive surface expression of CD4 and CD8 molecules. $CD4^-CD8^+$ T-cells are selected for class I MHC molecules while $CD4^+CD8^-$ T-cells are selected for class II MHC recognition and both types have been described in cattle (Ellis *et al.*, 1996; Howard *et al.*, 1989). $CD8^+$ T-cells show major cytotoxic activity against tumour cells and cells infected with intracellular pathogens, and some regulatory, mainly suppressive, effects. $CD4^+$ T-cells are designated as helper T-cells, which assist the activation of humoral and cellular immune responses. In young ruminants, double negative, $CD4^-CD8^-$ T-cells comprise up to 80% of the lymphocyte population (Ayoub and Yang, 1996).

Both $CD4^+$ and $CD8^+$ T-cells differentiate into functional effector cells after exposure to antigen. Early following stimulation by antigen and APC, $CD4^+$ T-cells secrete IL-2 and are designated Th0, while the APC mature to produce IL-12. As the Th0 cells continue to respond to the activating stimulus, they progress towards two polar extremes of differentiation: Th1 and Th2 cells (Mosmann and Coffman, 1989). This progression is illustrated in Figure 1.1. Th1 cells produce the inflammatory cytokines: IL-2, IL-12, IL-18 and IFN-γ, and are associated with predominantly CMI responses while Th2 cells, by releasing IL-4, IL-5, IL-9, IL-10, IL-13, are associated with expansion of regulatory T-cell numbers in concert with B-cell proliferation and differentiation thereby inducing an antibody response (Constant and Bottomly, 1997).

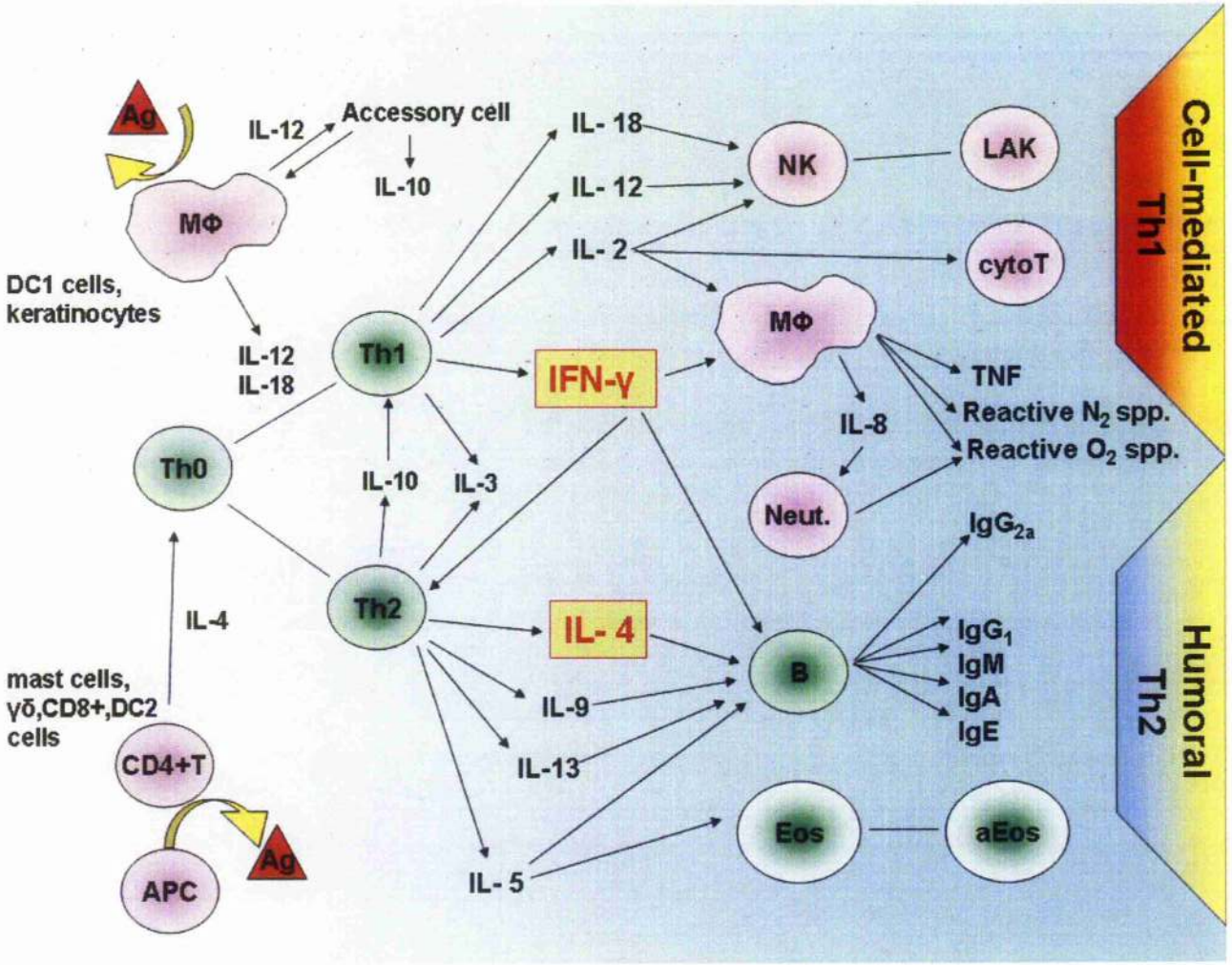


Figure 1.1 The innate and adaptive components of the immune system. Ag (antigen), APC (antigen presenting cell), B (B-lymphocyte), Th (helper T-lymphocyte), MΦ (macrophage), NK (natural killer cell), LAK (lymphocyte activated killer cell), Eos (eosinophil), aEos (activeated eosinophil), TNF (tumor necrosis factor), IFN (interferon) all cytokines (eg. IL-5) as presented. Cells primarily part of the innate immune response in purple, cells primarily part of the acquired immune response in green.

Commonly, helper T-cells show a combination of Th1 and Th2 features, although following prolonged stimulation in mice the response will polarise, becoming dominantly Th1 or Th2. Such polarisation appears to be much less evident in cattle (Estes and Brown, 2002). Simultaneous induction of regulatory and effector cytokines may represent a normal strategy of the immune system to control immune responses and reduce inflammation as an over-exuberant Th1 response is often the underlying cause of the observed pathology and illness.

In cattle, IFN-γ and IL-4 have been proposed as the respective signature cytokines for Th1 and Th2 responses (Estes and Brown, 2002). The specific IgG subclass pattern expressed is dictated by the availability of these two molecules: Bovine IL-4 (Th2) induces IgG_1 and IgE preferentially over IgG_2 while bovine IFN-γ (Th1) induces IgG_2 over IgG_1 production (Estes and Brown, 2002). Potent Th1 response and subclass IgG_{2a} inducers in rodents, such as bacterial DNA-based vaccines and adjuvants, elicit predominantly IgG_1 responses in cattle (Arulkanthan *et al.*, 1999). The cytokines IL-12 and IL-13 are thought to both modulate and augment the respective type Th1 and Th2 responses, channelling antibody production in particular directions (Trigona *et al.*, 1999; Tuo *et al.*, 1999). For viral infections in mice, Th1 cytokines inducing an early cytotoxic lymphocyte response are more important in recovery from infection while Th2 responses are better for prevention (Karupiah, 1998).

1.4.1.4 The major histocompatability complex

Genes in the Major Histocompatability Complex (MHC) produce proteins that determine which antigens are presented and processed by the rest of the immune system (Engelhard, 1994). Overall mammalian MHC genetic structure is well conserved, with three broad classes: (regions of DNA) MHC I, II and III, each with different types of genes. Class I [BTA23 q22; (McShane *et al.*, 2001)] and class II [BTA23 q12-q13; (Hess *et al.*, 1999)] genes code for cell surface glycoproteins while class III [also BTA23] genes code for other immune proteins such as the complement proteins, lymphotoxin and isoforms of TNF (Nonnecke and Harp, 1989).

Class I MHC proteins combine with β_2-microglobulin [BTA10; (Clawson *et al.*, 2004)] to form the class I MHC receptor found on virtually all mammalian nucleated cells (Kelm *et al.*, 2001). Class I MHC receptors communicate with

cytotoxic T-cells, expressing endogenously produced peptides and critically identifying cells infected with intracellular pathogens, e.g. viruses. Class II MHC receptors are found primarily on APC. Exogenous proteins ingested by the APC are displayed in association with the highly polymorphic MHC class II receptors which are recognised by $CD4^+$ helper T-cells (Chaplin, 2003). The nomenclature of the MHC is species–specific and is known as the bovine lymphocyte antigen system (BoLA) in cattle (Spooner *et al.*, 1979), largely mapping to bovine chromosome 23 (BTA23; (Fries *et al.*, 1986)).

Class I MHC molecules consist of one heavy chain and a very much smaller β_2 microglobulin chain (Engelhard, 1994). The loci coding for class I MHC can be divided into those that are highly polymorphic: class Ia loci and those which show little polymorphism: class Ib, Ic, and Id loci (Amills *et al.*, 1998). Non-MHC genes occupy a common framework - in the same order relative to each other - with classical class I genes filling the variable spaces between (Amadou, 1999).

This level of high polymorphism is maintained by mutation, gene conversion, recombinational "hot spots" and unequal crossing over of genes (Paterson *et al.*, 1998) and is driven by selective pressure from rapidly evolving pathogens (Yeager and Hughes, 1999). The polymorphism is concentrated in the floor and sides of the antigen-binding groove in the MHC molecule. In humans, there are three major class Ia molecules, termed A, B and C, each highly polymorphic, so that heterozygous individuals have a possible six distinct peptide-binding grooves. As each class I protein can bind many different peptides, this system can cope with a wide diversity of smaller antigenic peptides (Stern and Wiley, 1994).

The endogenous peptides bound to the peptide–binding groove in MHC class I molecules are generally synthesized within the actual cell bearing the class I molecule (Niedermann, 2002). The complex of class I MHC molecule and antigenic peptide result in a composite structure that is the molecular target for the T-cell TCR. The peptide alone or the MHC molecule plus a less compatible peptide will have negligible affinity for the TCR and so are not recognised by the T-cell, this is termed "MHC restriction" (Zinkernagel and Doherty, 1997). In cattle, there are between 10 and 20 genes involved in the class I region of the BoLA [BTA23 q22; (Lewin, 1996)] (Lindberg and Andersson, 1988).

1.4.1.5 Bovine lymphocyte antigen system

The pattern of expression of BoLA class I genes appears to be similar to that of humans (Di Palma *et al.*, 2002) with early studies describing expression of six or more classical class I loci depending on haplotypes (Ellis, 2004).

However the genetic structure of BoLA class II has some atypical features such as its division into two distinct regions IIa and IIb (van Eijk *et al.*, 1992). Other unusual elements include expression of ruminant specific DY genes (Baillingall *et al.*, 2004a), a chromosomal inversion (Band *et al.*, 1998) and variable numbers of DQ genes (Lewin *et al.*, 1999). Eleven class II BoLA loci have been identified but only protein products encoded at the DR and DQ loci in the MHC class IIa region (*DRA, DRB3, DQA, DQB1* and *DQB2*) have been demonstrated in cattle. The BoLA-DR genes and their protein products are among the best characterised of the MHC genes in cattle (Ledwidge *et al.*, 2001). The BoLA-DRB3 [BTA23 q21; (Russell *et al.*, 2000)] is a highly polymorphic gene, with more than 170 haplotypes having been identified in the major dairy and beef cattle breeds (Lewin, 1996). Recent work has demonstrated breed-specific levels of heterozygosity on this chromosome (Weimann *et al.*, 2003). Expression of BoLA class II has been documented on a wide range of bovine cell-types including B-cells (Lewin *et al.*, 1985), activated T-cells (Taylor *et al.*, 1993) and mammary epithelial cells (Fitzpatrick *et al.*, 1992).

1.4.2 Soluble acquired immunity

There are a multitude of cytokines secreted by a multitude of cell types acting as the soluble mediators of acquired immunity. However, IFN-γ and IL-4 are taken as the signature cytokines of Th-1 and Th-2 type immunological responses.

1.4.2.1 Interferon–γ

Interferon–γ is produced by a subset of T-cells (Th-1), some CD8+ T-cells and NK cells and activates T-cells, B-cells, NK cells and macrophages. Interferon-γ [BTA5 q22-q24; (Chaudhary *et al.*, 1993)] is a major stimulatory signal for macrophages (Foss and Murtaugh, 2000). It enhances NK cell activities and stimulates B-cell production of the immunoglobulin IgG_{2a} while lowering

production of IgG_3, IgG_1, IgG_{2b} and IgE in mice (Finkelman *et al.*, 1988). Production of IL-4 by helper T-cells is inhibited by IFN-γ while T-cell production of MHC Class I but not Class II molecules is enhanced (Billiau, 1996). Interferon–γ also activates macrophages, greatly promoting the destruction of ingested microorganisms.

1.4.2.2 Interleukin-4

Interleukin-4 (IL-4) is secreted by activated Th-2 cells and targets B-cells, T-cells, macrophages, endothelial cells, fibroblasts and mast cells. IL-4 [BTA7 q15-q21; (Buitkamp *et al.*, 1995)] is required for stimulation and differentiation of B-cells (Estes *et al.*, 1995) and is important for the progression of resting T-cells into cytotoxic T-cells (Klein and Horejsi, 1997).

1.4.2.3 Humoral acquired immunity

Having invaded the body, pathogens may replicate in two main sites. If they become intracellular, they tend to be controlled by cell-mediated immunity whereas if they multiply in the extracellular fluids, they are dealt with by humoral immunity via antibody.

1.4.2.4 B-cells

B lymphocytes (B-cells) are phenotypically defined by expression of the B-cell receptor (BCR), effectively membrane-anchored immunoglobulin (Chaplin, 2003). The majority of B-cells are concentrated within the tissues, especially the cortex of lymph nodes, the splenic marginal zone, the bone marrow and intestinal Peyer's patches, with the remainder found in the circulation (von Bochmer and Kisielow, 1990). B-cells defend against extracellular pathogens by (1) producing antibodies (Morafo *et al.*, 1999) and (2) endocytosis with subsequent antigen processing and expression of peptide fragments with MHC Class II proteins. B-cells are dependent on dendritic cells for activation and modulation at all stages of development, either directly or indirectly via helper T-cells (Banchereau and Steinman, 1998).

The BCR is Y-shaped, just like immunoglobulin consisting of both heavy and light chains. Another B-cell receptor, the FcR, has two general classes: activation (ITAM) and inhibitory (ITIM). Both types of FcR bind antibody with similar

affinity and specificity so that a functional balance operates and they are often found co-expressed on the B-cell surface (Kurosaki, 1999).

Random genetic rearrangement during B-cell differentiation, including allelic exclusion and editive processes on self-reactive proteins, leads to the assembly of the mature BCR antigen receptor (Bassing *et al.*, 2002). As Figure 1.2 shows, when a foreign multivalent antigen binds to the BCR, four processes are triggered: (1) B-cell proliferation, (2) B-cell differentiation into antibody-producing plasma cells, (3) memory cell formation and (4) antigen presentation to T-cells (Alam and Gorska, 2003).

Lymph node germinal centres are areas of intense B-cell division, with constant selective mutative pressures for improved antibody affinity (Przylepa *et al.*, 1998). Activated T-cells "help" B-cells to mature, by releasing cytokines and by direct cellular interaction via the CD40 receptor (Chaplin, 2003). This engagement facilitates B-cell proliferation and differentiation, the development of immune memory, controlled somatic hypermutation, antibody secretion and antibody isotype switching. Naïve B-cells express only IgM antibody in cattle, but following stimulation by helper T-cells, other antibody isotypes, IgG, IgE and IgA, are produced with identical antigen specificity (Alam and Gorska, 2003). The primary humoral immune response is characterized by detectable serum IgM and reactive IgM-expressing B-cells (Abbas *et al.*, 1996).

Following clonal expansion, most B-cells differentiate into plasma cells and enter into the tissues but a small yet critical memory cell subset migrate centrally to the lymph node germinal centres (Berek, 1992). Any persistent antigen fragments sequestered onto follicular dendritic cells will continue to drive memory cell expansion even after resolution of the initial infection (Campos and Godson, 2003). The anti-apoptotic factor BCL2 [BTA24; (Larsen *et al.*, 1999)] and the associated pro-apoptotic factor BAX [BTA18; (Reyes and Cockerell, 1998)] are important molecules at this stage. Effector memory cells tend to concentrate in tissues at or near the initial site of antigen encounter (Picker *et al.*, 1993), where they produce a broader spectrum of cytokines than naïve cells (Kaech *et al.*, 2002) and proliferate and differentiate much faster than naïve cells (Rogers *et al.*, 2000). This means that memory cells can respond more quickly to

lower levels of antigen, presented by more types of APC than naive cells, which translates to a faster, more effective secondary humoral response. However, there is a transitionary delay as memory cells convert to effector cells, which depending on the pathogen may prove significant (Zinkernagel, 2002).

As effector B-cells can bind only one antigen they make very effective APC, capable of activation with 1000-fold less antigen than non-specific APC such as macrophages. Following initial priming, the existence of so many identical B-cell clones makes secondary antibody responses much stronger and more rapid (as shown in Figure 1.2). With age, effector B-cell clonal populations expand and contract according to antigen exposure but B-cell memory populations persist (Maruyama *et al.*, 2000). Many cytokine and receptor molecules are involved in this process but CD40 and CD154 (Manoj *et al.*, 2003), and CD28 [BTA2 q31; (Parsons *et al.*, 1996)] are of particular importance.

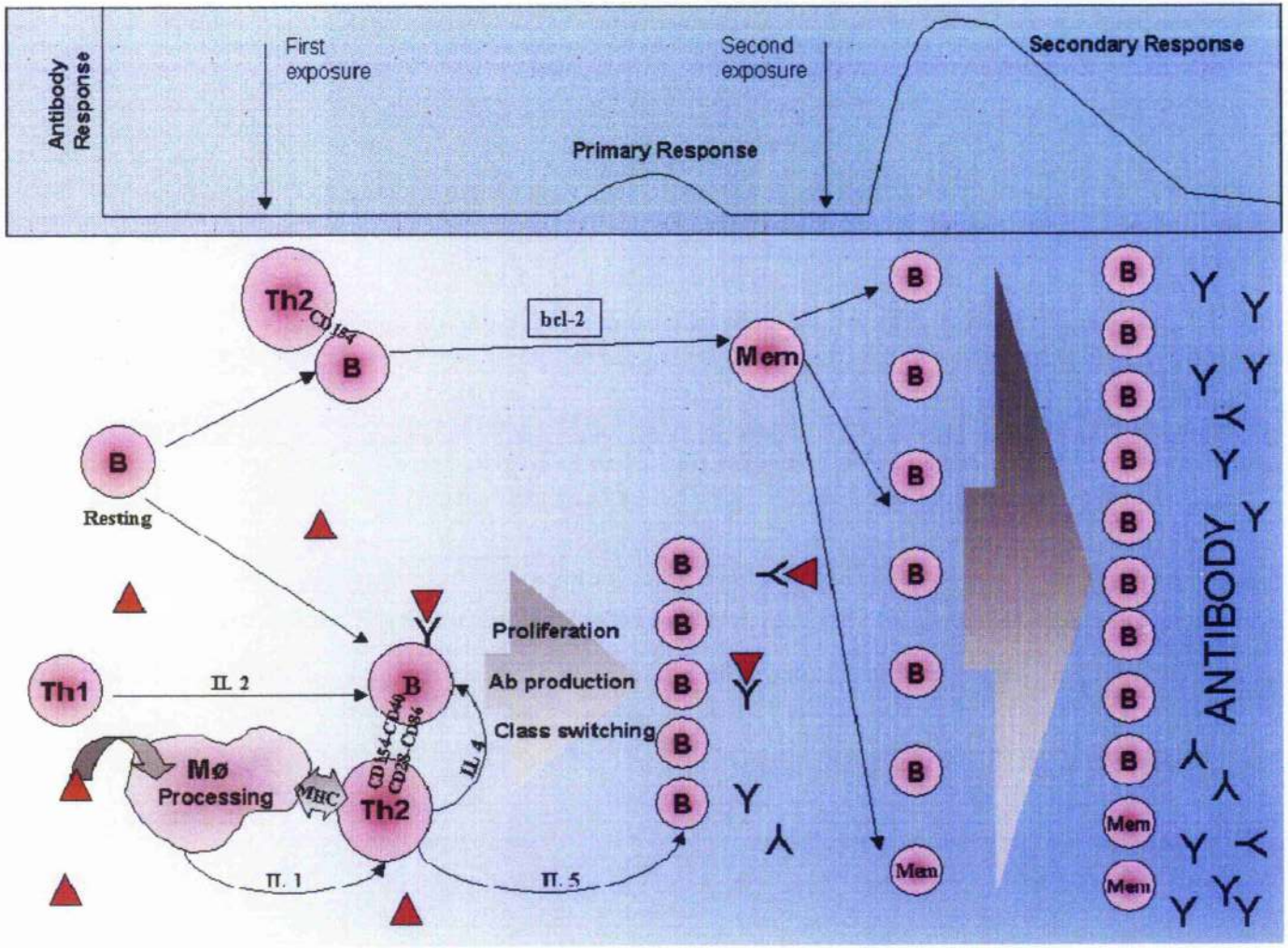


Figure 1.2 The humoral immune response: antibody production and associated cellular developments. Th1, Th2 (helper T-cells), MΦ (macrophage), Mem (Memory B-cell), B (B-cell), major histocompatability complex (MHC), cytokines as presented (IL1 to 5). Lymphocyte receptors/ligands (CD40, CD154, CD28, CD86), ▲ (antigen), **Y** (antibody)

The provoking antigen and the prevailing cytokine environment determine how the B-cell reacts and the immune response is produced. T-cell-independent mechanisms of B-cell activation do exist for polymeric antigens such as bacterial lipopolysaccharides (eg. *Escherichia coli, Salmonella* flagellin) but produce IgM only and result in weak immune memory. Without helper T-cell support, antibody isotype class-switching is not triggered. The Th2 subpopulation of helper T-cells promote IgG_1, IgA and IgE specific immunoglobulin by secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 whereas Th1 cells secrete Il-2 and IFN-γ which produces a much more generalised B-cell proliferation and polyclonal antibody secretion (Tizard, 2000).

Using human influenza virus as an infection model, Ada (2001) found that systemic levels of infectious virus and numbers of CD8+ cytotoxic T-cells rise in the first four days post-infection. Serum levels of infectious virus fall as the number of CD8+ cytotoxic T-cells peaks but both fall to low levels by day 10. Large numbers of memory cytotoxic T-cells are present between 14 and 40 days, some persisting for up to a year. After 4 days, the levels of effector B-cells begin to increase, IgM-producing cells developing first followed by IgG- and IgA-producers, peaking at about 2 months, again persisting systemically for about a year (Ada, 2001).

1.4.2.5 Immunoglobulins

Immunoglobulins are soluble B-cell receptors or antibodies found unbound in plasma and other secretory fluids. Immunoglobulins are products unique to B-cells, glycoproteins that bind antigen, the interaction being non-covalent (reversible) and highly specific (Tizard, 2000). Bound BCR or free antibody differ from TCR (of T-cells) in that the former can bind unprocessed antigen molecules.

The humoral response is based on antigen epitopes. An epitope is the portion of an antigen which is targeted and bound by an antibody. Binding affinity is the strength of the interaction between a single antigen binding site on the antibody and its specific antigen epitope, i.e. valency = one. Avidity is the total affinity of a single multivalent antigen molecule with a single multivalent antibody molecule. Antibody affinity intensifies with repeated antigen exposure while

antibody avidity increases with the number of repeating identical epitopes per antigen molecule. The ten antigen binding fragments (*Fab)* of an IgM molecule may have only moderate affinity individually but due to the large number of antigen-binding sites the molecule has high overall avidity. In murine viral models, neutralizing IgG antibodies are required in serum concentrations of 1 X 10^{-8} M and with an avidity of at least 1 X 10^{8} M to be protective (Zinkernagel, 2003).

All immunoglobulins have a similar basic monomer structure comprised of two heavy chains (50 kDa) (α, γ, δ, ε, μ) and two light (25 kDa) chains (κ or λ). Heavy chains [BTA21 q23-q24; (Chowdhary *et al.*, 1996)] contain 4-5 domains each of about 110 amino acids while light chains [BTA17; (Tobin-Janzen and Womack, 1992)] have only about 220 amino acids.

Genes identified within the heavy chain cluster include *IGHM, IGHG1, IGHG2, IGHG4, IGHA and IGHE.* There are three expressed cattle Cγ genes, each different in structure and by implication, function (Symons *et al.*, 1987; Symons *et al.*, 1989).

The amino acid sequence at the amino (N) terminal portions of the heavy (H) and light (L) chains vary greatly from one antibody molecule to another and are termed V_H and V_L, respectively. It is the juxtaposition of one hypervariable V_H segment and one V_L segment that creates the *Fab* domain, duplicated on each arm of the molecule. This determines the specificity of the antibody. There are three hypervariable regions in both the heavy and light chains which through folding give the molecule specific and unique antigen binding pockets (Chaplin, 2003). At the other end of the immunoglobulin molecule, the carboxyl (C) terminal portions of the heavy and light chains are constant within each subclass. The heavy chain constant regions link to form the *Fc* (crystalizable fragment) domain of the molecule, responsible for the biological activity of that antibody.

There are five distinct classes or isotypes of immunoglobulin dependent on the constituent heavy chains (in brackets): IgA (α), IgD (δ), IgE (ε), IgG (γ) and IgM (μ), with distinct structural and functional characteristics, each optimised for a specific group of pathogens or deployment within a specific environment (Tizard, 2000).

Serum IgM has a pentamer structure, ~5 day half-life, and potentially ten epitope-binding sites. It is too large to leave the circulation but is a potent

complement activator. Monomeric IgG, with two epitope-binding sites, has the longest half-life (~20 days), enters and is effective in tissues and is involved in complement activation and antigen opsonization. In total, more IgA is produced than any other antibody. Dimeric IgA has a half-life of ~5 days and predominates in mucosal secretions with the *Fc* portion attaching firmly to the mucous matrix. The polymeric immunoglobulin receptor (pIgR) [BTA16 q13; (Kulseth *et al.*, 1995)] is important to the transcytosis of IgA at mucosal surfaces. Immunoglobulin-E is a monomer with two epitope binding sites and an Fc portion which readily binds with eosinophils. It mediates parasitic and allergic reactions, triggering mast cell and basophil activation in skin and mucosae.

The isotype of an immunoglobulin molecule can change; "class switching" is the process by which a B-cell alters expression of heavy chain isotype thereby altering the *Fc* domain and function of the antibody (Estes, 1996). Class switching is coordinated by T-cell derived cytokines and T-cell/B-cell interactions via the CD40 receptor (Estes, 1996). Each B-cell can produce only one light chain isotype. Minor diversity in the actual amino acid sequence of a particular isotype produces different antibody allotypes, i.e. $IgG2_a$, $IgG2_b$.

During development, the random shuffling of the many variable-region genes confers each B-cell with a capacity to produce a distinctive and unique antibody (Matsuda and Honjo, 1996). The binding site consists of at least five fragments of random peptides, encoded and blended from a library of genes allowing very diverse structures to be created from a limited genetic "hardware". Germline antibodies are those produced based on recombination of inherited genetic information. In addition, selective somatic mutation of the B-cell's variable–region genes continues within the germinal centres of secondary lymphoid tissues, to force the evolution of B-cells that produce higher affinity antibodies (Schwartz, 2003). Together, these processes ensure the diversity of antibody repertoire critical to animal survival (Butler, 1998). In mice, quantitative trait loci (QTL) for IgG antibody against exogenous rhodopsin were mapped to murine chromosomes 1, 5 and 13 – all non-MHC immune response genes (Wu *et al.*, 1996).

Actual binding of antibody to antigen occurs by two competitive models; lock-and-key and induced-fit forces, with hydrogen bonds, ionic bonds, hydrophobic and Van-der-Waals interactions all involved (Braden and Poljak, 1995). On

initial encounter, a large germline antibody molecule modifies and re-conforms its shape to flexibly grasp antigens by a process of induced-fit. As the interaction continues, somatic mutation produces antibody molecules with binding sites precisely complementary to the antigen in shape and electronic charge so that the grasp becomes fixed and affinity increases (Mariuzza *et al.*, 1987).

Infection is controlled by circulating IgG and IgM antibodies by: (1) agglutination of infectious agents and opsonization of particles, both of which facilitate phagocytosis, (2) serum neutralisation of viruses and toxins, (3) binding to pathogen thereby blocking host cell attachment or initiating the classical complement pathway with membrane-attack-complex (MAC) formation, (4) mediating antibody-dependent cell-mediated cytotoxicity and (5) immobilisation of bacteria/protozoa (Roth and Perino, 1998).

Once released systemically, levels of IgG negatively feedback to regulate their own production. This IgG-mediated suppression is antigen-specific, non-epitope-specific in most cases and stronger in higher affinity antibodies than lower affinity (Heyman, 2003). The inhibition may act by (1) antigen/antibody complexes co-binding to specific BCR and FcγRIIB receptors on B-cells to directly down-regulate antibody secretion or (2) antibodies "masking" antigen from B-cells, blocking interaction and subsequent immunostimulation. In addition antigen/antibody immunocomplexes may be taken up by B-cells more efficiently than antigen alone with either positive or negative feedback effects (Heyman, 2003).

Bovine humoral immunity is unusual, as cattle appear to lack functional IgD antibody (Zhao *et al.*, 2002) and have a unique distribution of antibodies within specialised secretions such as colostrum (Butler, 1983). The levels of IgG_1 and IgG_2 in normal adult bovine sera are almost equivalent (Butler *et al.*, 1994), the latter being the most important opsonin for neutrophil and macrophage phagocytosis. As with most mammalian species, IgM and IgA form a small proportion of overall serum immunoglobulins, but unlike most species, IgA in cattle exists in a dimeric form. Cattle are unusual in having light chains with 20-fold more λ-chains than κ-chains (Butler, 1998). Allotypic variants of bovine immunoglobulins exist for IgG_1, IgG_2 and IgG_3 (trace) but only appear to be functionally significant for IgG_1 (Bastida-Corcuera *et al.*, 1999).

It should always be recalled that due to the transient and compartmentalised dynamics of immune reactions, the processes and genes detected as important by any particular experimental investigation will vary according to what, when and how measurements are made (Hawken *et al.*, 1998). For each immunostimulatory event or infection, a unique host/pathogen/environment combination occurs which may defy exact field repetition or experimental replication (Horin, 1998).

1.5 Colostrum

Due to the syndesmochorial arrangement of the ruminant placenta, calves are born agammaglobulinemic (Redman, 1979). Bovine colostrum contains large amounts of immunoglobulins derived from the maternal bloodstream (Sasaki *et al.*, 1977), which the calf absorbs intact into its systemic circulation, after ingestion. As endogenous production is neglible, levels of passive serum antibody in calves are therefore determined within 24 hours of birth (Besser *et al.*, 1987). However this passive immunity does not provide complete protection from infection (Baker *et al.*, 1989), but rather attentuates neonatal disease allowing a form of physiological vaccination to occur (Zinkernagel, 2003).

1.5.1 Colostrum metabolism and function

Bovine colostrum contains highly enriched levels of fats, proteins and peptides, fat-soluble vitamins, minerals, nucleotides, polyamines, enzymes, hormones, growth factors and cytokines (Campana and Baumrucker, 1995) and most crucially, greatly elevated concentrations of antibodies (Morin *et al.*, 1997). Typically bovine colostrum contains 100-700 mg/dl IgA, 300-1300 mg/dl IgM and 3400-8000 mg/dl IgG, thus IgG accounts for 65-90% of the total antibody content. Colostrum quality is affected by the balance between colostrogenesis and lactogenesis, parity of the dam, vaccination programmes, previous wild-type infections, length of dry period and prepartum milking (Quigley and Drewry, 1998).

Transport of colostral antibodies occurs through a transiently permeable intestinal wall, then via the lymphatic system into the calf's general peripheral circulation (Arthington *et al.*, 2000), with absorption localised primarily to the

jejunum and ileum (Bush *et al.*, 1971). A combination of high abomasal pH, colostral trypsin-inhibitors causing low intestinal proteolysis and unique pinocytic activity of the neonatal intestinal epithelial cells permits absorption of intact protein macromolecules (Clover and Zarkower, 1980). On contact, colostral antibody binds to a specific Fc receptor (*FcRn*) on the neonatal intestine (Mayer *et al.*, 2002), exciting a limited burst of pinocytosis. Macromolecule internalisation halts after exhaustion of this pinocytic activity, and maturation of the enterocyte's intracellular digestive apparatus has occurred (Stott *et al.*, 1979). The *FcRn* [BTA18 81cM; (Dolcschall *et al.*, 2005)] receptor has a molecular structure very similar to the MHC class I molecule and in adult life protects IgG from catabolism and degradation, acting as a chaperone protein inside the phagocytic lysosome (Ghetie and Ward, 1997).

Antibody isotypes are absorbed by different mechanisms: IgG absorption has been shown to be linearly proportional to colostral concentration up to a ceiling value (Stott and Fellah, 1983). Antibody transfer is thought to be highly enhanced from serum to colostrum in the dam, with no significant difference in rate reported in BRSV and BHV-1 specific colostral antibody levels, in titre-matched vaccinated and unvaccinated dams (Ellis *et al.*, 1996). Vann *et al.* (1995) ranked absorption efficiencies as IgM > IgG_2 > IgA > IgG_1 with an overall effective immunoglobulin uptake of ~22% after six hours.

Immunoglobulin-G absorption decreases linearly from 6 to 48 hrs post-calving in bovine neonates, effectively ceasing after 36 hours (Abel Francisco and Quigley, 1993; Vann *et al.*, 1995). The decline is due to a combination of enterocyte maturation, intensifying secretion of digestive enzymes (Guilloteau *et al.*, 1984) and bacterial colonisation of the sterile gut (James *et al.*, 1981). However, up to 30% of neonatal calves which ingest colostrum remain hypogammaglobulinaemic (Burton *et al.*, 1989b), with serum IgG_1 levels less than 10 mg/ml post-absorption (Rea *et al.*, 1996).

Passive serum antibody levels have peaked at 24-26 hours postpartum in suckled beef calves (Logan and Gibson, 1975). From then to ~10 weeks old, total serum antibody declines with negligible endogenous production evident (Rajala and Castren, 1995). Maternally-derived antibodies become undetectable between 95 and 231 days (Brar *et al.*, 1978).

In addition to a transient proteinuria *post-natum*, antibody clearance occurs primarily due to resecretion into the small intestine. This process allows significant functionality to be retained, providing useful local but short-term protection from enteritis (Besser *et al.*, 1987). Some antibody clearance is also accounted for by dilution effects due to increasing body size and blood volume and general protein catabolism and turnover.

1.5.2 Other colostral functions

Although the primary purpose of colostrum is the passive transfer of antibodies, it contains a substantial and viable cellular element, predominantly lymphocytes. This component may be at concentrations of $1x10^6$ per ml, the majority of which are T-cells, the remainder containing a high macrophage component (Ellis *et al.*, 1996). These cells can survive up to 36 hrs once ingested, often penetrating the intestinal wall and migrating to mediastinal lymph nodes (Liebler-Tenorio *et al.*, 2002). Calves which received leukocyte-depleted colostrum had significantly lower IgA and IgM levels than calves which ingested whole colostrum (Riedel-Caspari and Schmidt, 1991).

Beyond its immunomodulatory role, the volume, quality and timing of colostrum intake greatly affects many metabolic and endocrine factors in neonatal calves, significantly the levels of insulin, glucagon, prolactin and growth hormone (Blum *et al.*, 1997; Hadorn *et al.*, 1997; Hammon *et al.*, 2000; Rauprich *et al.*, 2000).

1.5.3 Environmental effects on colostrum

Final levels of absorbed colostral antibody depend on many highly variable factors: antibody isotype (Selman *et al.*, 1971a), volume and quality of colostrum, time of first feeding, feeding method, environmental temperature (Donovan *et al.*, 1986; Olson *et al.*, 1980), seasonal effects (Gay *et al.*, 1983), dystocia (Weaver *et al.*, 2000), calf stress (Johnston and Stewart, 1986), and calf birthweight, vigor, and dam behaviour (Selman *et al.*, 1970). Heifers produce lower quality colostrum than cows (Chelack *et al.*, 1993) and lactation number or parity has been linked to colostral quality (Pritchett *et al.*, 1991). Hammon and

Blum (1998) observed prolonged elevation of serum IgG levels from extended feeding of colostrum. Poor dam *pre-partum* nutrition reduced IgG absorption in the resulting calf (Hough *et al.*, 1990). Many of these factors combine to contribute to the wide range of maternally-derived serum antibody levels in calves, even within identical management systems.

The regime of colostrum feeding can greatly influence resulting efficacy of antibody uptake (Quigley and Drewry, 1998). Calves that suckle their dams tend to begin consumption later and consume a smaller critical volume than nipple-fed animals, so reducing the total efficiency of passive antibody transfer (Brignole and Stott, 1980; Logan *et al.*, 1981; Selman *et al.*, 1970). In contrast, Suh *et al.* (2003) described significantly higher serum IgG levels in beef calves compared to dairy calves. Use of oesophageal feeding as a method of colostrum administration is contentious. Lee *et al.* (1983) found marginally lower IgG serum levels with this method, the rationale being that oesophageal feeding places colostrum into the neonatal rumen delaying entry into the abomasum and intestine (Lateur-Rowet and Breuink, 1983). However other authors reported no detrimental immune effects (Adams *et al.*, 1985; Molla, 1978). Best practice was described as four litres of colostrum supplied inside the first six hours of life (Besser *et al.*, 1991). The reported half life of bovine maternally derived passive IgG_1 ranges from 16 to 26 days (Besser *et al.*, 1987; Brar *et al.*, 1978; Gay *et al.*, 1983; McGuire *et al.*, 1976; Sasaki *et al.*, 1977).

1.5.4 Animal effects on colostrum

Holstein calves were described as being more efficient at absorbing colostral antibody than Ayrshire calves (Baumwart *et al.*, 1977). Jersey calves were reported as having twice the passive antibody levels of comparable Holsteins (Tennant *et al.*, 1969) and Friesian/Ayrshire crossbreds have been documented as having greater antibody absorption than purebred Ayrshires (Selman *et al.*, 1971a).

Bradley *et al.* (1979) found Hereford calves to have significantly greater levels of antibody than Simmental calves, with offspring from multiparous dams having significantly more passive serum antibody than those from primiparous dams. Halliday *et al.* (1978) recorded Shorthorn/Galloway crossbred calves as having

higher concentrations of colostral IgG_1 and IgM than Hereford/Friesian crossbred calves. Similar differences between crossbreds were demonstrated by O'Kelly (1991). Offspring vigour may be important in these findings as less vigorous (very heavy or very light) calves consume less colostrum during the first crucial hours. Muggli *et al.* (1984) described significant variation due to calf breed in neonatal passive serum antibody levels in Angus, Red Poll and Hereford breeds, ranked as listed. A general increase in calf post-natal activity in the former cattle breeds may increase colostrum consumption, explaining this difference (Muggli *et al.*, 1987).

No significant differences in colostral IgG_1 concentration were reported for calf sex, calf sire, sire-line or dam-age (Gilbert *et al.*, 1988). However sire-within-line and level of dam inbreeding were found to be highly significant at 24 and 36 hours. Levels of absorbed serum IgG_1 were found to decrease as inbreeding increased but this effect may be confounded by management differences (Gilbert *et al.*, 1988).

In another study, Norman *et al.* (1981) found calf sex was not generally significant apart from a dilution effect in male calves (commonly larger), but did find dam-age as significant. The study reported that crossbred dams produced calves with higher IgG and IgM concentrations than purebred dams with heritabilities of 0.52±0.28 and 0.69±0.30 for IgG_1 at 24 and 36 hrs with equivalent values of 0.30±0.26 and 0.35±0.26 for IgM. Two further studies also found levels of calf IgG_1 positively correlated with dam age (Frerking and Aeikens, 1978; Mueller and Elleinger, 1981). Muggli *et al.* (1987) described no difference in serum IgG_1 levels related to sex of calf but found antibody negatively correlated with birthweight in Herefords and positively correlated in Angus calves. Later studies found that cows producing crossbred calves had greater total immunoglobulins and total IgG and IgA concentrations in colostrum than cows producing purebred calves (Muggli *et al.*, 1987; Vann *et al.*, 1995), termed the sire-of-foetus effect on the dam's lactation.

A negative correlation has been described for birthweight and neonatal IgG_1 levels in both cattle and sheep (Cabello and Levieux, 1981; Selman *et al.*, 1971b). Positive selection for this production trait may indirectly lead to reductions in transferred IgG_1 levels, due simply to dilution effects. However other authors found this association not significant (Muggli *et al.*, 1984). That

study found dam (1) dystocia ($p<0.05$); (2) calf-age ($p<0.05$); (3) dam-age ($p<0.05$) and calf-breed ($p<0.01$) as significant for levels of maternally-derived calf serum IgG_1. Heritabilities for sire and maternal effects were established as 0.09±0.09 and 0.27±0.17 respectively (Muggli *et al.*, 1984). Levels of IgG_1, IgG_2 and IgM were found to be positively correlated with daily weight gain until 42 days old; thereafter the association subsides (Halliday *et al.*, 1978).

Laegreid *et al.* (2002) described a significant association with poor colostrum absorption and specific haplotypes (especially haplotype 3) for the *FCGRT* gene (coding for the FcRN receptor) on BTA18. A strong linkage between failure of transfer of passive antibody and the β_2M gene on BTA10 was reported by Clawson *et al.* (2004). Calves with the β_2M marker *AII27-3* were 10-fold less likely to absorb adequate passive antibody than other genotypes.

1.5.5 Immune effects of colostrum

Neonatal immune unresponsiveness has many causes including phenotypic and functional immaturity in CD4+ T-cells, low expression of MHC class II in neonatal dendritic cells and actual numbers of T-cells and APC being several orders lower in infants compared to adult humans (Jafarzadeh and Shokri, 2003). A similar immunological immaturity is described in young calves (Barrington and Parish, 2001).

Receiving antibodies passively via colostrum actually inhibits the development of the endogenous immune system across many species (Crowe, 2001; Fitch, 1975; Smith and Ingram, 1965). Both systemic and mucosal immunoglobulin responses are all down-regulated by feeding of colostrum (Kimman *et al.*, 1987a). In colostrum-deprived calves low level antibody synthesis begins within one week, but this is delayed up to one month in colostrum-fed calves (Clover and Zarkower, 1980). The mechanism is believed to act by specific antibody binding to the B-cell FcγRIIB surface receptor signalling inhibition of antibody production (Heyman, 2003) or by formation of antigen-antibody complexes leading to the removal of the stimulus, without reaction from the host immune system (Crowe *et al.*, 2001). The immune suppression exerted by passive antibody may also be due to "hiding" of antigenic sites by maternal passive IgG, diminished helper T-cell activity or increased suppressor T-cell activity, a

general immaturity of the immune system or any combination of these factors (Kimman *et al.*, 1987a).

Bovine virus diarrhoea virus challenge experiments have found anamnestic responses at second challenge in calves previously challenged when they had high serum levels of passive serum antibody (Ridpath *et al.*, 2003). Maternally-derived antibody may permit limited viral replication at the first challenge. This viral replication occurs below the threshold that would cause clinical disease but is high enough to produce a weakly protective T-cell and B-cell lymphocyte response. Evidence of such physiological vaccination is consistent with organised perinatal vaccination studies in calves (Ellis *et al.*, 1996).

In mice and humans, the relative contribution of cellular immunity, primarily $CD4^-$ and $CD8^+$ T-cells, has been found to increase according to circulating levels of passive serum antibody (Crowe *et al.*, 2001). This type of short duration immunity is an important substitute until induction of a more sustained antibody response occurs (Connors *et al.*, 1991).

1.6 Vaccination

Vaccination has had a greater economic impact on livestock and poultry production than all other therapeutic and prophylactic disease treatments combined (Babiuk *et al.*, 1999) and remains one of the most cost-effective method of reducing economic loss and suffering from animal disease (Bowersock and Martin, 1999).

Ideally vaccines should mimic natural infection by inducing strong, lifelong protective immunity against infection, in particular stimulating immunological memory (Terpstra and Kroese, 1996). Immunological memory results from both antigen-specific and non-specific signals during the primary immune response, producing memory cells from naïve precursors in the host (Campos and Godson, 2003).

Four properties of vaccines are critical to their effectiveness: (1) antigen-presenting cells must be stimulated to process antigen efficiently and induce appropriate cytokine production (2) both T-cells and B-cells should be stimulated

in order to establish large populations of their respective memory cells (3) helper and effector T-cells must be generated to several epitopes in the vaccine to overcome individual animal variation in MHC class II polymorphism and epitope properties (4) antigen should persist in appropriate lymphoid sites so that a population of antibody producing cells is established, providing medium-term protection (Tizard, 2000).

For viruses in particular, vaccines can act to (1) prevent initiation of primary infection by viral field strains, (2) reduce virus replication at site of entry, (3) prevent or minimise systemic spread, (4) reduce virus shedding and (5) prevent establishment of viral foci which can lead to second waves of infection (Terpstra and Kroese, 1996).

As most infectious agents enter via mucosal surfaces, oral or intranasal vaccine delivery produces a local immunity with the greatest chance of preventing infection (Babiuk *et al.*, 1999). Individual host factors such as extremes of age, poor nutritional status and pregnancy reduce the immunogenicity of a vaccine but the intrinsic pathogenesis of the infection and biological constraints of the immune system also limit vaccine effectiveness (Campos and Godson, 2003). Completely perfect protection without any pathogen replication is rarely achieved (Zinkernagel, 2003).

1.6.1 Types of vaccine

Live viral vaccines are based on avirulent mutants, produced by treatment with mutagens, chemicals, radiation or multiple passage. Vaccine strain and inoculum concentration are critical factors dictating the immune response especially in the face of maternally derived passive immunity (Hanly *et al.*, 1995).

Classically, inactivated vaccines contain virus rendered non-infectious by chemical inactivation (Van Oirschot, 2001). They are stable and safe but generally require adjuvant bases and repeat inoculations. As adjuvants improve, inactivated vaccines may provide a more prolonged immunological stimulus inducing higher booster effects than currently possible (Kerkhofs *et al.*, 2003). Inactivated vaccines have no replication phase in the host, so minimal cytotoxic cellular responses are triggered, and often, secretory IgA is not induced. Overall,

inactivated vaccines tend to provide a shorter and slower immunity than modified live vaccines (Hjerpe, 1990).

Live vaccines need fewer inoculations, no adjuvants and usefully induce IFN but do have disadvantages: (1) they are more labile, (2) are potentially open to recombinational events (Ridpath and Bolin, 1995), (3) may be contaminated with extraneous virus, (4) tend to be more pathogenic than inactivated vaccines and (5) can induce a carrier state (Roth, 1999).

Inactivated vaccines act as exogenous antigens, generally processed by CD4+ helper T-cells. The inclusion of adjuvants primes for a Th2 response producing an enhancement of the humoral response, while intranasal live vaccines induce predominantly a Th1 response (Karupiah, 1998; London *et al.*, 1998). By definition, live virus vaccines infect cells and undergo replication, antigen being processed endogenously by host cells. Cytotoxic T-cells have a dominant role in the subsequent immune reaction. As IFN-γ is induced by live vaccines (Woolums *et al.*, 2003), protection can be very rapid in susceptible animals even in the face of an outbreak (Makoschey and Keil, 2000). Activated T-cell clones and non-MHC restricted NK cell cytotoxicity act as the major sources of this IFN-γ production (Woolums *et al.*, 1999). Polarisation by IFN-γ production towards a Th1 response has not been described in cattle to date (Brown *et al.*, 1998). If a live vaccine is boosted with an inactivated vaccine, the immune effect is enhanced, perhaps due to the Th1 priming (Kerkhofs *et al.*, 2003).

1.6.2 Factors controlling immune responses to vaccines

Farm management influences, such as nutrition, animal density and environment can all physiologically confound immune responses (Ott, 1996). In addition, the physiological status of the individual, i.e. pregnancy, puberty, or lactation, tremendously influences the transcription of genes related to the immune response (Kelm *et al.*, 2001).

As previously discussed, maternally-derived passive antibody suppresses the immune response induced by vaccination, the effect being dose-dependent, and isotype- and antigen-specific (Ellis *et al.*, 2001a). Colostrum ingestion eliminates IgG-positive B-cells from mesenteric and peripheral lymph nodes (Aldridge *et*

al., 1998) which may facilitate immunotolerance of maternally-derived antibody and increase its longevity.

The immune response of animal populations, influenced by genetic and environmental factors, follows a Gaussian distribution so that 100% protection in all animals is an aspirational goal rather than realistic objective (Tizard, 2000). Using vaccines in outbred populations will always result in considerable variability in the level and type of immune response and the subsequent protection generated (Lofthouse and Kemp, 2002). Commercial cattle producers use vaccines to control disease at a population level rather than at individual animal level with herd immunity being the primary aim so reducing overall disease transmission rates.

Similarly, the critical dose of vaccine necessary to stimulate effective protection varies among individual animals. Using an inactivated BRSV vaccine, Ellis *et al.* (2001b)reported substantial differences in immune response by varying the inoculated antigen concentration. The minimal vaccinal dose required for effective individual immunity within any given population also has a normal distribution, termed the "tolerance distribution" (Terpstra and Kroese, 1996). Within any genetically heterogenous population, vaccine responses are a continuous, quantitative phenomenon due to both environmental and host factors. It is oversimplistic to divide vaccine responses into success or failure, as a rich spectrum of responses is much more appropriate (Ovsyannikova *et al.*, 2004a).

Sex may affect vaccine response. The onset of puberty in cattle is strongly influenced by nutrition (Sejrsen, 1994) and is also heritable (Splan *et al.*, 1998). Hypothalmic gonadotrophin-releasing-hormone has been implicated in the modulation of humoral and cellular immune responses and the hormone oestradiol is immunostimulatory while testosterone is immunosuppressive (Tanriverdi *et al.*, 2003).

Vaccine non-responsiveness has been explored previously. Expression of specific MHC haplotypes causing defects in antigen presentation, deficient antigen-specific T-cell or B-cell repertoires, weak helper T-cell function and various degrees of immunological antigen tolerance are all possible reasons for failure of a humoral response (Jafarzadeh and Shokri, 2003). Concurrent stimulation by several antigens seems to have no deleterious effect. No significant differences were reported in clinical signs or immune response

between weanling heifers, which received a live BRSV vaccine alone or in conjunction with 18 other polyvalent live or inactivated antigens (Carmel *et al.*, 1992).

1.6.3 Antibody response to vaccination

Generally, the levels of antibodies induced do not depict the extent, efficacy or entirety of the immune response and are a crude measure of the degree of immunoactivation (Martin and Bohac, 1986). Although vaccination and serum antibody levels are strongly associated, the humoral component represents only a portion of total immune mechanisms induced by the respiratory pathogens: BHV1 (Denis *et al.*, 1994), BRSV (Sandbulte and Roth, 2002) and PIV3 (Henrickson, 2003). A study by Bryson *et al.* (1999) showed little correlation between the extent of pneumonic lesions and levels of serum PIV3 antibody. That study concluded that the level of antibody in nasal secretions provides a better index of host resistance to PIV3 infection than serum antibody levels (Bryson *et al.*, 1999).

Protection against BRSV has been reported without demonstrable antibody response (Ridpath *et al.*, 2003) but most human and bovine RSV studies suggest accumulative acquisition of resistance to lower respiratory disease associated with increasing levels of serum antibody (Ellis *et al.*, 1990). Recent work in cattle has suggested that although IgA dominates in nasal, lacrimal and salivary secretions, IgG is found in higher concentrations in the lower respiratory tract (Kalina *et al.*, 2005). Hammond *et al.* (1999) found that, in ponies, although no single *in vitro* parameter provided an adequate measure of protection, a composite of several antibody characteristics such as serum level, avidity index and molecule conformation ratio could be used as a reliable measure of challenge resistance following vaccination against Equine Infectious Anaemia. Serum antibody concentrations do form an objective, reliable method, capable of assessing the immunogenic properties of different vaccine products (Tollis *et al.*, 1996). There are few workable alternatives: it is scientifically laborious and seems to be economically prohibitive to assess clinical efficacy of vaccines using field challenge trials beyond that required for drug registration.

1.6.4 Adverse effects

Vaccine failure can occur for many reasons, with unsatisfactory administration, immunosuppression, or inhibition by maternally-derived antibody being the most common. Management activities such as dehorning, weaning, handling, forced exercise and transportation are stressful to cattle. Stress has important immunosuppressive effects including decreased antibody response to primary vaccination, reduced phagocytic cell migration and inhibited lymphocyte function (Roth and Perino, 1998). In pigs, both cell-mediated and antibody-based immune responses are influenced by basal levels of circulating corticosteroid which in turn are dictated by the animals social rank (Hessing *et al.*, 1995).

Although live vaccines are in the main safe, some adverse effects have been reported. Some viral transmission from vaccinated to unvaccinated animals was recorded using live BHV1 vaccines (Baker *et al.*, 1989). In addition, live BHV1 vaccines have been found to enhance the clinical severity of infectious keratoconjunctivitis (George *et al.*, 1988), to induce meningoencephalitis (Furuoka *et al.*, 1995) and were implicated in vaccine-induced outbreaks of infectious bovine rhinotracheitis in cattle (Whetstone *et al.*, 1986).

1.7 Bovine Respiratory Disease

Bovine respiratory disease (BRD) has been extensively investigated in recent years with numerous vaccines and antibiotics developed for its control. Bovine respiratory disease was described as the principal health problem for calf producers worldwide (Lekeux, 1995). It is particularly common during the initial 45 days after calves have been weaned, transported and mixed with other animals (Roth and Perino, 1998), with cattle being highly susceptible when passive serum antibody wanes at three to four months old (Bryson *et al.*, 1978).

In a Northern Ireland bovine mortality study, encompassing 38% of the total cattle population, respiratory disease was the most common cause of death in animals aged between 1 and 24 months (Menzies *et al.*, 1996). An Irish feedlot survey based on 6400 cattle over 6 months found respiratory disease to be the most frequent cause of morbidity and mortality (Healy *et al.*, 1993). In US

feedlot cattle, BRD morbidity can reach 75% with case mortalities up to 50% (Rivera *et al.*, 2002). Gardner *et al.* (1999) found that steers treated for BRD had lower live and carcase weights, lower average daily weight gain and poorer grading scores. Substantial economic losses occur, as demonstrated by a Scottish study which found costs of £21 per animal at risk for 152 outbreaks (Gunn and Stott, 1996). The potential for viral disease will only increase as cattle populations grow larger and production intensification continues.

Ninety per cent of bacterial bovine pneumonias develop subsequent to a viral infection (Babiuk *et al.*, 1988). Viruses are obligatory, intracellular parasites with limited genomes that code for functions they cannot adopt from host cells (Strauss *et al.*, 1991). Viruses evolve by gene mutation, recombination and duplication and have very short generation intervals and high mutation rates (Gavora, 1996). A high reproduction capacity, short generation time and high mutation rate allow rapid changes in genotype. DNA viruses such as bovine herpesvirus 1 are more stable than RNA viruses (BRSV and PIV3) and tend to produce persistent or latent infections unlike the rapidly moving pandemics typical of RNA viruses (Strauss *et al.*, 1991). The latter tend to mutate at a much higher rate than DNA viruses, allowing their populations to contain a good deal more variability, so that many RNA virus quasi-species may exist within a single host (Domingo *et al.*, 2000). The cyclic emergence of virulent new strains of the avian pathogen Marek's Disease virus, capable of overcoming previous generations of vaccines, is typical of this constant viral evolution (Nair, 2005). Figure 1.3 illustrates the breakdown of bovine respiratory disease cases as submitted to the Scottish Agricultural College, Veterinary Service in 2002.

Viruses have multiple effects on antibacterial defences including impairment of mucociliary clearance, suppression of phagocytic cell function, and interference with lymphocyte function (Roth and Perino, 1998). Indeed, lung pathology is often enhanced due to the synergistic effects of superinfection by more than one respiratory virus (Kelling *et al.*, 1996). In particular, BVDV infection in cattle affects lymphocyte, macrophage and neutrophil function (Ketelsen *et al.*, 1979; Roth *et al.*, 1986) with immunosuppressive effects leading to enhanced disease from other concurrent infections (Elvander *et al.*, 1998).

No antiviral pharmaceutical drugs are currently marketed for large animal use and their deployment into such farm animal species appears unlikely. Currently in the UK, commercial vaccines are commonly used against many viral pathogens of cattle including BRSV, BHV1, PIV3, BVDV, bovine coronavirus (BCV) and bovine rotavirus.

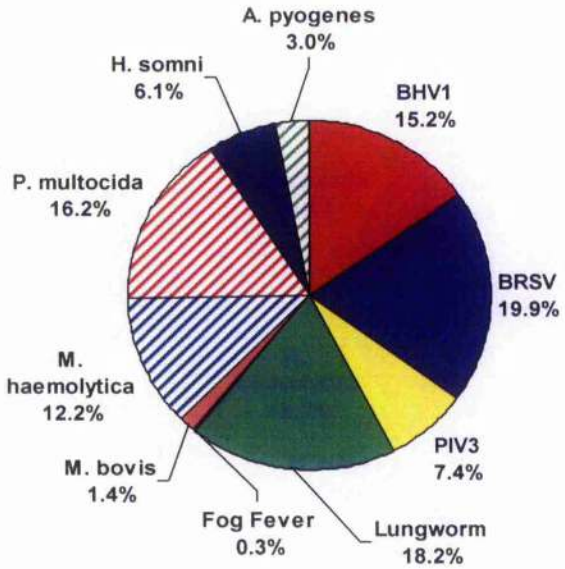


Figure 1.3 Breakdown of confirmed causes of respiratory disease in cattle submitted to Scottish Agriculture College (SAC) over 2002. Data from SAC Veterinary Service monthly report, October 2002. Also shown are incidence levels of *Mycobacterium bovis, Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* and *Actinomyces pyogenes.*

Vaccination will remain central to managing respiratory virus disease in cattle. Breeding for increased resistance to clinical viral disease and/or greater responses to vaccines is likely to be a feasible and productive approach to this problem, rather than attempting complete pathogen eradication. A strategy of optimising animal genetics to respond to vaccination has many advantages, already discussed, including: public health and ethical issues, sustainability,

product quality and animal welfare. It is a system readily adaptable to a wide range of management practices and is open-ended, encouraging host immunity to match the relentless evolution of pathogens they must overcome.

1.8 Genetic Control of Disease Resistance

Resistance to infectious disease is extremely complex. It involves innate and acquired humoral and cell-mediated mechanisms, all of which are under genetic control (Wilkie, 1984). Genetic control suggests effects on phenotype should be predictable, permanent and transmissible. It is this transmissibility that implies immune effects could be a target for selection. Ideally, breeding strategies should enhance both the humoral and cell-mediated components of immunity in a balanced manner so that extreme, harmful phenotypes are avoided (Mallard *et al.*, 1992).

The development and maintenance of resistance mechanisms has an energetic cost, i.e. energy is then not available for reproduction and survival, otherwise genes underlying host resistance would rapidly fix within a population. Counterintuitively, extreme dimorphism in innate resistance is a likely natural outcome as highly resistant animals compete successfully, then lose their competitive advantage over highly susceptible animals as pathogen exposure diminishes with the result that their resistance advantage declines (Boots and Bowers, 2004). Strong immune responses are favoured by extensive antigenic heterogenicity and sustained pathogen evolution but autoimmunity and auto-aggressive reactions favour individuals genetically programmed to have lower immune responsiveness (Wu *et al.*, 1996). These twin pressures are antagonistic, which, in addition to intricate host-pathogen relationships, means that no single genetic solution exists for all "real-world" scenarios. Functional diversification of immune responses is optimal in natural populations and, to some degree, is a constructive objective of mass breeding programmes (Wu *et al.*, 1996).

Although species-specific and breed-specific resistances are important, it is the variability in an individual's disease resistance that has the most potential for inclusion in future breeding programmes (Horin, 1998). Within any breeding programme, deliberate efforts should be made not to over-emphasise currently

important but nonetheless singular aspects of the phenomenon of disease resistance (Horin, 1998). Enhancing resistance by breeding for single genes is possible, as exemplified by immunity to *Escherichia coli* diarrhoea in piglets (Edfors-Lilja and Wallgren, 2000), BLAD in cattle (Nagahata, 2004) and the prion diseases (Hunter *et al.*, 1997), but this is uncommon at present. The *NRAMP1* gene identified in cattle (Feng *et al.*, 1996) is responsible for resistance against intracellular pathogens in mice (Vidal *et al.*, 1993). Mutations in this bovine gene may predispose to diseases associated with *Brucella abortus, Mycobacterium paratuberculosis* and *Salmonella* spp. (Adams and Templeton, 1998). Adverse effects are also possible – a defiency of IgG_2 antibody resulted in chronic non-responsive pneumonia in a young Holstein heifer (Francoz *et al.*, 2004) and in a young Holstein bull, an X-chromosome mutation affecting mucous gland function was described, predisposing to repeated rhinotracheitis (Seeliger *et al.*, 2005).

Heritable effects are also detectable for more complex phenotypes. Glass *et al.* (2005) described differences in the kinetics of responses to *Theileria annulata* infection based on cattle-breed (Holstein vs Sahiwal) in the acute phase protein, α_1 acid glycoprotein, as well as peripheral blood parasitaemia. Hansen *et al* (2003) described non-zero heritabilities for postnatal mortality among Danish Holstein calves due to non-specific infectious diseases. An association between polymorphisms at the defensin loci [BTA27 q13-q14; (Tarver *et al.*, 1998)] and somatic cell counts was reported in Holstein-Friesian cows (Ryniewicz *et al.*, 2003). Abdel-Azim *et al.* (2005) described heritabilities (±s.d.) of 0.14 (±0.07) and 0.16 (±0.08) for uterine infections and udder health, respectively, in a four year study of milking Holstein cows (n =14473).

With particular reference to bovine respiratory disease, heritabilities (±sd) for incidence of pneumonia were estimated as 0.07 (±0.02), 0.29 (±0.03) and 0.17 (±0.05) in Danish Red, Danish Friesian and Danish Jersey cattle breeds, respectively (Wassmuth *et al.*, 2000). In a long-term study of beef calves, Snowder *et al.* (2005) found a peak heritability (±se) estimate of 0.26 (±0.04) for resistance to bovine respiratory disease in composite cross-bred calves. However

Muggli-Cockett *et al.* (1992) were unable to detect significant heritable effects in a similar though smaller study of bovine field respiratory infections.

Continuation of the existing tightly controlled and intensive breeding of cattle has the potential to cause the loss of immunological fitness, due to restriction of genetic *BoLA* polymorphisms, with particular vulnerability to new emerging pathogens (Ballingall *et al.*, 2004b). Selection based on production traits alone may inadvertently lead to animals which cannot produce appropriate infection or vaccine responses (Newman *et al.*, 1996). Incorporating immunological traits, when designing classical biometrical genetic improvement programmes for farm animals is one solution (Soller and Andersson, 1998). This process is facilitated by the availability of new genomic techniques such as QTL analysis.

1.8.1 MHC control of immunity

An association between the MHC and resistance/susceptibility to disease has been described in many species (McDevitt, 2002). In man, the genetic effects are significant but never absolute and such MHC-linked diseases tend to have prominent autoimmune characteristics (Sonderstrup and McDevitt, 2001). Major histocompatability complex genotype can also underlie responsiveness to vaccination, as demonstrated in human hepatitis B vaccines by *MHC-DR3* (Alper *et al.*, 1989; Desombere *et al.*, 1998). Ovsyannikova *et al.* (2004b) reviewed associations between human MHC and antibody responses to measles, mumps, rubella and hepatitis B. In particular, loci *HLA-B8, HLA-B13* and *HLA-B44* were correlated with seronegativity and *HLA-B7* and *HLA-B51* were associated with seropositivity.

Associations have been reported between both class I and class II *BoLA* genes and the incidence of some bovine diseases including persistent lymphocytosis (Stear *et al.*, 1988), ketosis (Mejdell *et al.*, 1994) and mastitis (Oddgeirsson *et al.*, 1988; Park *et al.*, 2004; Sharif *et al.*, 2000). Different *BoLA* haplotypes have been associated with resistance/susceptibility to mastitis in different breeds of cattle (Mallard *et al.*, 1995; Mejdell *et al.*, 1994; Park *et al.*, 2004). Particular *BoLA* alleles have been linked with enhanced neutrophil function (Weigel *et al.*, 1991), resistance to bovine leukosis (Xu *et al.*, 1993), dermatophilosis (Maillard

et al., 1996) and vaccine response/protection (Garcia-Briones *et al.*, 2000). Many authors have described positive correlations between *BoLA*-associated genes and resistance to clinical or subclinical mastitis in cattle (Aarestrup *et al.*, 1995; Kelm *et al.*, 1997; Sharif *et al.*, 1998; Sharif *et al.*, 2000). Substitutions at the *DRB3* locus have been shown to significantly influence mastitis resistance in many breeds of cattle (Dietz *et al.*, 1997). A successful reduction in prevalence of dermatophilosis was achieved by Maillard *et al.* (2003) by selection against a BoLA susceptibility allele in the cattle population native to the island of Martinique.

Some *BoLA* haplotypes express products of single loci which, if promoted within intensive selective breeding programmes, could result in populations with limited MHC diversity and therefore questionable immunological fitness (Ellis, 2004).

1.8.2 Genetic control of humoral immunity - all species

The genetic control of antibody response has been studied in many species, in addition to cattle. In a seminal experiment in selectively-bred mice, Biozzi *et al.*, (1982) reported that although antibody-dependent immunity (against extracellular pathogens) improved, macrophage-dependent immunity (against intracellular pathogens) decreased in mice selected for high serum antibody concentrations. It was concluded that in this group, extended persistence of antigen, due to a diminished macrophage function, prolonged the period of antibody stimulation so allowing serum antibody levels to increase but,by defintion, decreasing CMI (Biozzi *et al.*, 1984; Ibanez *et al.*, 1988). By the sixteenth mouse generation, antibody levels in the high (H) antibody line were 200-fold those of the low (L) antibody line with 14% of this variation due to MHC gene effects (Mouton *et al.*, 1988). However, a comparable study in the pig failed to show a similar effect (Buschman, 1982).

Again, in selected lines of mice Covelli *et al.* (1989) and Puel *et al.* (1995) linked H and L antibody responses back to the genome, in particular to markers on Mmu 12 (*Igh* locus), Mmu 17 (*H-2* locus) and Mmu 6 (*lyt-3*, *Tcrb* or *IgK* loci). To a lesser extent, Mmu 4, Mmu 6 and Mmu 8 also showed involvement. Another study estimated at least three additional loci controlling anti-rabies antibody production in mice previously bred for H and L antibody responses to

sheep erythrocytes (De Franco *et al.*, 1996). Wu *et al.* (1996) tested IgM and IgG responses to bovine rhodopsin in F2-crossbred mice and calculated 67% of the total variance as genetic in origin. Strong QTL associated with antibody responsiveness were identified on Mmu 1 (*D1Mit14* locus), Mmu 5 (*D5Mit122* locus) and Mmu 13 (*D13Mit35* locus). The authors suggested that polymorphisms from several loci contribute to each specific antibody response so that the inheritance of humoral response to any single antigen is, at least partially, dependent on transfer of these loci to progeny.

The more intensively farmed species have been investigated most in this area. Two experiments assessing levels of antibody in chickens induced by sheep erythrocytes (SE) (Pinard *et al.*, 1992) and two bacterial antigens (Kean *et al.*, 1994) reported heritabilities of 0.31 and 0.21, respectively. Further poultry studies showed that females had greater anti-SE antibody levels than males suggesting sex-linkage in this species (Parmentier *et al.*, 2001), and heritabilities of 0.15 and 0.28 in chickens selected for H and L antibody responses to SE, respectively (Bovenhuis *et al.*, 2002). Quantitative trait loci for SE-induced antibody responses in a second-generation F2-cross of H and L lines were identified on chicken (*Gallus gallus* autosome); *GGA6*, *GGA9*, *GGA15*, *GGA16* (MHC-linked), and *GGA27* (Siwek *et al.*, 2003b). The same study highlighted distinct sets of QTL operating for primary and secondary antibody responses and further investigation by Siwek *et al.* (2003a) differentiated exclusive antigen–specific QTL e.g. primary antibody responses to SE on *GGA5* and *GGA23*, from general non-specific QTL, on *GGA3* and *GGA14*, for SE and keyhole limpet hemocyanin antibody responses.

In four generations, Krausslich *et al.* (1983) produced a high-antibody line of pigs with a 64% improvement in antibody responses over non-selected animals. A heritability of 0.27 was estimated for antibody responses induced by keyhole limpet hemocyanin within chosen pig lines (Joling *et al.*, 1993). Strong evidence suggests positive production qualities associated with pigs selected for higher immune responses to chicken albumen (Mallard *et al.*, 1998). Pigs selected for high (H) immune response had larger litters, increased weight gains, and better vaccine responses than pigs selected for low immune responses (Magnusson *et al.*, 1997; Mallard *et al.*, 1992). This was a general enhancement, with both vaccines formulated on carbohydrate and lipospolysaccharide antigens inducing

higher antibody levels in the H group, itself selected earlier on responses to another antigen. The frequency of non-responders to both viral and bacterial vaccination was significantly greater in the L pig group as opposed to the H pig group (Wilkie and Mallard, 1998). Heritabilities for IgG response to *Escherichia coli* antigens O149 and K88 were estimated as 0.29 and 0.45, respectively, in a Yorkshire/Landrace crossbred population (Edfors-Lilja *et al.*, 1994). Furthermore, QTL with strong effects on IgG levels to these *Escherichia coli* antigens, O149 and K88, were identified on pig chromosomes SSC6 and SSC5, respectively (Edfors-Lilja *et al.*, 1998).

Some researchers have investigated associations between production traits and immune responsiveness. Mashaly *et al.* (2000) reported that chickens selected for low (L) antibody response to SE were heavier than chickens with high (H) antibody respones while Cheng *et al.* (2001) demonstrated a negative correlation between levels of total IgG and productivity/survivability in selected chicken strains. In turkeys, selection for increased body weight lowered immune responses (Bayyari *et al.*, 1997). Cattle become more susceptible to bovine leukaemia virus as milk yields rise (Simianer *et al.*, 1991), and in an extension of the pig study described above (Magnusson *et al.*, 1997), mycoplasmal arthritis was more severe, but mycoplasmal pleuritis and peritonitis less severe, in the high antibody response pigs.

1.8.3 Genetic control of humoral immunity - cattle

A study by Lie (1979) in young Norwegian Red bulls vaccinated with human serum albumin found that primary antibody responses were influenced by stronger genetic control than secondary responses, with heritabilities (h^2 (±s.e.)) of 0.56 (±0.33) and 0.15 (±0.19), respectively. The same author reported associations between *BoLA-W16* and high antibody response to human serum albumin, and *BoLA-W2* and low antibody responses to the same antigen (Lie, 1985). In adult dairy cows, Mazengera *et al.* (1985) calculated mean heritabilities of 0.10 (±0.06), 0.10 (±0.07), 0.09 (±0.06) and 0.14 (±0.07) for naturally occurring levels of IgG_1, IgG_2, IgM and IgA, respectively.

Immunisation of three-week old calves with the antigens human erythrocytes and ovalbumin (OVA) produced antibody responses with h^2 of 0.51 (±0.42) and 0.87

(±0.50) respectively (Burton *et al.*, 1989a). Calves ranked consistently as high, moderate or low antibody producers to both antigens. Secondary responses to both antigens appeared more heritable than primary responses, although there was no correlation between responses to each antigen (Burton *et al.*, 1989a).

In the peripartum period, Detilleux *et al.* (1994) described mean h^2 of between 0.55 to 0.84 for naturally occurring levels of IgM, 0.24 to 0.63 for IgG_2 and up to 0.43 for IgG_1 in adult Holstein cows. Newman *et al.* (1996), identified significant effects of both sire and *BoLA* type (in particular *BoLA-W4*, *BoLA-W6* and *BoLA-W7*) on levels of IgG antibody, induced by immunisation against the bacterium *Brucella abortus* in juvenile beef heifers. An association between the bovine allele *IgG2^c (A2)* and mastitis-based breeding values has been recorded (Kelm *et al.*, 1997) and Dietz *et al.* (1997) reported that the expression of allele *BoLA DRB3.2*24* correlated with increased levels of serum IgG_2.

The antibody responses of Holstein cows to the antigens OVA and *Escherichia coli* J5 were evaluated and h^2 were estimated as between 0.32 to 0.50, and 0.50 to 0.58, respectively, varying significantly around parturition (Wagter *et al.*, 2000). Furthermore, García-Briones *et al.* (2000) described associations between allelic variability in specific *BoLA* genes and the humoral response of Hereford steers, induced by vaccination against foot-and-mouth disease. In this study, *BoLA* gene types *DRB3.2*12* and **18* were linked to high antibody levels without protection while *DRB3.2*1, *3,* and **7* were correlated with increased clinical protection to the virus. Recently, microarrays have been used to identify gene variability associated with high and low antibody responsiveness, in adult cattle to chicken albumin (Hernandez *et al.*, 2003), e.g. genes for IL-1β, IL-11 and NK-cell (protein 4 precursor) were linked to low antibody responders.

Breed-associated differences in humoral responses have also been described. Following injection of porcine erythrocytes, Angus calves had significantly higher antibody responses than Simmental calves (Engle *et al.*, 1999). Cole *et al.* (2001) linked dam-breed with antibody responsiveness to immunisation with a live BHV1 vaccine. Purebred Angus dams had calves which responded more strongly to the vaccine than those from crossbred or purebred Brahma dams. Kashino *et al.* (2005), investigating antibody response to tick saliva (*Boophilus microplus*), described significant differences in specific IgG_1 and IgG_2 between Nelore (resistant) and Holstein (susceptible) cattle breeds. Further bovine

humoral research has revealed a selective IgG_2 deficiency among Danish Red cattle which has been associated with reduced disease resistance (Jensen *et al.*, 1981).

It should be noted that a wide variety of techniques were used to measure antibody levels in the above studies including serum neutralisation, radial immunodiffusion, microhaemagglutination, flow cytometry and ELISA. This reflects not only progress in laboratory practice and technology but also the individual strengths and weaknesses of each assay.

1.9 Project aims and objectives

Vaccination against bovine viral respiratory pathogens elicits a systemic antibody response in juvenile cattle. It was assumed that this is a polygenic, quantitative trait resulting in a wide range of response phenotypes. In this project, a large crossbred population of juvenile cattle was immunised with three commercial respiratory viral vaccines. Using specific antibody response as a phenotypic trait, detailed models were fitted. It was hypothesized that this immune response could be modelled using multivariate analysis so that it would be possible to decompose and quantify intrinsic, extrinsic and genetic influences. Using detailed pedigrees for the animals involved, in conjunction with genetic marker data, quantitative trait loci (QTL) were sought for antibody based immune function. It was hoped that any QTL identified could be incorporated into selective breeding programmes, to anchor desirable immune response characteristics in conjunction with improvements in production traits. Improving or maintaining immunological performance, by this method, will have valuable survival and welfare benefits.

- Fit an accurate multivariate model of antibody responses induced by a live intramuscular cattle vaccine against bovine respiratory syncytial virus (BRSV).

- Fit an accurate multivariate model of antibody responses induced by a live intranasal cattle vaccine against bovine parainfluenza 3 virus (PIV3).

- Fit an accurate multivariate model of antibody responses induced by a live intranasal cattle vaccine against bovine herpesvirus 1 (BHV1).

- Metrically determine correlations, relationships and associations between the many environmental factors involved for these phenotypes.

- Identify and assess QTL relevant to the genetic component of the phenotypes observed.

Chapter 2

Demographics, management and health of the crossbred cattle population

2.1 Introduction

Cattle breeds have been discrete socioeconomic entities for hundreds of years, evolving to cope with a diversity of climatic, disease and nutritional pressures, a process consolidated recently by restrictions imposed by pedigree registration and the widespread use of artificial insemination. Modern cattle breeds have been developed for distinct production objectives, perhaps the most obvious being the division into dairy and beef types. This functional separation is best typified by the Holstein Friesian, the predominant milk producing cattle breed worldwide and the Charolais, principally a beef producing breed (Bellmann *et al.*, 2004).

Although European *Bos taurus* cattle breeds appear to be much more closely related than the *Bos indicus* breeds of Africa and Asia (MacHugh *et al.*, 1994), the Charolais breed was reported as a genetic outgroup based on the analysis of 12 microsatellite markers among six European breeds (MacHugh *et al.*, 1994). Further research (Blott *et al.*, 1998) found two major groupings of cattle breeds: a Southern continental group which included Charolais, Limousin, Simmental and Jersey cattle and an Anglo-Northern continental group which included the Holstein Friesian, Belgian Blue, Hereford and Aberdeen Angus breeds. Those authors also found that the Charolais and Holstein-Friesian breeds had little evidence of "genetic bottle-necking" i.e. low levels of heterozygosity and small numbers of alleles. Another study using a combination of microsatellite and biochemical (e.g. blood groups) markers identified similar findings, positioning the Charolais cattle breed (together with the Parthenis breed) some distance on phenograms from the Holstein breed (Moazami-Goudarzi *et al.*, 1997). As Charolais and Holstein breeds are genetically significantly distant, they may be expected to have larger potential differences in gene frequencies. A study by Weiner *et al.* (2004), based on native British cattle breeds, emphasized the importance of including phenotype alongside genotype in all inter-breed comparisons.

The Roslin Bovine Genome Programme (RoBoGen) is a collaborative project between the Roslin Institute, the University of Nottingham, the University of Bristol and the University of Glasgow (SLP LINK Project LK0618). The primary goal was to map the bovine genes controlling traits relevant to the efficient and healthy production of higher quality meat and milk. An experimental herd was established according to a controlled breeding programme and a very large number of traits were measured under standardised conditions, generating phenotypic data on a per-animal basis. The

traits examined ranged from feed conversion efficiency, growth rate, carcase conformation, meat quality, meat tenderness to immune responsiveness associated with disease resistance (Work Package 6). All of the work documented here was completed under Work Package 6.

Charolais and Holstein-Friesian breeds were chosen as founder breeds for the RoBoGen project. It was postulated that such genetically distinct breeds will have differences not only in production traits but also immune capacity due to the selective pressure of the distinct sets of environmental factors and pathogen exposures which beef and dairy cattle encounter. As a result, different alleles or genes may have become fixed or appear at different rates in the two different cattle breeds. Subsequently, when crossed in a structured breeding programme, such contrasting genetic pools should permit maximum information from the experimental population. This in turn will facilitate the detection of QTL relevant to the particular phenotype under investigation. Studies such as that by Heaton *et al.* (2001) have demonstrated breed-based differences in the frequencies of immunity related gene haplotypes in Holstein and Charolais cattle.

As the Charolais-Holstein is a typical terminal cross used by commercial producers by breeding beef sires on non-elite dairy cows, all empirical results for immune response are of direct practical relevance too, especially in relation to the many production traits assessed. In addition, the design of the project allowed the influence of both traditional dairy and beef calf rearing systems on immunological function to be examined. As respiratory disease is a foremost cause of morbidity and mortality among dairy and beef calves, responses to vaccination against three principal respiratory viruses were evaluated. In the same population, other immune function traits measured included T-cell responses to the bacterial mastitis pathogen, *Staphylococcus aureus* (Young, 2002; Young *et al.*, 2005).

The three viral pathogens selected for detailed investigation were Bovine Respiratory Syncytial Virus (BRSV; Chapter 3), Bovine Parainfluenza-3 virus (PIV3; Chapter 4) and Bovine Herpesvirus 1 (BHV1; Chapter 4). Respiratory disease in cattle is often due to multiple pathogens, and commonly presents as a disease complex rather than due to a single agent. To assess interactions and the influences which could affect antibody response phenotype under test, the population was also screened for Bovine Viral Diarrhoea Virus (BVDV; section 2.3.1) and Bovine Coronavirus (BCV; section 2.6.2). Such data, collected on circulating wild-type infections, could give some

indication of baseline levels of heritability for antibody response expected when naturally occurring pathogens affect the study animals.

2.2 Materials

2.2.1 Cattle

The study population was a herd of second-generation Holstein-Charolais calves, founded from four purebred Charolais (F0) sires and eight first-cross Holstein-Charolais (F1) sires, maintained at Blythbank Farm, The Roslin Institute, Edinburgh. This resource herd had been established primarily to identify QTL associated with commercially relevant production traits such as growth rate, meat quality, puberty onset and carcass conformation.

The four F0 Charolais sires used were coded: CH108, CH158, CH200 and CH219 and the eight F1 Holstein-Charolais sires were coded R01, R02, R05, R11, R12, R15, R19 and R21. These twelve sires were used to produce an interline resource that included 91 Holstein backcross [F1 ♂ X Holstein ♀] (**BH**), 80 Charolais backcross [Charolais ♂ X F1 ♀] (**BCH**) and 292 second-cross [F1 ♂ X F1 ♀] (**F2**) calves. Relative breed-cross percentages were therefore BH (20%), BCH (17%) and F2 (63%). The breeding programme is shown schematically (Figure 2.1).

Figure 2.2 shows how the calves were subdivided on sex, breed-cross and year-of-birth. A total of 463 (53% male, 47% female) calves entered the study over four successive years: relative cohort sizes being 57(12%), 120(26%), 141(30%) and 148(32%) in 1998, 1999, 2000 and 2001, respectively.

Four [sire X dam] pairings: [R15 X R09], [R19 X R110] (both 2000 cohort), [R02 X R59] and [R02 X R98] (both 2001 cohort) were selected for multiple ovulation and embryo transfer resulting in 7, 8, 10 and 11 calves, respectively. Excluding these four dams, the study used 197 dams averaging 2.22 calves each over the study period.

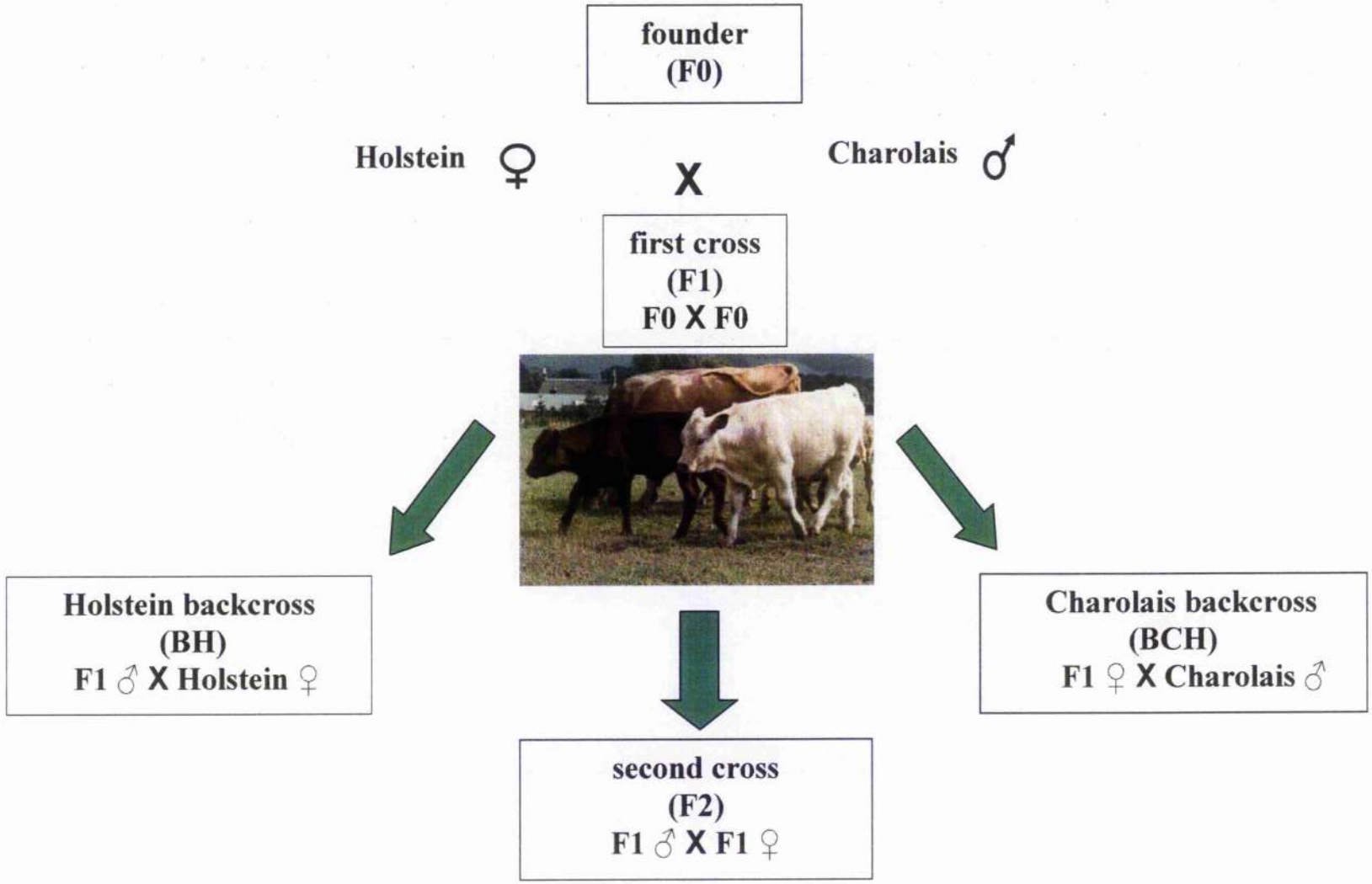


Figure 2.1 The Robogen breeding programme and herd structure. BH (Holstein backcross), BCH (Charolais backcross), F0, F1 and F2 represent founder, first and second intercrosses, respectively.

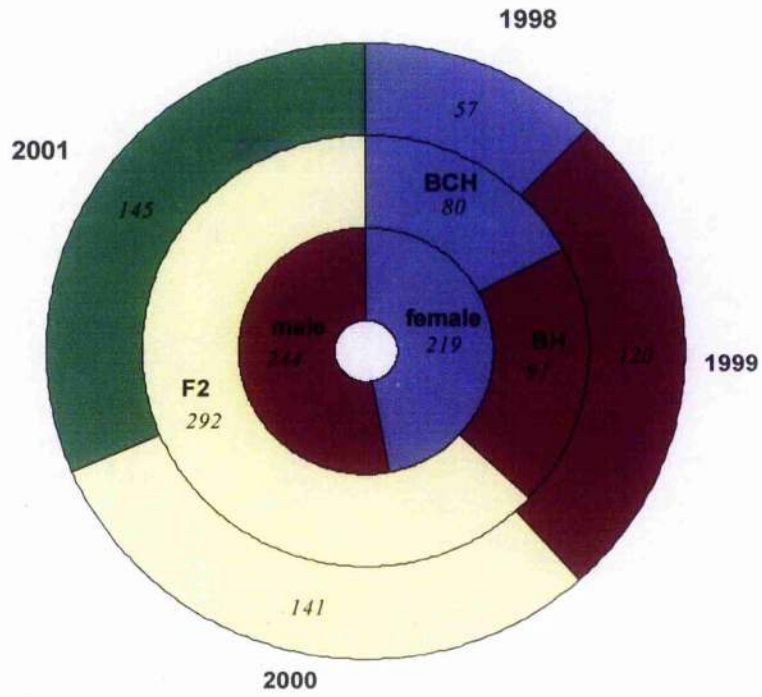


Figure 2.2 Breakdown of Robogen herd grouped on main parameters outer circle (**year-of-birth**: 1998, 1999, 2000 and 2001), middle circle (**breed-cross**: BCH, BH and F2) and inner circle (**sex**: female and male). Actual number of calves per group included in italics. All groups sum to 463 calves.

2.2.2 General management

All calves received ~2 litres of birth-dam colostrum via stomach-tube, before six hours of age. Calves were weighed at birth, tagged and assigned to one of two groups, dependent on *sex*.

All male calves were returned immediately to their birth dams and had unrestricted suckling with their dams at grass, until approximately six months

old. Males were not castrated as they were to be finished as entire bulls with a target weight of 550 kg (for other aspects of the Robogen project). Accurate bodyweight and other conformational data was collected from the male calves regularly at ~60 day intervals, in addition to being linear-type scored by a Holstein Breed Society inspector for conformation and locomotion.

All female calves were weaned by 36 hours, segregated from the rest of the herd and raised indoors, initially on milk-replacer then switched early onto a proprietary compound diet. Female calves continued to be housed separately until the start of the vaccine study. Any differences in results between sexes were, therefore, confounded by differences in the management of calf rearing, essentially representative of the beef and dairy systems of calf production.

2.2.3 Vaccination protocol

For the 77-day duration of the study, all calves were housed in single-sex groups of similar weights. Both male and female calves were treated just before the vaccine study commenced with ivermectin (Ivomec, Merial Animal Health, Essex, UK) to treat for both internal and external parasites. Over the experimental study period, all calves were housed and fed an age-appropriate concentrate diet, with water and straw supplied *ad libitum*.

On Day 0, each calf received 2 ml of the attenuated BHV1 and PIV3 vaccine, Imuresp RP (Pfizer Animal Health, Surrey, UK), according to the manufacturer's recommendations. Rehydrated vaccine was administered intranasally in a 2 ml dose, half of which was placed in each nostril. On Day 28, each calf received 2 ml of Rispoval RS, (Pfizer Animal Health, Surrey, UK), an attenuated BRSV vaccine, by prescapular intramuscular injection, according to the manufacturer's recommendations. All calves were re-immunized with 2 ml of Rispoval RS, intramuscularly on Day 49 after primary vaccination.

Blood samples were collected by jugular venepuncture on Days 0, 14, 28, 42, 63 and 77, providing six longitudinal samples per calf (Figure 2.3). Approximately 3-5 ml of serum were separated by centrifugation within two hours of sampling and stored at -20°C until testing.

Following the completion of the vaccine study, both sets of calves entered feed trials which lasted for six months in the case of the females, and until slaughter for the males.

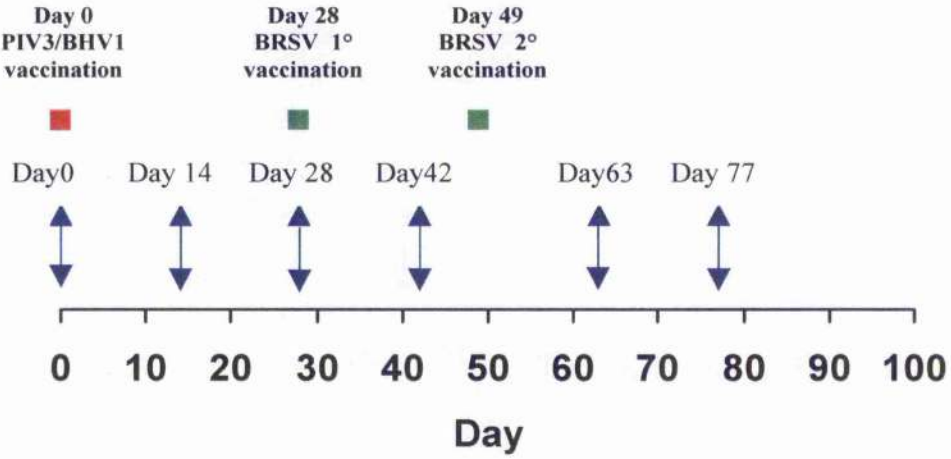


Figure 2.3 Respiratory virus vaccination and sampling time-line. Sera collected on sampling days: Days 0, 14, 28, 42, 63 and 77, marked in blue. PIV3/BHV1 vaccination on Day 0, marked in red. BRSV vaccination on Days 28 and 49, marked in green.

2.2.4 Date-of-birth

Calving seasons remained almost constant between cohorts. Table 2.1 shows the range of birth dates and overall size of the cohorts.

Cohort	First birth	Last birth	Length (days)	No. of calves
1998	31/03/98	03/07/98	94	57
1999	28/03/99	04/07/99	98	120
2000	22/03/00	22/06/00	92	141
2001	22/03/01	20/06/01	90	145

Table 2.1 First and last dates-of-birth, length of calving season and number of calves, per cohort in years 1998 to 2001.

There was no evidence of significant differences of date-of-birth between cohorts (Figure 2.4), tested using one-way analysis of variance (ANOVA; $p > 0.1$). Births were evenly spread over each calving season, with no evidence of clustering in any of the four cohorts. The coefficients of variation were relatively stable at 19.8, 24.4, 21.7 and 20.6 for years 1998, 1999, 2000 and 2001, respectively.

At the start of sampling, calves ranged between 60 and 167 days old. The experiment began (Day 0) on 01/09/1998, 01/09/1999, 05/09/2000 and 04/09/2001 for each of the respective cohorts. There were small but statistically detectable differences between the mean cohort ages (ANOVA; $p < 0.005$) with 1998, 1999, 2000 and 2001 having mean ages of 148.4, 154.2, 162.5 and 158.1 days, respectively. However there was no significant difference between the mean age of male and female calves (t-test; $p > 0.1$).

All statistical analysis was performed using *age* as continuous variate. To facilitate graphical visualisation of results, the continuous variate *age* was recoded into the factor *age,* categorised into five 21-day intervals. Calves aged 60-81 days were classified as age-group I, those aged 82-103 days as age-group II, those aged 104-125 days as age-group III, those aged 126-147 days as age-group IV and those aged 148-169 days as age-group V. There were 23, 62, 70, 128 and 184 calves in age-groups I, II, III, IV and V, respectively.

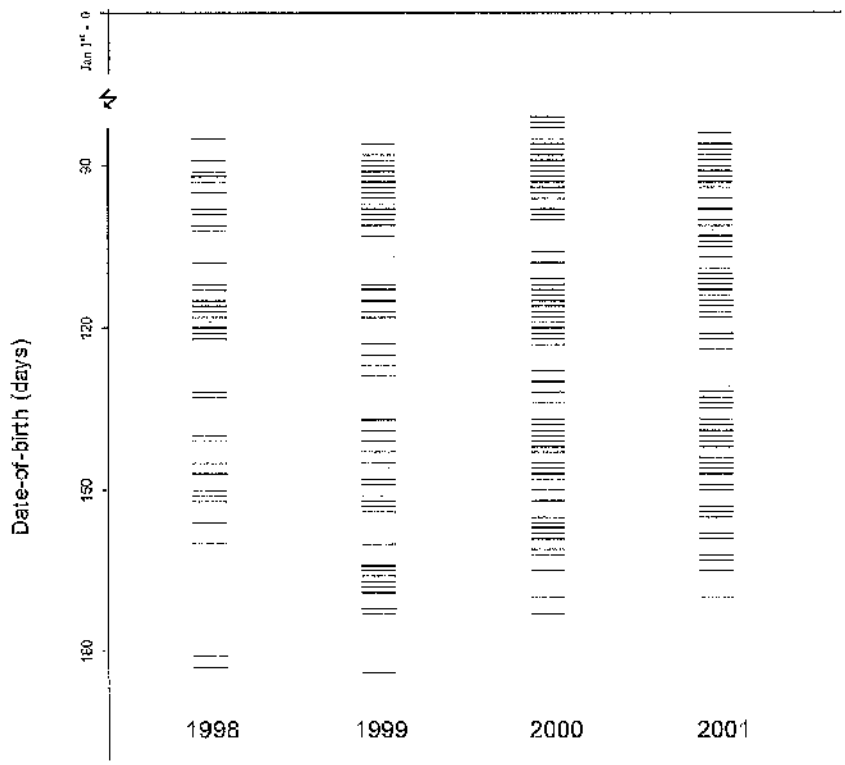


Figure 2.4 Robogen calf date-of-birth, grouped on cohort: 1998 to 2001. Each birth event is represented by a horizontal line. Dates shown in days from January 1st (Day 0) for each respective year.

2.2.5 Dam-age

Date-of-birth was not recorded for 14 of the birth-dams used in the study and these were excluded from the analysis below.

The mean age of cows at calving was 3.91 years (~47 months), with a minimum of 1.93 years (~23 months) and a maximum of 7.41 years (~89 months). There were 45, 115, 128, 132, 30 and 3 birth-dams which had calves in their 2nd, 3rd, 4th, 5th, 6th and 7th years, respectively. The factor *dam-age* was defined as the age at calving of the birth-dam (rounded to the nearest year).

2.2.6 Birthweight

Twenty-three calves had unrecorded birthweights and were excluded from the analysis below.

Male calves were heavier than female calves at birth (t-test; $p < 0.001$), with mean weights of 45.5 kg and 41.6 kg, respectively. There was no evidence of significant differences in birthweight due to breed-cross (ANOVA; $p > 0.1$). There was some evidence (ANOVA; $p < 0.01$) that birthweight increased over the four years in the study with means of 40.6 kg, 42.6 kg, 43.7 kg and 45.5 kg for cohorts 1998, 1999, 2000 and 2001, respectively. Figure 2.5 shows that within each cohort, male calves tended to be heavier than female calves in addition to the gradual overall increase in birthweight over the four cohorts. Within cohorts, there was some evidence of a positive relationship (correlation $r = 0.236$; $p < 0.001$) between date-of-birth and birthweight, i.e. calves born later in the year were marginally heavier than those born earlier. Figure 2.6 shows that there was a small increase in birthweight up to dam-age = four, the effect stabilising thereafter at ~47.4 kg in the male and ~43.7 kg in the females.

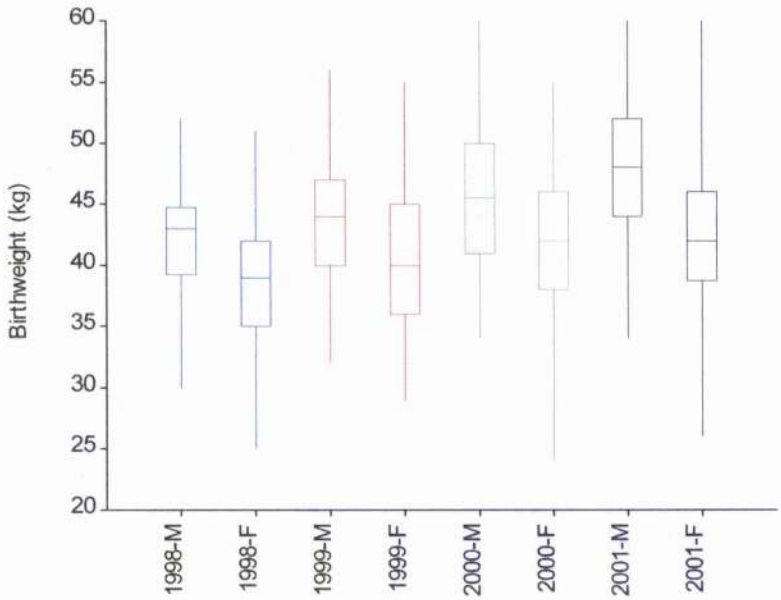


Figure 2.5 RoboGen calf birthweights (kg) grouped by **year-of-birth**: 1998 (blue), 1999 (red), 2000 (green) and 2001 (black) and by **sex**: male (M) and female (F) as marked.

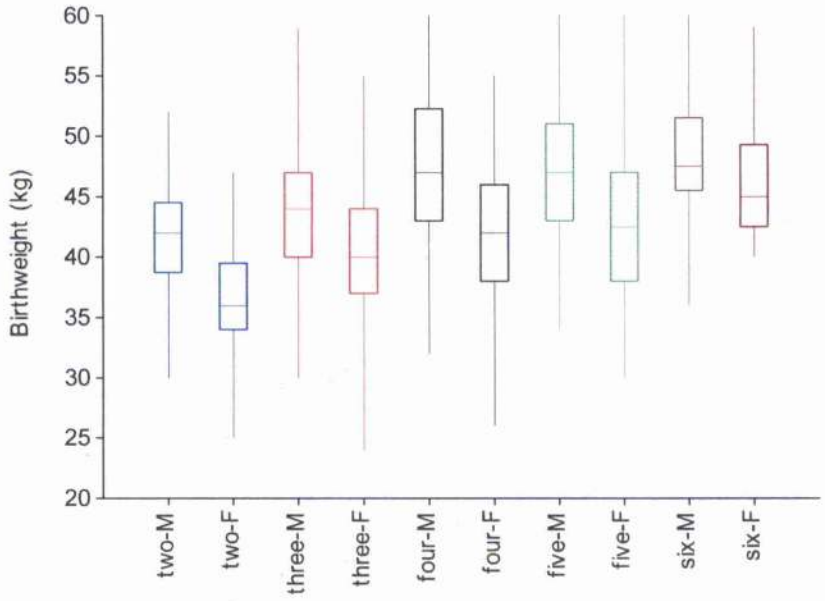


Figure 2.6 Calf birthweights (kg) grouped by **dam-age**: two (blue), three (red), four (black), five (green) and six (brown) and by **sex**: male (M) and female (F) as labelled. (Dam-age seven not shown as n=3 in this group).

2.3 Pathogen screening

The Robogen cattle population was screened for antibodies to two common bovine pathogens found in the UK. Both of these pathogens have been implicated in the bovine respiratory disease complex (Ellis, 2001).

2.3.1 Bovine viral diarrhoea virus

The first, bovine viral diarrhoea virus (BVDV), is an endemic cattle pathogen in the British Isles (Paton *et al.*, 1998), responsible for a disease complex which includes abortion, diarrhoea, lameness, pneumonia and immunosuppression (Graham *et al.*, 1998a). Any interference with immune capability could have serious consequences for the current study, thus prior exposure to BVDV was assessed.

Sera were tested quantitatively by solid-phase antibody capture ELISA specific for BVDV-IgG according to the manufacturer's guidelines (Svanovir BVDV-Ab, SVANOVA Biotech, Uppsala, Sweden). Technical methodology was according

to the manufacturer's instructions without modification and is described in detail later (Chapter 3 – section 3.2.2).

2.3.2 Bovine coronavirus

Paton *et al.* (1998) reported that bovine coronavirus (BCV), the second pathogen screened for here, was almost ubiquitous in the UK cattle population. Bovine coronavirus can act as a respiratory pathogen (Storz *et al.*, 2000) in addition to being potentially enteropathogenic for cattle. As such, BCV may influence the immune responses to vaccination against the three other respiratory pathogens (BRSV, PIV3 and BHV1) assessed later in the current study.

Sera were tested quantitatively by solid-phase antibody capture ELISA specific for total BCV-IgG according to the manufacturers guidelines (Svanovir BCV-Ab, SVANOVA Biotech, Uppsala, Sweden). Technical methodology was according to the manufacturer's instructions without modification and is described in detail later (Chapter 3 – section 3.2.2).

2.4 Results

Only five calves had detectable levels of BVDV antibody above relative optical density (ROD) =10% with no discernible patterns based on sex, year-of-birth, breed-cross, age, dam-age, dam or sire.

However, mean levels of BCV IgG were high (Table 2.2). Antibody to BCV was detected evenly across all groups except the male calves in cohort 2001 (Figure 2.7). Female calves had higher levels of BCV antibody than male calves (t-test; $p < 0.001$) across all cohorts but there was no evidence of breed-cross effects (ANOVA; $p > 0.1$).

	BCV-IgG				
Trait	*(ROD)* **mean**	**variance**	*(ROD)* **median**	*(ROD)* **dQ**	**skewness**
Day 77 BCV overall	121.7	3436.0	129.9	58.8	-0.5
Day 77 BCV females	155.5	1531.9	146.5	58.7	0.3
Day 77 BCV males	90.7	3176.0	104.3	103.2	-0.2

Table 2.2 Levels of BCV-IgG on Day 77 relative to PIV3/BHV1 vaccination. BRSV vaccine administered on Day 0 and Day 21. **dQ** (interquartile range); **ROD** (relative optical density). All data is non-transformed and presented as overall and grouped on *sex*.

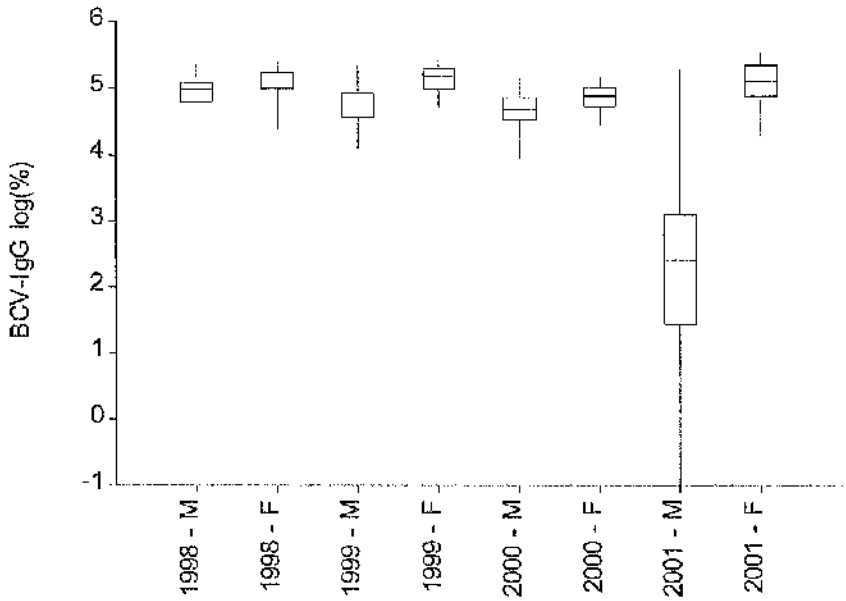


Figure 2.7 Levels of BCV-IgG antibody, grouped on **year-of-birth** (1998 to 2001) and **sex** (male (M) and female (F)). Results are log transformed relative optical density (ROD) data.

Figure 2.8(a) shows how levels of BCV antibody largely tend to be almost constant among all age-groups and sexes, a pattern common when virus is actively circulating within a population. Such high levels of antibody against BCV indicate that the virus was widely disseminated across the four cohorts of the Rohogen herd. However in one sub-group: the male calves of cohort 2001, levels of BCV antibody were much lower. Therefore, within this specific sub-group, there is much less evidence of active BCV infection, emphasizing the unpredictability of field viral infections. In this sub-group, antibody levels against BCV tended to be lower in older calves (Figure 2.8(b)), a pattern consistent with declining levels of maternally-derived antibody.

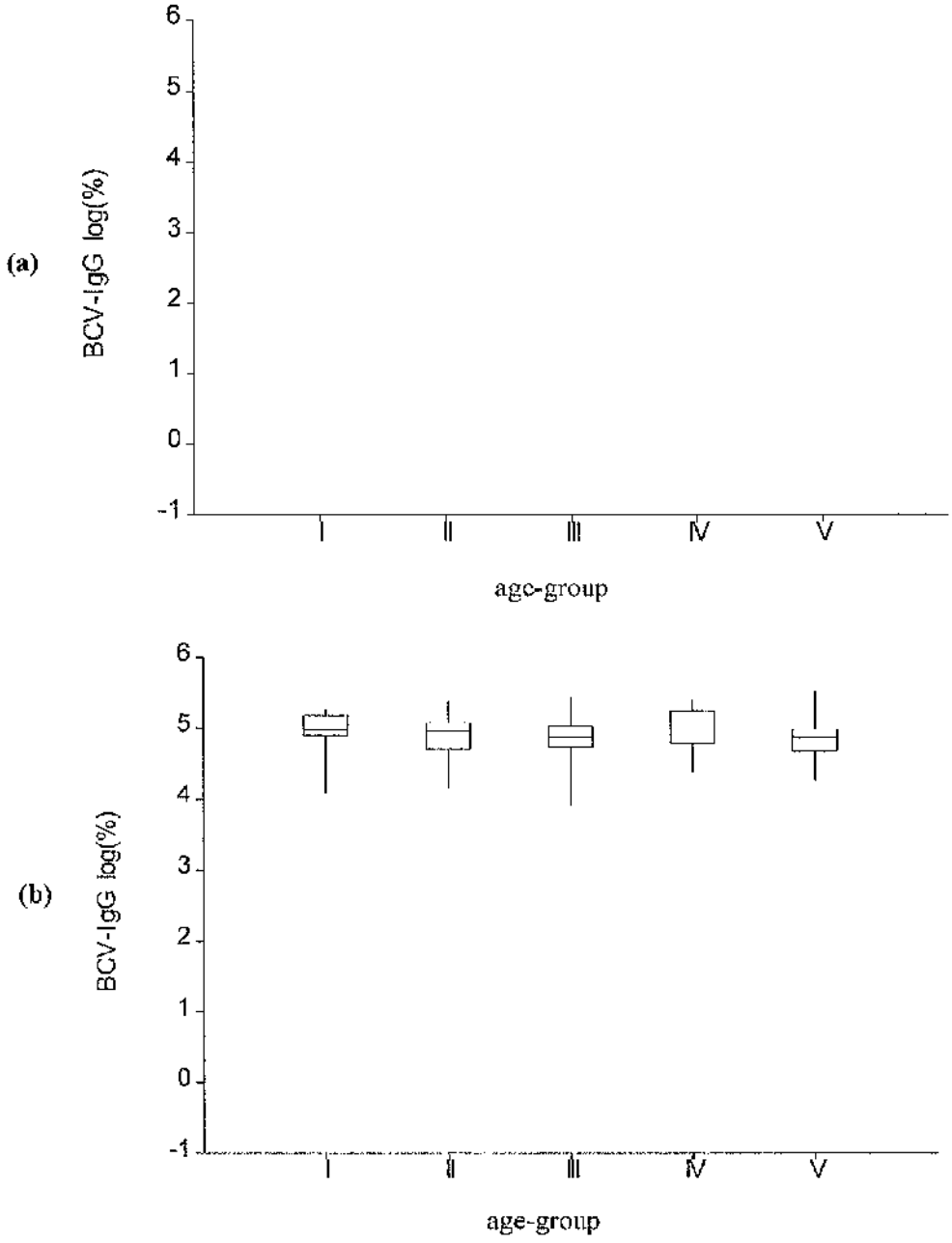


Figure 2.8 Levels of BCV-IgG antibody, grouped on **age**. Results shown for **(a)** 2001 males only and **(b)** all other remaining calves. Age-group I ~ 60-81 days, age-group II ~ 82-103 days, age-group III ~ 104-125 days, age-group IV ~ 126-147 days and age-group V ~ 148-169 days. Results are log transformed relative optical density (ROD) data.

2.5 Discussion

It has been demonstrated that the Robogen cattle population used in this study, although an intensely investigated experimental herd, shares many features in common with commercial UK cattle units. Data were collected across four different years, to reduce one-off seasonal effects. Within the study, both dairy and beef calf-production systems were examined. The population studied was large and calves were tested over a 100-day age range, to allow comprehensive assessment of the important variable, age. All of these features contributed to the broad scope of the study, making it suitable for detailed assessment of the most important variables controlling antibody responses to vaccination in young cattle.

Male calves are larger than female calves at birth (McDermott *et al.*, 1992). For Holstein calves this sex effect was quantified as 8.5% (Kertz *et al.*, 1997) which closely reflects the pattern seen in the current study. Primiparous Holstein cows have smaller calves than Holstein multiparous cows partially because first-parity cows have shorter gestation lengths than later-parity cows (Johanson and Berger, 2003). In the current study, dam-age positively influenced calf birth-weight until four years of dam-age. Kertz *et al.* (1997) made a very similar finding in Holstein calves with a 7-8% increase per-year of dam-age, the effect persisting up to the fourth parity. A study of beef breeds including Charolais cattle also came to the same conclusion (Van Vleck and Cundiff, 1998). The subject is comprehensively reviewed by Rumph and Van Vleck (2004). Other research regarding birth weights in calves has identified influential areas on the bovine genome such as QTL on BTA2 (Grosz and MacNeil, 2001) and BTA23 (Elo *et al.*, 1999).

The Robogen herd operated under normal commercial levels of biosecurity and was not pathogen-free. Screening for viral pathogens was not exhaustive. Adenoviruses, rhinoviruses, reoviruses, enteroviruses, bovine parvoviruses and other bovine herpesviruses are not unusual isolates on UK cattle farms (Stott *et al.*, 1980). Undoubtedly, there will be other pathogens which have remained unreported here. There was however evidence of the virtual absence of one pathogen, BVDV, and the confirmed presence of another pathogen, BCV, two of the more prevalent bovine infections in the UK. Both of these have been

demonstrated previously as components of the bovine respiratory disease complex.

Low levels of herd seroprevalence indicate virtually no active circulation of BVDV within the test population. Those BVDV-specific antibodies detected in this study are probably of colostral origin, most likely due to birth-dam exposure to the virus, prior to joining the Robogen herd. Another possibility is low-level horizontal transmission of pestivirus from the adjoining Blythbank sheep flock.

There may be potential risks in over-interpretation of the BCV results as the antibody levels were due to field infection without standardisation of timing, inoculate dose, route or content. However, they do act as a useful comparison with antibody kinetic patterns obtained in the respiratory vaccine experiment described in later chapters. In addition, they demonstrate that the Robogen herd operated under pressure from multiple bovine pathogens, representative of typical UK farming conditions, and therefore any results from later chapters should be applicable to commercial production environments.

Chapter 3

Antibody to bovine respiratory syncytial virus

3.1 Introduction

3.1.1 Background

Bovine Respiratory Syncytial Virus (BRSV) is a major cause of respiratory disease in both dairy and beef cattle worldwide, acting either as a primary pathogen or synergistically with other agents (Larsen, 2000). Bovine Respiratory Syncytial Virus appears to be endemic in most areas of intensive cattle production (Uttenthal *et al.*, 1996) with morbidity ranging from 80 to 100 percent and mortality up to 10 per cent (Bryson *et al.*, 1978). Bovine Respiratory Syncytial Virus was found to be the virus most commonly implicated in outbreaks of bovine respiratory disease in animals under one year old in the UK (Veterinary Investigation Diagnosis Analysis, 2002). Recent research has shown UK isolates to be more closely related to US variants than those from continental Europe (Nettleton *et al.*, 2003). In Northern Europe, BRSV outbreaks tend to be seasonal, occurring most frequently in early winter (van der Poel *et al.*, 1993).

The peak incidence of severe disease occurs commonly in young animals, less than six months old, often in the presence of maternally-derived antibody (Patel and Didlick, 2004). Poor immunogenicity (Larsen *et al.*, 2001) and silent re-infections among seropositive adults (van der Poel *et al.*, 1993) may explain annual resurgences on "closed" dairy farms. It has been proposed that immunity is more transient in the upper respiratory tract than in the lungs (Stott and Taylor, 1985). Within any herd, passive and active BRSV antibody levels in calves are dictated by a seasonally periodic BRSV recirculation among the adult bovine population (van der Poel *et al.*, 1997). Bovine Respiratory Syncytial Virus often leads to severe disease in young beef and dairy calves (Verhoeff and van Nieuwstadt, 1984) with clinical signs rarely seen at less than two weeks old, most severe between one and five months old and virtually absent in cattle over nine months (Larsen, 2000). Signs of BRSV disease appear between two and five days post-infection and persist for up to eight days (Belknap *et al.*, 1991). Typically these include coughing, lacrimation, nasal discharge, depression, anorexia, pyrexia and tachypnoea often progressing to dyspnoea and interstitial pneumonia (Bryson *et al.*, 1978).

Vaccination is a common control measure, with modified live vaccines considered the safest and most efficacious (Ellis *et al.*, 1995; West *et al.*, 1999), stimulating both mucosal and systemic, humoral and CMI responses (Sandbulte and Roth, 2002). Rispoval RS (Pfizer Animal Health, Surrey, UK) a vaccine used commonly in Europe, based on the RB-94 strain of BRSV (a 1969 Belgian isolate) has been successfully employed commercially since 1978 (Zygraich, 1982). Within any given population, however, all BRSV vaccination regimes induce a broad spectrum of antibody responses in individual animals (Stewart and Gershwin, 1990; Kubota *et al.*, 1992; Outteridge, 1993). Although vaccination does limit clinical disease, it seems not to suppress transmission and recirculation of wild-type BRSV (Piazza *et al.*, 1993; Schrijver *et al.*, 1997).

Neutralizing antibody, induced by either vaccination or natural infection, is critical for subsequent protection against BRSV (West *et al.*, 1999a). Specific BRSV antibody, in acute or convalescent sera therefore provides useful indirect evidence of infection and vaccination. The use of serum antibody levels as a correlate of vaccine-induced protection has been discussed previously (Plotkin, 2001), with the suggestion that for BRSV, antibody may be more important in the prevention of infection than clearance of the virus (McInnes *et al.*, 1999). The development of a BRSV-specific ELISA (Kimman *et al.*, 1987b; Langedijk *et al.*, 1996) has permitted quantitative, repeatable, evaluation of isotype-specific antibody responses to both BRSV vaccination and natural infection in cattle (Elvander *et al.*, 1995; Hazari *et al.*, 2002). These studies have identified a number of factors which influence the level of immune response generated, such as inoculate content and infection site (Kimman *et al.*, 1989), maternally-derived antibody (Uttenthal *et al.*, 2000) and calf age (Thomas *et al.*, 1986).

3.1.2 Genetic Factors

In addition to non-genetic effects, there is growing evidence of a predetermined association between genotype and immunological response to both infection and to vaccination. In humans, significant genetic control of vaccine-induced antibody responses has been reported for the viral diseases hepatitis B (Poland and Jacobson, 1998), measles, mumps and rubella (Tan *et al.*, 2001). Similar evidence extends to the responses of humans to the human equivalent of BRSV,

human Respiratory Syncytial Virus (hRSV), where polymorphisms in human cytokine genes have been demonstrated to modulate both immune response and disease severity (Gentile *et al.*, 2003; Hoebee *et al.*, 2004). The severity of the bronchiolitis caused by hRSV infection is influenced by polymorphisms in the IL-10 gene (Wilson *et al.*, 2005) and a genetic locus controlling susceptibility to viral bronchiolitis has been identified near the human IL-8 gene (Hull *et al.*, 2004). The CC chemokines – RANTES (regulated on activation, normal T-cell expressed and secreted) and macrophage inflammatory protein (MIP)-1α and their associated receptor CCR5 have been implicated as determining the severity of bronchiolitis caused by hRSV (Hull *et al.*, 2003). Bovine homologues of the human CCR5 receptor have been identified though as yet are unmapped. Stark *et al.* (2002) also described 15-fold differences in susceptibility to hRSV challenge between strains of inbred mice. Also in mice, hRSV post-vaccination eosinophilia was found to be influenced by MHC haplotype (Hussell *et al.*, 1998).

Although there is no comparable evidence published regarding BRSV specifically, the influence of genetics on endogenous serum antibody in cattle has been established (Burton *et al.*, 1989b; Lie, 1979; Mazengera *et al.*, 1985). Cumulatively, these findings and others reviewed by Glass (2004) strongly imply that a significant component of variation in protective immunity dependent on vaccine-induced antibody is genetically determined.

The proposed hypothesis is that in a sufficiently large, fully pedigreed population, genetic influences on immune responses are detectable and the size of this genetic component will be computable. In the current study, a commercially available live BRSV vaccine was used on a juvenile crossbred bovine population and the specific IgG antibody responses were measured by ELISA. Subsequent analysis allowed the separation of heritable factors, such as additive and maternal genetic effects, from non-transmissible factors, such as year-of-birth, age and sex effects. Accurate compartmentalisation of the observed variation permits their respective contributions to the overall immune response to be assessed and quantified.

3.2 Methods

3.2.1 Vaccination and sampling

The study population was the Robogen herd. This experimental population and the vaccination protocol are described in detail in Chapter 2, section 2.2.3. Blood samples were collected on Days -28 and -14 pre-vaccination; Day 0, the day of vaccination and Days 14, 35 and 49 post-vaccination, providing six longitudinal samples per calf.

3.2.2 ELISA for detection of total BRSV-specific IgG antibody

Sera were tested quantitatively by solid-phase antibody capture ELISA specific for total BRSV-IgG according to the manufacturer's guidelines (SVANOVA Biotech, Uppsala, Sweden). Briefly, samples were added at a dilution of 1/25 in PBS-Tween buffer (0.01 M pH 7.4 phosphate buffered saline (PBS) containing 0.05% Tween 20) to each of two wells, one coated with viral antigen and the other with control antigen, to make a final volume of 100 µl. Common positive and negative control sera were included on each 96-well microtitration plate also at a dilution of 1/25. Each plate was shaken, sealed and incubated for one hour at 37°C, then washed three times with PBS-Tween buffer. After the third wash, each plate was tapped dry and 100 µl of horse-radish peroxidase-conjugated anti-bovine IgG added to each well. Each plate was then incubated for a further hour at 37°C and washed as previously described. One hundred µl of substrate chromogen solution (3',3',5',5'-tetramethylbenzidine and hydrogen peroxide) were added to each well and the plate was incubated at room temperature for ten minutes after which the reaction was stopped by adding 50 µl of 2 M H_2SO_4 per well. After shaking, the optical density (OD) of each well was measured immediately at 450 nm on a MRX microplate plate reader (Dynex Technologies, Chantilly, USA). The corrected optical density (COD) value for each sample and the assay control sera were calculated by subtraction of the OD value of each control antigen-coated well from that of the corresponding viral antigen-coated well, and the relative optical density (ROD) value for each sample calculated as a percentage of the highly positive control serum on a per-plate basis:

$$\text{ROD (\%)} = \frac{\text{COD serum sample} \times 100}{\text{COD positive control}} \qquad \text{[EQ 3.1]}$$

Samples were tested in duplicate and repeated if these pairs of ROD values were >10% discordant. The intra-plate and inter-plate repeatabilities for the BRSV-specific IgG ELISA were 97.6% and 98.9%, respectively.

3.2.3 ELISA for detection of BRSV-specific IgG_2 antibody

Total BRSV-IgG_2 levels were tested using a modified form of the above assay. All dilutions were as described above but 100 µl of 1/10,000 horse-radish peroxidase-conjugated anti-bovine IgG_2 (Acris Antibodies GmBH, Hiddenhausen, Germany) was used as the secondary antibody. Following assay optimisation by cross-titration, samples were again included at a dilution of 1/25 in PBS-Tween buffer. Levels of BRSV-IgG_2 antibody were tested for Days 0, 14, 35 and 49 and the corresponding ROD values, calculated as above. The intra-plate and inter-plate repeatabilities for the BRSV-specific IgG_2 ELISA were 98.7% and 97.2%, respectively. This assay did not discriminate between allotypes IgG_{1a} and IgG_{2b} (Williams, 1990)

3.2.4 Serum neutralisation test for detection of BRSV antibody

For validation and comparison, serum antibody levels specific to BRSV were determined independently (Biobest Laboratories Ltd, Pentlands Science Park, UK) using another *in vitro* method in addition to the ELISA, the serum neutralisation test (SNT; (Gillette, 1983)). A subset of 30 sera, five selected from each of the six sampling days, were analysed by SNT. Sera were chosen to represent the range of BRSV-IgG responses on each sampling day. Correlations between SNT results and the corresponding ELISA results were calculated.

3.3 Statistical Methods

3.3.1 Data preparation

Means, medians, variance and interquartile range for all traits were calculated using Genstat 7.0 (VSN International Ltd, Herts, UK). The same software was used to calculate skewness (a measure of distribution symmetry, Appendix A.1) for each trait, with normality set as a skewness of 0.0. All data collected on total BRSV-IgG and BRSV-IgG_2 antibody levels was subsequently normalised by $\log_e$ transformation.

Deviations (Δ) in antibody levels between all days post-vaccination (Days 14, 35 and 49) and the day of vaccination (Day 0) were calculated for both antibody isotypes - so typically for Day 35:

$$\Delta b\text{IgG (D0 to D35)} = \text{Day 35 BRSV-IgG - Day 0 BRSV-IgG} \quad \text{[EQ 3.2]}$$

The total area under both BRSV-IgG antibody curves was calculated pre-vaccination (between Days -28, -14 and 0) and post-vaccination (between Days 0, 14, 35 and 49), inclusive. The trapezoidal rule (Appendix A.2) was used to calculate a good working approximate of this summary trait (Abramowitz and Stegun, 1972). So typically:

$$\text{3-day-area } b\text{IgG}_{(\text{prevne})} = \text{area under IgG curve for Days -28, -14 and 0} \quad \text{[EQ 3.3]}$$

3.3.2 Non-regressive statistical methods

Depending on the number of factor levels, either unpaired Students t-test (if 2 levels) or one-way analysis of variance (ANOVA; (if $\geq$ 3 levels)) were used to compare factor effects. Tests were confirmed as significant if $p < 0.05$ and highly significant if $p < 0.001$. Pearson product moment correlations between combinations of factors and variates were evaluated for levels of total BRSV-IgG and BRSV-IgG_2 on all sampling days and r values calculated. Excel 2003 (Microsoft Corp., Seattle, USA) was used for collation and preparation of data, Genstat 7.0 (VSN International Ltd, Herts, UK) was used for statistical analysis,

as stated and ASREML (VSN International Ltd, Herts, UK) used for statistical analysis, as stated.

3.3.3 Logistic regression

By introducing a binary coding system whereby **Δ*b*IgG (D0 to D14), Δ*b*IgG (D0 to D35)** and **Δ*b*IgG (D0 to D49)** were converted into either 1 (positive Δ) or 0 (negative Δ), a variate suitable for analysis by logistic regression was generated. Linear binomial models with these binary variates as the response variable (y) and the level of Day 0 BRSV-IgG antibody as the explanatory variable (χ) were fitted to the data using a logit link function, $\left(\log\left(\frac{p}{1-p}\right)\right)$ and the associated model parameters (α and β) estimated by logistic regression methods (Genstat 7.0).

Using

$$y = \alpha + \beta\left(\chi - \bar{\chi}\right) \qquad \text{[EQ 3.4]}$$

where p is the probability of binary 1, (i.e. a positive Δ); χ is the population mean level of Day 0 BRSV-IgG antibody. Assuming that when $p = 0.5$, a positive or negative antibody response was equally likely, it was possible to provide an empirical estimate of the threshold at which pre-existing BRSV antibody begins to block the BRSV vaccine response. This mechanistic approach is exploratory in nature, greatly simplifying the modelling of this relationship. No other relevant factors were included at this stage. If the first threshold (p =0.5) can also be envisaged as the point at which 50% of the calves showed a positive antibody response, a second useful threshold may be estimated when p =0.9 or when a positive vaccine response may be expected in 90% of the population.

3.3.4 Univariate mixed linear regression

Mixed linear models were fitted to the data using Residual Maximum Likelihood (REML) methods, with Genstat procedures (Genstat 7.0, VSN International Ltd). The impact of a number of explanatory factors was evaluated and the remaining variation decomposed into variances of either transmissible or environmental

origin. The fixed effects in the model, with appropriate degrees of freedom (df), were *breed-cross* (BCH, BH, F2; 2df), *sex* (male, female; 1df), *year-of-birth* (1998, 1999, 2000, 2001; 3df), *dam-age* (2, 3, 4, 5, 6, 7; 5df) and *age* at first sampling (continuous variate), with calf *sire* included as a random effect. As male and female calves were reared separately and under different management, the effect *sex* incorporates management and environmental components, in addition to its physiological effect. To improve model clarity, the fixed effect *breed-cross* was further resolved into *Holstein* and *recombination-loss* fractions in accordance with: *breed-cross* **BCH** = (0.25 *Holstein*, 0.50 *recombination-loss*); *breed-cross* **F2** = (0.50 *Holstein*, 1.0 *recombination-loss)* and *breed-cross* **BH** (0.75 *Holstein*, 0.50 *recombination-loss)*. *Holstein* represents the expected genetic contribution of the Holstein cattle breed, while *recombination-loss* represents the expected fractional loss in the phenotype of progeny, arising from the non-productive recombination of genetic loci at fertilisation which previously interacted in either of the two parents (Fries *et al.*, 2002).

Thus the linear model was:

$$Y_{ijklmnp} = \mu + \alpha_i + \beta_j + \delta_k + \pi_l + \gamma_m + \varepsilon(s_{ijklmnp} - \hat{s}) + u_{ijklmn} + e_{ijklmnp} \qquad \text{[EQ 3.5]}$$

where

$Y_{ijklmnp}$ - observation of phenotypic trait,
μ - population mean,
α_i - effect (fixed) of sex i (i = 1,2),
β_j - effect (fixed) of Holstein fraction j (j = 1,2,3),
δ_k - effect (fixed) of recombination-loss fraction k (k = 1,2,3),
π_l - effect (fixed) of dam-age l (l = 2,3,4,5,6,7),
γ_m - effect (fixed) of year-of-birth m (m = 1,2,3,4),
$\varepsilon(s_{ijklmnp} - \hat{s})$ - effect (fixed) of sampling age $s_{ijklmnp}$(age of calf *ijklmnp*) expressed as a deviation from the population mean age $\hat{s}$,
u_{ijklmn} - direct heritable effect (random) of sire $n \sim N(0, \mathbf{I}\sigma_s^2)$,
$e_{ijklmnp}$ - residual error (random) $p \sim N(0, \mathbf{I}\sigma_e^2)$.

Where both u_{ijklmn} and $e_{ijklmnp}$ were assumed to have multivariate normal distributions with means **0** and (co)variances $\mathbf{I}\sigma_s^2$, and $\mathbf{I}\sigma_e^2$, respectively. Estimates of direct heritability ($\mathbf{h^2}$) were calculated using:

$$h^2 = 4\frac{\sigma_s^2}{\sigma_p^2} \qquad \text{[EQ 3.6]}$$

where phenotypic variance σ_p^2 was estimated as $\sigma_p^2 = \sigma_s^2 + \sigma_e^2$, as defined above. The basic model was extended for post-vaccination Days 14, 35 and 49 by

adding the appropriate level of pre-existing BRSV-IgG antibody on the day of vaccination (Day 0 BRSV) as a covariate (q).

The significances of the fixed effects and their interactions were estimated by the generalised Wald test. Only those interactions achieving statistical significance ($p < 0.05$) were retained in the final best-fit model. The significance of random effects in the model was determined using log-likelihood ratio tests. The robustness of each model produced was verified by examining the linearity of standardised deviance residuals against normal order statistics (Belsley *et al.*, 1980) as exemplified in Appendix A.5.

3.3.5 Bivariate mixed linear regression

To evaluate heritable maternal effects, a two step approach was adopted. To account for the potential scale of variation between male and female calves kept in different environments, a bivariate mixed linear analysis was made using ASREML procedures (ASREML, VSN International Ltd) for each sampling day with male and female responses treated as separate traits (Gilmour *et al.*, 2000). However given the limitations imposed by the number of observations, these analyses assumed that the fractions of variance explained by maternal or additive variance were the same in each sex/environment and that maternal and additive effects were perfectly and equally correlated across both sexes. Although such assumptions would be implicitly made if the BRSV-IgG responses of both sexes were analysed as the same trait, the above approach gave an opportunity for homogeneity of variances across the sex/environment boundary to be tested.

Thus the bivariate linear model was:

$$Y_{ijklmnp} = \mu_i + \alpha_{ij} + \beta_{ik} + \gamma_{il} + \varepsilon_i (s_{ijklmnp} - \hat{s}) + u_{ijklm} + a_{ijklmn} + e_{ijklmnp} \qquad [EQ\ 3.7]$$

where

$Y_{ijklmnp}$ - observation of phenotypic trait,
μ_i - effect (fixed) of sex i (i = 1,2),
α_{ij} - effect (fixed) of year-of-birth j for sex i (j = 1,2,3,4),
β_{ik} - effect (fixed) of cross k for sex i (k = 1,2,3)
γ_{il} - effect (fixed) of embryo transfer l for sex i (l = 1,2)
$\varepsilon_i (s_{ijklmnp} - \hat{s})$ - effect (fixed) of ε_i, regression coefficient for sex i on sampling age $s_{ijklmnp}$ expressed as a deviation from the population mean age $\hat{s}$.
u_{ijklm} - maternal effect (random) of birth-dam m ~ multivariate N(0,**I** $\sigma^2_m(i)$), with $\sigma^2_m(i)$ constrained to be $m^2\sigma^2_p(i)$ for sex i,
a_{ijklmn} - additive effect (random) of calf n ~ multivariate N(0, **A** $\sigma^2_a(i)$), with $\sigma^2_a(i)$ constrained to be $h^2\sigma^2_p(i)$ for sex i,
$e_{ijklmnp}$ - residual error (random) p ~ N(0, **I** σ^2_e (i)).

Where $\sigma^2_p(i)$ was the phenotypic variance for sex i, **I** is the identity matrix and **A** is the additive genetic relationship matrix, calculated from the pedigree traced back to the purebred founder sires.

The constraints on the variance components were achieved by fixing the maternal and additive genetic correlations for the effect in males and in females to be both 0.999 and by constraining the additive and maternal transmissible variance components in each sex to have identical linear relationships with the residual variance for that sex e.g. $\sigma^2_m(i) = c_1$, $\sigma^2_e(i) = 0$ :: $\sigma^2_a(i) = c_2$, $\sigma^2_e(i) = 0$ where c_1 and c_2 were the same constants for both sexes. This approach necessitated multiple runs to find the maximum likelihood, made by searching across a grid of values for c_1 and c_2, and the maximum located to an accuracy of ±0.005. The validity of the assumption of equal direct heritable and maternal heritable fractions of variance was tested. The magnitude of phenotypic variances and components for the two sexes/environments were found to be sufficiently similar to allow pooling. Subsequent models therefore dropped dependence of the variance components on sex i.

The second step examined the correlations of the different components across the different periods using multi-trait linear models. For reasons made clear in the results this was done in several stages: joint analysis of BRSV-IgG levels on pre-vaccination Days -28, -14, and 0; joint analysis of BRSV-IgG levels on post-

vaccination Days 14, 35 and 49; joint analysis of PRE and BRSV-IgG levels on Day 14, (where PRE is the mean of the three observations on Days -28, -14 and 0); and joint analysis of PRE with BRSV-IgG levels on Days 35 and 49.
The linear models then fitted were:

$$Y_{hijklmnp} = \mu_{hi} + \alpha_{hij} + \beta_{hik} + \gamma_{hil} + \tau_{hi}(s_{hijklmnp} - \hat{s}) + u_{hijklm} + a_{hijklmn} + e_{hijklmnp} \quad [EQ\ 3.8]$$

where the model terms differ from those of the previous model only by the addition of subscript $_h$ denoting each trait *h*. The variance covariance matrices for the distributions of u_{hijklm}, $a_{hijklmn}$ and $e_{hijklmnp}$ were then direct products $\mathbf{V}_m \otimes \mathbf{I}$, $\mathbf{V}_a \otimes \mathbf{A}$ and $\mathbf{V}_e \otimes \mathbf{I}$ where $\mathbf{V}_m$ was the (co)variance matrix for the maternal transmissible components across traits, with $\mathbf{V}_a$ and $\mathbf{V}_e$ similarly defined for the additive transmissible and residual effects across traits.

3.4 Results

3.4.1 Descriptive analysis

Substantial variation in the levels of total BRSV-IgG and BRSV-IgG_2 antibody were demonstrated in this population of young cattle of mixed age, sex, breed and management history (Table 3.1). Generally, a steady reduction in mean and median levels of pre-existing BRSV-IgG occurred throughout the population in the four-week pre-vaccination period. There was no detectable difference (t-test, $p > 0.1$) between mean ΔbIgG(D-28 to D-14) and ΔbIgG(D-14 to D0) suggesting that pre-vaccination, the rate of decay may be constant. Both measures of spread; variance and interquartile range also tended to decline pre-vaccination, as increasing numbers of calves approached undetectably low levels of pre-existing BRSV-IgG. For similar reasons, the distribution of BRSV-IgG became progressively more skewed with the later pre-vaccination samples.
More complex patterns were apparent in the levels of BRSV-IgG and BRSV-IgG_2 post-vaccination. Vaccine efficacy was good - on post-vaccination Days 14, 35 and 49, respectively 64.8%, 80.6% and 75.1% of calves had increased levels of BRSV-IgG above Day 0 levels. The equivalent percentages for increases in levels of BRSV-IgG_2 were 71.0%, 87.6% and 83.4% on Days 14, 35 and 49, respectively. Overall mean levels of both BRSV-IgG and BRSV-IgG_2 antibodies

increased, 6.4-fold and 5.4-fold, respectively, between Day 0 and Day 35 (Figure 3.1(a+b)). Both BRSV-IgG variance and interquartile range dropped on the earliest post-vaccination sampling day (Day 14) then increased again on Days 35 and 49. The distribution of BRSV-IgG levels approached normality on Day 35 but had returned to a more skewed pattern by Day 49. BRSV-IgG_2 variance and interquartile range were much higher on post-vaccination Days 35 and 49, than the earlier Days 0 and 14. Post-vaccination, BRSV-IgG_2 levels were 6.8-fold, 4.7-fold, 3.2-fold and 2.5-fold lower than BRSV-IgG levels on Days 0, 14, 35 and 49, respectively. The distribution of BRSV-IgG_2 levels remained markedly skewed throughout the study.

(a) total BRSV-IgG

Trait	(ROD) mean	variance	(ROD) median	(ROD) dQ	skewness
Day -28 *b*IgG	26.9	774.7	16.9	39.3	1.1
Day -14 *b*IgG	20.4	636.2	10.2	25.8	1.5
Day 0 *b*IgG	13.8	417.6	4.8	16.7	2.2
Day 14 *b*IgG	20.0	238.6	16.1	16.0	2.0
Day 35 *b*IgG	38.9	365.3	37.5	27.6	0.5
Day 49 *b*IgG	31.3	419.4	27.1	29.4	0.9
Δ*b*IgG(D-28 to D-14)	-6.6	67.1	-4.8	10.6	0.5
Δ*b*IgG(D-14 to D0)	-6.5	78.9	-3.4	9.3	1.9
Δ*b*IgG(D0 to D14)	6.2	245.1	5.3	21.7	-0.1
Δ*b*IgG(D14 to D35)	18.7	468.7	17.6	38.5	0.2
Δ*b*IgG(D35 to D49)	-7.7	226.1	-7.1	11.8	1.4
3-day-area *b*IgG $_{(prevac)}$	81	9213	42	106	1.0
3-day-area *b*IgG $_{(postvac)}$	192	7947	178	106	1.0

(b) BRSV-IgG$_2$

Trait	(ROD) mean	variance	(ROD) median	(ROD) dQ	skewness
Day 0 *b*IgG$_2$	2.2	6.6	1.3	2.2	2.7
Day 14 *b*IgG$_2$	4.5	16.7	3.4	4.4	2.0
Day 35 *b*IgG$_2$	12.6	115.4	9.7	12.7	1.9
Day 49 *b*IgG$_2$	13.1	175.0	9.5	13.6	2.3
Δ*b*IgG$_2$(D0 to D14)	2.4	20.0	1.5	4.5	1.5
Δ*b*IgG$_2$(D14 to D35)	8.0	93.9	5.3	11.0	1.9
Δ*b*IgG$_2$(D35 to D49)	0.6	92.7	-1.0	5.1	4.2
3-day-area *b*IgG$_2$ $_{(postvac)}$	58.0	1858.9	47.4	52.5	1.7

Table 3.1 Levels of **(a)** total BRSV-JgG on Days -28 to 49 and **(b)** BRSV-JgG$_2$ on Days 0 to 49, relative to first vaccination. BRSV vaccine administered on Day 0 and Day 21. BRSV-IgG$_2$ levels not measured before Day 0. **dQ** (interquartile range); **ROD** (relative optical density). Values based on non-transformed ROD data.

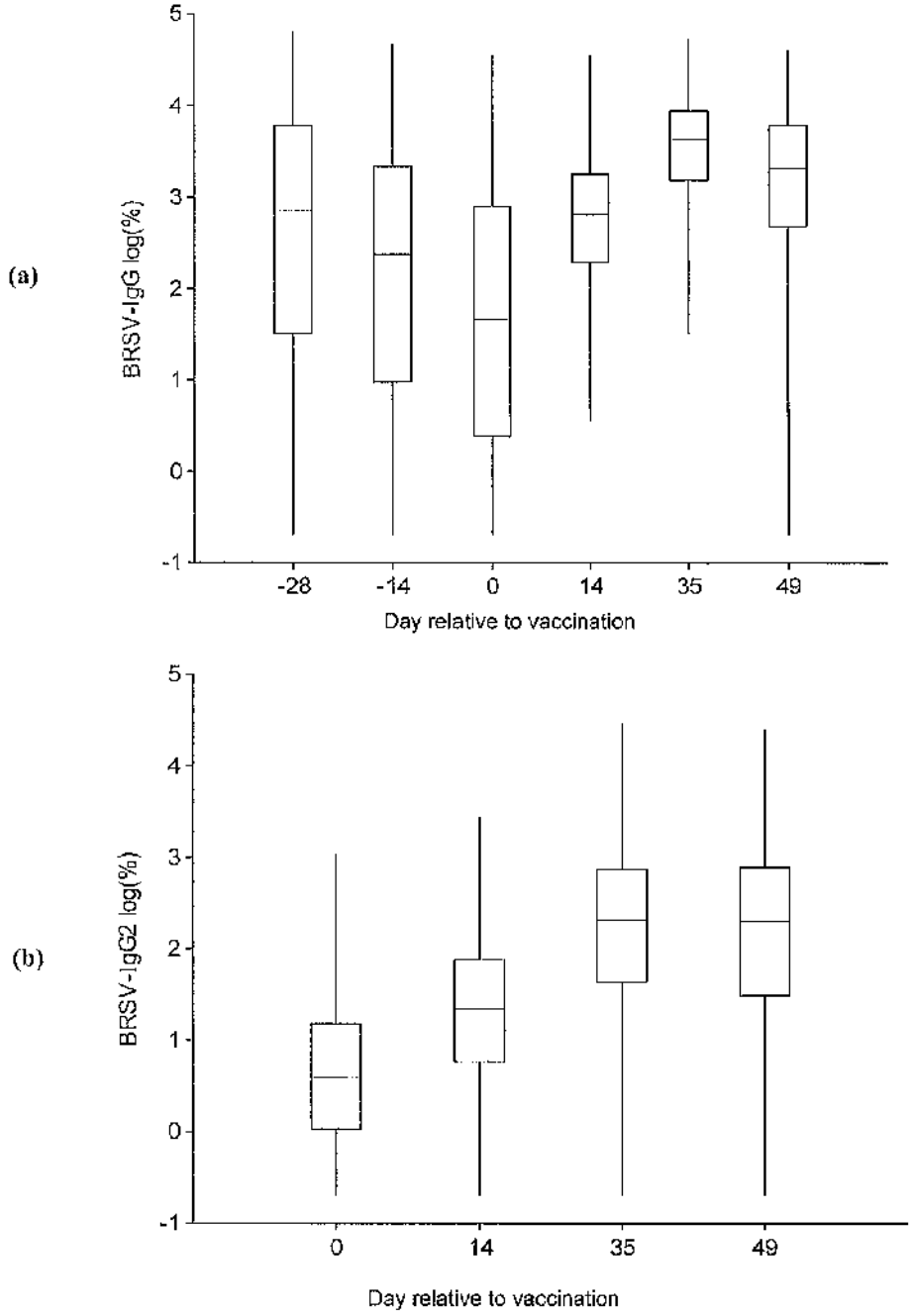


Figure 3.1 Antibody levels of **(a)** BRSV-IgG on Days -28 to 49 and **(b)** BRSV-IgG_2 on Days 0 to 49, relative to first vaccination. Median (central horizontal line), quartiles (outer horizontal lines), and range (outer vertical lines) shown. BRSV vaccine administered on Day 0 and Day 21. BRSV-IgG_2 levels not measured before Day 0. Values are log transformed ROD data.

3.4.1.1 BRSV-IgG and BRSV-IgG_2 antibody half-lives

Based on the regression coefficients for Days -28, -14 and 0, the half-life of total BRSV-IgG was estimated as 24.7 days (95% confidence interval: 22.4 – 27.0 days). Based on the regression coefficient for Day 0, the antibody half-life of BRSV-IgG_2 was longer, estimated to range between 52 and 93 days.

3.4.1.2 BRSV-IgG and BRSV-IgG_2 antibody correlations

Pre-vaccination, BRSV-IgG levels for each sampling day were strongly correlated with BRSV-IgG levels for previous sampling days with (r =0.958) between Day -14 and Day 0, (r =0.928) between Day -28 and Day -14 and (r =0.908) between Day -28 and Day 0. BRSV-specific IgG_2 levels were measured on only one pre-vaccination day (Day 0).

The strongest post-vaccination correlations were found between the same antibody isotype collected on consecutive sampling days (Figure 3.2) with slightly weaker correlations for Day 49 BRSV-IgG than the other BRSV-IgG and BRSV-IgG_2 sampling days. Moderate correlations (r =0.560; r =0.526) were found between serum levels of Day 35 BRSV-IgG and BRSV-IgG_2 on Day 35 and 49, respectively (Table 3.2). Other correlations between various isotypes and post-vaccination sampling day pairings were weak (r <0.250). All reported correlations are $p < 0.001$.

The raw data for levels (untransformed) of BRSV-IgG on Days -28 to 49 and BRSV-IgG_2 on Days 0 to 49, relative to vaccination, are shown in Appendices A.3 and A.4, respectively.

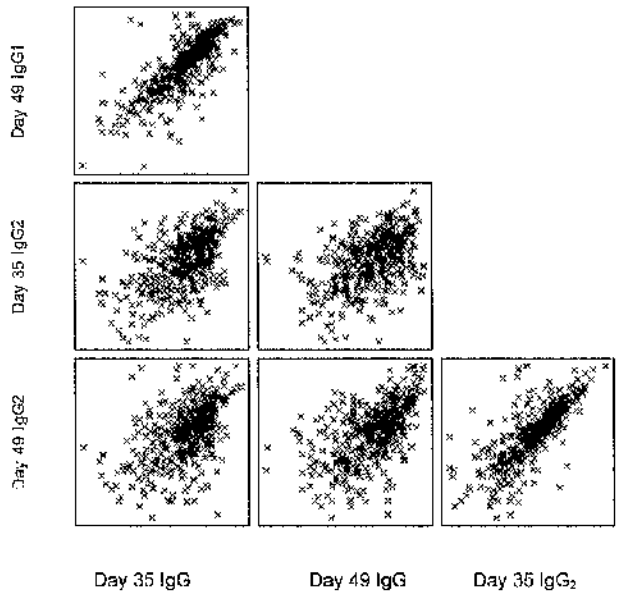


Figure 3.2 Correlations ($p<0.001$) between levels of total BRSV-IgG and BRSV-IgG_2, on Days 35 and 49, relative to first vaccination. BRSV vaccine administered on Day 0 and Day 21. BRSV-IgG_2 levels not measured before Day 0. All values are log transformed relative optical density (ROD) data.

	Day 14 IgG	Day 35 IgG	Day 49 IgG	Day 14 IgG_2	Day 35 IgG_2	Day 49 IgG_2
Day 14 IgG	1					
Day 35 IgG	0.222	1				
Day 49 IgG	0.167	0.785	1			
Day 14 IgG_2	0.359	0.298	0.267	1		
Day 35 IgG_2	0.001	0.555	0.487	0.441	1	
Day 49 IgG_2	0.022	0.630	0.571	0.398	0.768	1

Table 3.2 Pearson correlations between levels of total BRSV-IgG and BRSV-IgG_2 on Days 14, 35 and 49, relative to first vaccination ($p < 0.001$). Analysis based on log transformed ROD data. BRSV-IgG_2 levels not measured before Day 0.

Mean Δ*b*IgG(D14 to D35) were ~3.0-fold greater than mean Δ*b*IgG(D0 to D14); a difference consistent with a transient lag period before full antibody production is attained. The mean Δ*b*IgG(D35 to D49) was negative, demonstrating a drop in total BRSV-IgG to this point. The correlation between Δ*b*IgG(D14 to D35) and Δ*b*IgG(D0 to D14) was low (r =+0.176) but positive. That between Δ*b*IgG(D35 to D49) and Δ*b*IgG(D14 to D35), was again low but negative (r =-0.186).

3.4.1.3 BRSV-specific serum neutralisation test

Pearson correlation coefficients between results from the BRSV-IgG and BRSV-IgG_2 ELISA and those obtained using BRSV-specific SNT were high, calculated to be 0.66 and 0.84, respectively ($p < 0.001$).

3.4.2 Analysis of fixed effects

3.4.2.1 Sex / Management

Sex influenced levels of BRSV-IgG on all sampling days (REML; $p < 0.005$) except post-vaccination Day 14. When taken over the entire sampling period, antibody patterns for male and female calves were similar. Figure 3.3(a) shows BRSV-IgG antibody responses when grouped on *sex*. Pre-vaccination, female calves had higher levels of BRSV-IgG than male calves (t-test, $p < 0.001$) with mean (antilog) levels of BRSV-IgG in the female calves being 1.9-fold, 2.0-fold and 2.1-fold greater than the corresponding levels of IgG in the male calves, on Days -28, Day -14 and Day 0, respectively (Figure 3.3(a)). An equivalent comparison for BRSV-IgG_2 antibody on Day 0, showed no significant difference between sexes (t-test; $p > 0.1$).

Post-vaccination on Days 35 and Day 49, levels of BRSV-IgG in the female calves were 1.2-fold (antilog) higher than those of the male calves on each day (t-test; $p < 0.001$). On Day 14 post-vaccination, there was no significant difference in levels of BRSV-IgG between the sexes (t-test; $p > 0.1$). By Day 35, post-vaccination, female calves had higher (1.2-fold) levels of BRSV-IgG_2 antibody than the male calves (t-test; $p < 0.001$). On the other post-vaccination days, *sex* did not influence levels of BRSV-IgG_2 (t-test; $p > 0.1$). BRSV-IgG variance fell 1.27-fold (antilog) at the vaccination event, although it appeared to be relatively stable before and after vaccination. There was no evidence of differences in variance based on *sex*, at any stage during the study (Figure 3.3(b)). Table 3.3 shows that pre-vaccination, *sex* was a significant factor (two-way interaction) for levels of BRSV-IgG, at a relatively stable level. *Sex* was not included in REML models for early Day 14, post-vaccination but once again featured (again as two-way interactions) on Days 35 and 49. *Sex* appeared to be important for levels of BRSV-IgG_2 on Days 35 and 49 (Table 3.4).

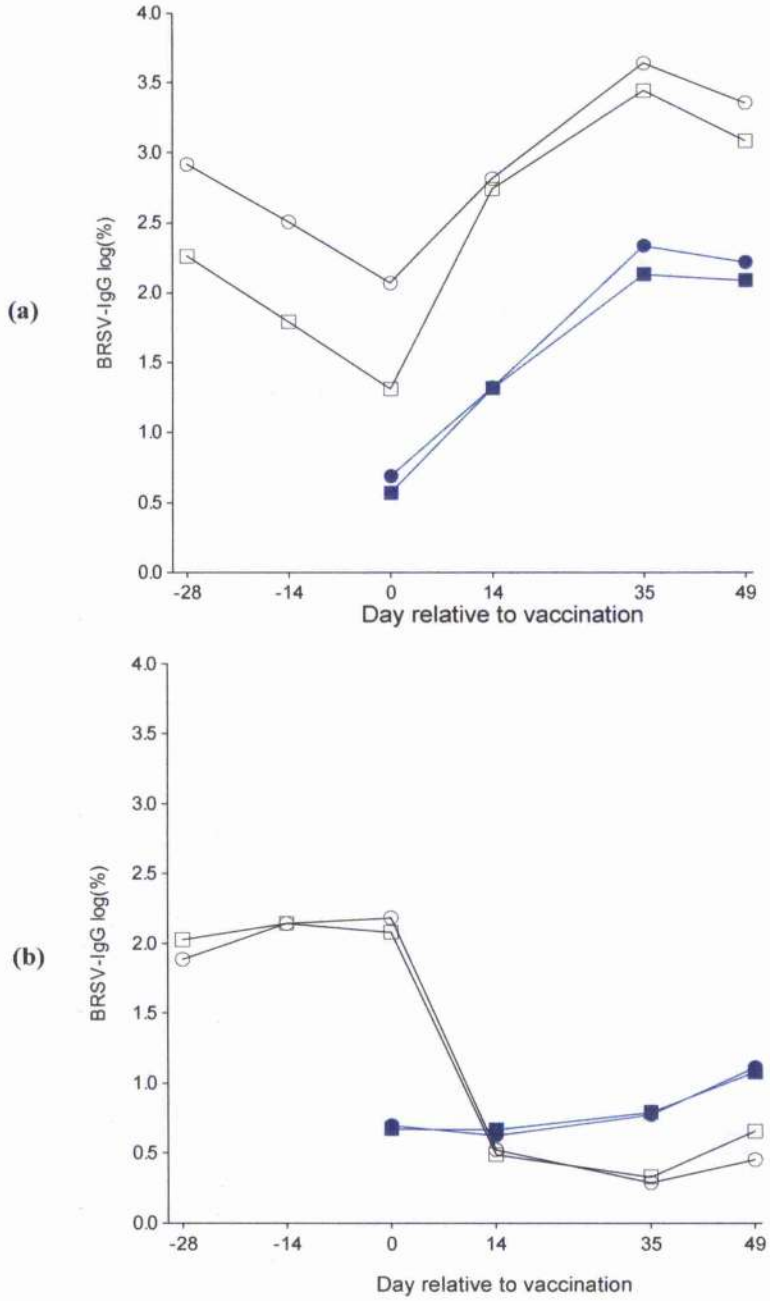


Figure 3.3 Levels of total BRSV-IgG (black) and BRSV-IgG_2 (blue) from Day -28 to Day 49, relative to vaccination. BRSV-IgG_2 levels not measured before Day 0. BRSV vaccine administered on Day 0 and Day 21. Means **(a)** and variances **(b)** presented. Values are log transformed relative optical densities (ROD%) and grouped on **sex:** female (○) and male (□)

BRSV-IgG by sex

Day	REML term	Wald statistic	d.f.	*P* value
-28	sex.year-of-birth	8.22	3	0.042
-14	sex.dam-age	12.82	5	0.025
0	sex.dam-age	11.25	5	0.047
14	-	-	-	-
35	sex.Day 0-BRSV	8.55	1	0.003
	sex.year-of-birth	9.35	3	0.025
49	sex.year-of-birth	17.14	3	< 0.001

Table 3.3 Data analyses using REML models. Significant inclusions of the fixed effect **sex**, either as main effects or interactions for levels of BRSV–IgG on Days -28 to 49, relative to vaccination. BRSV vaccine administered on Day 0. Values are log transformed ROD data. Day 0-BRSV (level of BRSV-IgG on Day 0)

BRSV-IgG$_2$ by sex

Day	REML term	Wald statistic	d.f.	*P* value
0	-	-	-	-
14	-	-	-	-
35	sex	18.44	1	< 0.001
49	sex	9.07	1	0.003

Table 3.4 Data analyses using REML models. Significant inclusions of the fixed effect **sex**, either as main effects or interactions for levels of BRSV–IgG$_2$ on Days 0 to 49, relative to vaccination. BRSV vaccine administered on Day 0. Values are log transformed ROD data.

3.4.2.2 Year-of-birth

Figure 3.4(a) shows mean BRSV-IgG and BRSV-IgG_2 antibody responses when grouped on *year-of-birth*. The effect of *year-of-birth* was unremarkable until Day 35 post-vaccination. At this point *year-of-birth* became significant (REML; $p < 0.001$), due largely to higher levels in year 2000 compared to the other three years. Mean BRSV-IgG levels in year 2000 were 1.3-fold (antilog) and 1.6-fold (antilog) the mean BRSV-IgG level of the other three years on Days 35 and 49, respectively. *Year-of-birth* was significantly associated with BRSV-IgG_2 levels on all three post-vaccination days, due largely to lower levels (0.8-fold; antilog) in 2001 compared to the mean BRSV-IgG_2 level for the other three years. Over the 4-year duration of the experiment, mean post-vaccination levels of BRSV-IgG antibody in the male group tended to gradually increase from cohort 1998 to cohort 2001, with overall 2001 levels being 1.6-fold (antilog) those of 1998. In contrast, mean post-vaccination levels of BRSV-IgG in the female group, gradually decreased over the same 4-year period, with overall 2001 levels being half those of 1998.

Although general patterns of BRSV-IgG variability were very similar between *year-of-birth* cohorts, the first cohorts, 1998 and 1999, tended to have the highest pre-vaccination variances while the later cohorts, 2000 and 2001, tended to have the lowest. This pattern of divergence may be stochastic or indicative of differences in the circulation of field BRSV infections between cohorts. There was a mean 1.2-fold (antilog) decrease in BRSV-IgG variance from Day 0 to Day 14 post-vaccination and post-vaccination BRSV-IgG variance was lower in 2000 than the other three cohorts (Figure 3.4(b)). *Year-of-birth* featured for levels of BRSV-IgG on all days (as two-way interactions) except Day 14, post-vaccination (Table 3.5). *Year-of-birth* was significant on all sampling days for levels of BRSV-IgG_2, apparently becoming more important for later days (Table 3.6).

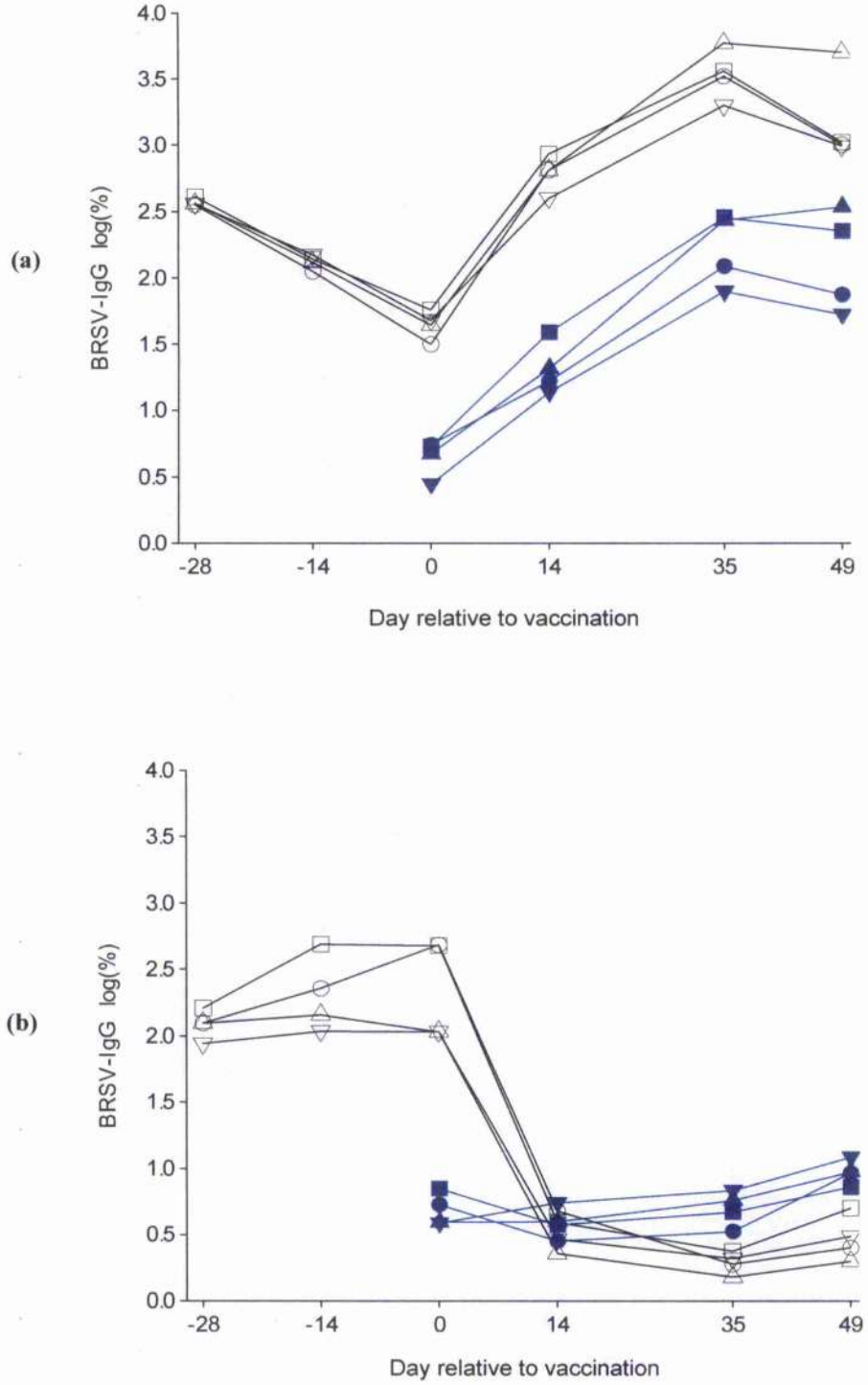


Figure 3.4 Levels of total BRSV-IgG (black) and BRSV-IgG_2 (blue) from Day -28 to Day 49, relative to vaccination. BRSV vaccine administered on Day 0 and Day 21. BRSV-IgG_2 levels not measured before Day 0. Means **(a)** and variances **(b)** presented. Values are log transformed relative optical densities (ROD) and grouped on **year-of-birth:** 1998 (○), 1999 (□), 2000 (△) and 2001(▽).

BRSV-IgG by year-of-birth

Day	REML term	Wald statistic	d.f.	*P* value
-28	year-of-birth.sex	8.22	3	0.042
-14	year-of-birth.Holstein	17.26	3	< 0.001
0	year-of-birth.Holstein	18.11	3	< 0.001
	year-of-birth.age	9.58	3	0.022
14	-	-	-	-
35	year-of-birth. Day 0-BRSV	14.15	3	0.003
	year-of-birth.sex	9.35	3	0.025
49	year-of-birth.sex	17.14	3	< 0.001

Table 3.5 Data analyses using REML models. Significant inclusions of the fixed effect **year-of-birth,** either as main effects or interactions for levels of total BRSV–IgG on Days -28 to 49, relative to vaccination. BRSV vaccine administered on Day 0. Values are log transformed relative optical density (ROD) data. Day 0-BRSV (level of BRSV-IgG on Day 0)

BRSV-IgG$_2$ by year-of-birth

Day	REML term	Wald statistic	d.f.	*P* value
0	year-of-birth.age	11.20	3	0.011
	year-of-birth	9.56	3	0.023
14	year-of-birth	12.21	3	0.007
35	year-of-birth	41.16	3	< 0.001
49	year-of-birth	57.95	3	< 0.001

Table 3.6 Data analyses using REML models. Significant inclusions of the fixed effect **year-of-birth,** either as main effects or interactions for levels of total BRSV–IgG$_2$ on Days 0 to 49, relative to vaccination. BRSV vaccine administered on Day 0. Values are log transformed ROD data.

3.4.2.3 Age

Figure 3.5 shows total BRSV-IgG and BRSV-IgG_2 antibody levels when grouped on the factor *age*. For Days -28,-14, 0 and 14, levels of BRSV-IgG for all age-groups were significantly different from each other (ANOVA, $p < 0.001$). However on post-vaccination Days 35 and 49, these differences were minimal, with the highest values being only 1.06-fold (ANOVA; $p < 0.01$) and 1.07-fold (ANOVA; $p < 0.05$) greater than the lowest, respectively.

Before and including the day of vaccination (Day 0), increasing *age* was associated with a steady decrease in the level of both BRSV-IgG and BRSV-IgG_2 i.e. younger calves have higher levels of antibody, older calves have lower levels of antibody. On Day 14 post-vaccination, the youngest calves (age-groups I and II) have highest levels of BRSV-IgG, age-group III calves have the lowest levels of BRSV-IgG, while older calves (age-groups IV and V) have mid-range levels of BRSV-IgG. A similar pattern was found for Day 14 levels of BRSV-IgG_2. By Day 35 post-vaccination, older calves generally had higher levels of BRSV-IgG than younger calves although age-group III had again the lowest levels of BRSV-IgG antibody. By Day 49 post-vaccination, increasing *age* was associated with an increase in the levels of both BRSV-IgG and BRSV-IgG_2 antibodies: the inverse to that found pre-vaccination.

After REML models were fitted, *age* at first vaccination was found to influence (REML; $p < 0.001$) levels of both subclasses of BRSV-IgG, on all pre-vaccination days. On Days -28, -14 and 0, pre-vaccination, calf *age* had a negative effect on levels of antibody. Post-vaccination, *age* combined with pre-existing Day 0 levels of BRSV-IgG was found to significantly influence (REML; $p < 0.005$) levels of antibody on Days 14, 35 and 49, the effect being negative for all three days but 2.3-fold (antilog) stronger on Day 14 than on Day 35 or 49.

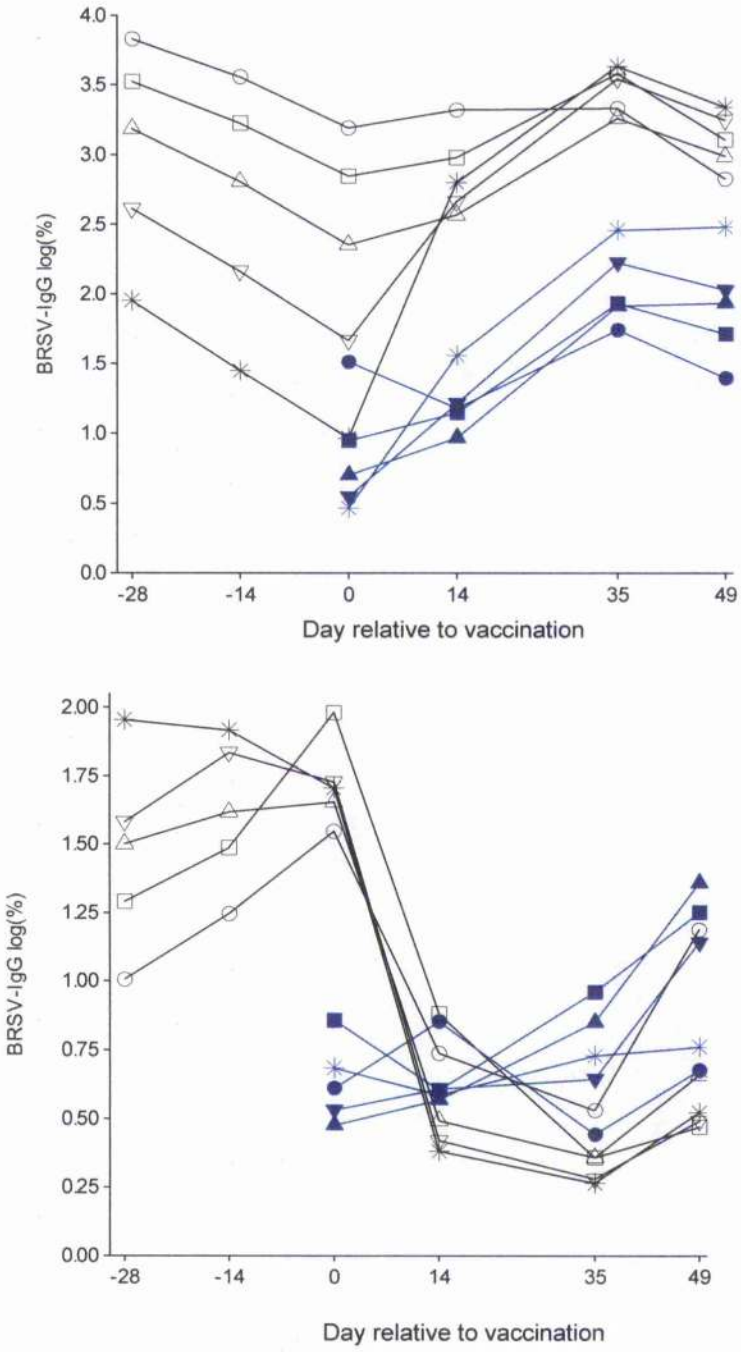


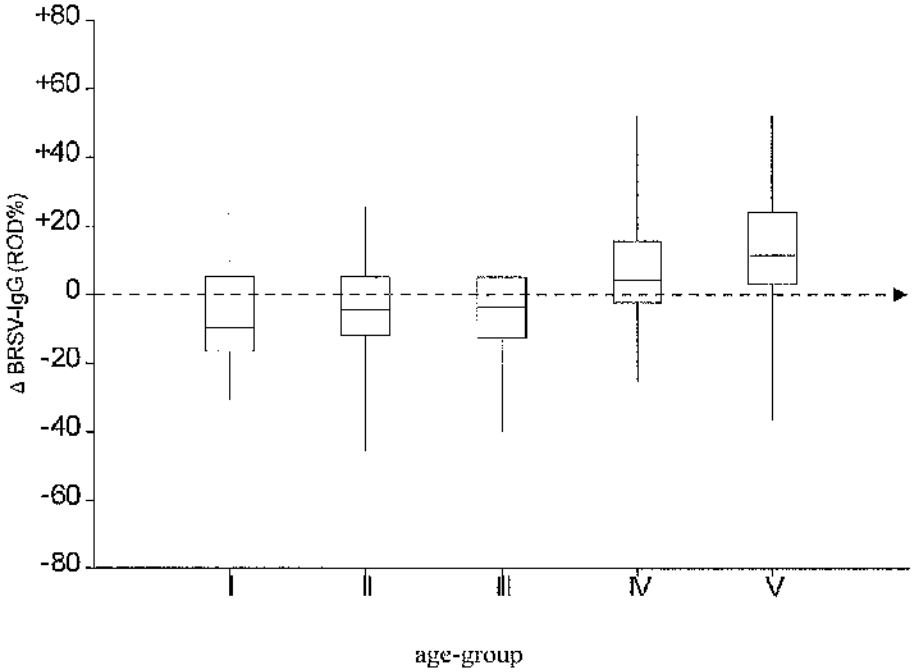
Figure 3.5 Levels of total BRSV-IgG (black) and BRSV-IgG_2 (blue) in a population of 463 calves from Day -28 to Day 49, relative to first vaccination. Vaccine administered on Day 0 and Day 21. BRSV-IgG_2 levels were not measured pre-vaccination. Mean **(a)** and variance **(b)** presented. Results are log transformed ROD data and presented grouped on **age**: Group I~ 60-81 days (○), Group II ~ 82-103 days (□), Group III ~ 104-125 days (△), Group IV ~ 126-147 days (▽) and Group V ~ 148-169 days (*)

Deviations in levels of BRSV-IgG between days: Δ*b*IgG(D0 to D14) and Δ*b*IgG(D0 to D35) tended to increase with increasing *age*. For Δ*b*IgG(D0 to D14), age-group V showed a mean deviation of (ROD =+13.3%) compared to a mean deviation of (ROD =-5.9%) for age-group I (Figure 3.6(a)). While for Δ*b*IgG(D0 to D35), age-group V showed a mean increase of (ROD =+35.4%) compared to a mean decrease of (ROD =-8.9%) for age-group I (Figure 3.6(b)). Age was important for levels of BRSV-IgG, pre-vaccination and early post-vaccination (up to Day 14), its significance appeared to decline for the later post-vaccination days (Table 3.7). Age was only significant on Days 0 and 14 for levels of BRSV-IgG_2 (Table 3.8).

By Day 35 post-vaccination, differences in levels of BRSV-IgG based on *age* were small, although levels of BRSV-IgG remained lowest in calves aged 104-125 days. By Day 49, older calves had the highest levels of BRSV-IgG, in an almost perfect inverse of the pre-vaccination scenario.

To summarise: Pre-vaccination, older calves have lower levels of BRSV-IgG (and less markedly, lower BRSV-IgG_2). Early post-vaccination (Day 14), calves younger than 103 days continued to have the highest levels of BRSV-IgG but showed the smallest response (Δ) to vaccination. Calves aged 104-125 days although they do respond moderately well to BRSV vaccination, do so from a lower starting point than the younger animals and therefore have lower peak levels of BRSV-IgG on Day 35. Calves older than 125 days, have marginally lower peak levels of BRSV-IgG on Day 35 than younger calves. To arrive at those peak levels however, these older calves show much greater antibody responses (Δ), the effect apparently positively associated with *age*.

(a)



(b)

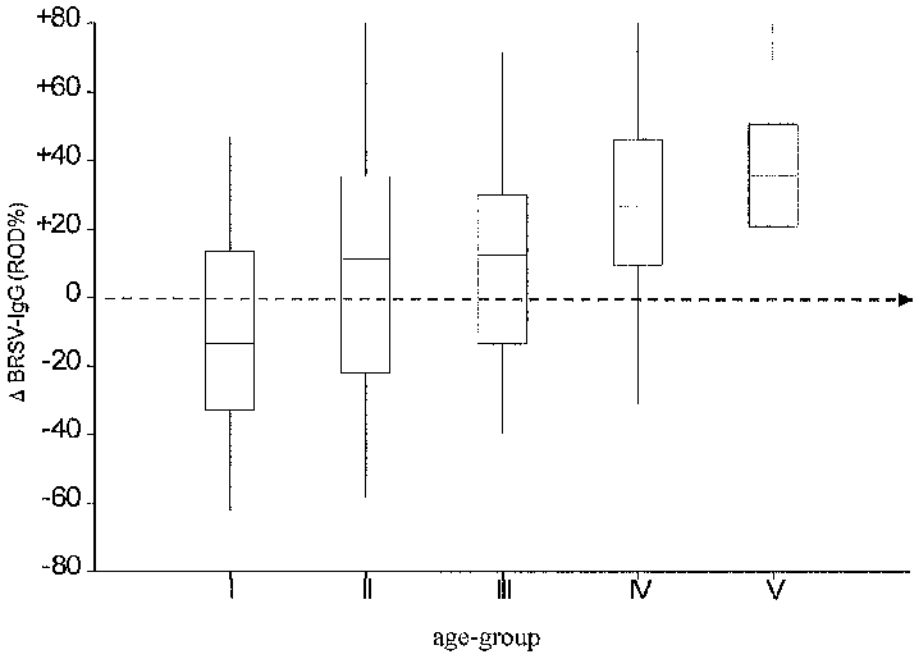


Figure 3.6 Deviation in level of BRSV-IgG on **(a)** Δ*b*IgG(D0 to D14) and **(b)** Δ*b*1gG(D0 to D35). Median (central horizontal line), quartiles (outer horizontal lines), and range (outer vertical lines) shown. Values are non-transformed relative optical density (ROD) data and presented grouped on **age**: age-group I ~ 60-81 days, age-group II ~ 82-103 days, age-group III ~ 104-125 days, age-group IV ~ 126-147 days and age-group V ~ 148-169 days.

BRSV-IgG by age

Day	REML term	Wald statistic	d.f.	*P* value
-28	age	85.36	1	< 0.001
-14	age	99.70	1	< 0.001
0	age. year-of-birth	9.58	3	0.022
14	age.Day 0-BRSV	82.41	1	< 0.001
	age	9.84	1	0.002
35	age.Day 0-BRSV	5.79	3	0.016
49	-	-	-	-

Table 3.7 Data analyses using REML models. Significant inclusions of the fixed effect **age**, either as main effects or interactions for levels of BRSV–IgG on Days -28 to 49, relative to vaccination. Vaccine administered on Days 0 and 21. Values are log transformed ROD data. Day 0-BRSV (level of BRSV-IgG on Day 0)

BRSV-IgG$_2$ by age

Day	REML term	Wald statistic	d.f.	*P* value
0	age.year-of-birth	11.20	3	0.011
14	age.Day 0-BRSV	21.49	1	< 0.001
	age	12.93	1	< 0.001
35	-	-	-	-
49	-	-	-	-

Table 3.8 Data analyses using REML models. Significant inclusions of the fixed effect **age**, either as main effects or interactions for levels of BRSV–IgG$_2$ on Days 0 to 49, relative to vaccination. Vaccine administered on Days 0 and 21. Values are log transformed ROD data. Day 0-BRSV (level of BRSV-IgG on Day 0)

3.4.2.4 Pre-existing antibody

In general, levels of pre-existing BRSV-IgG antibody on Day 0 suppressed subsequent humoral responses to vaccination. These effects were most apparent early post-vaccination. Figure 3.7 shows the BRSV-IgG deviation induced between Day 0 and Day 14 (ΔbIgG(D0 to D14)) against the level of pre-existing BRSV-IgG at vaccination.

Regression coefficients when levels of pre-existing antibody were included in REML models for induced BRSV-IgG and BRSV-IgG_2 levels on Days 0 to 49 are shown in Table 3.9. Across both sexes, pre-existing Day 0 levels of BRSV-IgG were negatively correlated with vaccine induced levels of BRSV-IgG on Days 35 and 49, but not those of BRSV-IgG_2 on the same days. Day 14 levels of BRSV-IgG were negatively associated with induced levels of BRSV-IgG on Days 35 and 49 in the female group but positively associated with corresponding Days in the male group. Day 14 levels of BRSV-IgG_2 were positively associated with induced levels of BRSV-IgG_2 antibody on Day 35 and Day 49, in both sexes.

Using logistic regression, it was possible to develop binomial models (parameters: α and β detailed in Table 3.10) and therefore calculate and graph the probability that the Day 0 level of pre-existing BRSV-IgG was associated with a positive deviation in BRSV-IgG. This process was performed for ΔbIgG(D28 to D42), ΔbIgG(D28 to D63) and ΔbIgG(D28 to D77). Consequently estimates were obtained of the 50% (p =0.5) and 90% (p =0.9) threshold levels were estimated and plotted for BRSV-IgG antibody (Figure 3.8).

For BRSV, the 50% inhibitory threshold on Day 0 was 11.7% for Day 42 responses, 25.9% for Day 63 responses, and 19.8% for Day 77 responses. For BRSV, the 90% inhibitory thresholds on Day 0 were 3.8%, 12.8% and 9.5% for Days 42, 63 and 77, respectively.

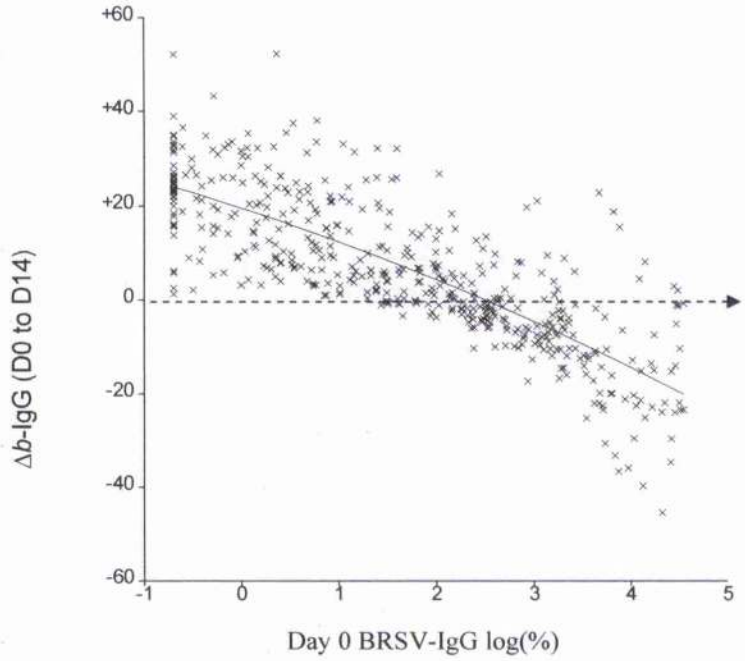


Figure 3.7 Deviations in level of total BRSV-IgG - **Δ*b*IgG(D0 to D14)** against level of pre-existing Day 0 BRSV-IgG. Best fit regression line (solid). Zero threshold (Δ*b*IgG =0) (dashed). Δ*b*IgG(D0 to D14) values are untransformed, Day 0 BRSV-IgG are log transformed ROD data.

Induced IgG antibody on Day

Pre-existing IgG_2 antibody on Day		0	14	35	49		Pre-existing IgG antibody on Day
49	F	-	0.92 (0.21)	-0.42 (0.07)	-0.48 (0.08)	F	0
	M	-	**n.s.**	-0.18 (0.08)[1]	-0.28 (0.08)	M	
35	F	0.90 (0.05)	-	-0.21 (0.04)	-0.23 (0.04)	F	14
	M	0.77 (0.05)	-	0.24 (0.06)	0.23 (0.07)	M	
14	F	0.32 (0.08)	0.35 (0.07)	-	0.87 (0.04)	F	35
	M	0.48 (0.07)	0.45 (0.06)	-	0.85 (0.05)	M	
0	F	**n.s.**	**n.s.**	0.32 (0.06)	-	F	49
	M	**n.s.**	**n.s.**	0.15 (0.06)[2]	-	M	
		49	35	14	0		

Induced IgG_2 antibody on Day

Table 3.9 REML analysis based on log transformed ROD data
Above diagonal (black): Regression coefficients (standard error) when level of pre-existing BRSV-IgG on Days 0 to 49 is included in REML models for BRSV-IgG on Days 0 to 49.
Below diagonal (blue): Regression coefficients (standard error) when level of pre-existing BRSV-IgG_2 on Days 0 to 49 is included in REML models for BRSV-IgG_2 on Days 0 to 49.
Results presented grouped on *sex*: female (**F**), male (**M**). All associations have $p < 0.001$ except [1]($p = 0.019$), [2]($p = 0.022$) and **n.s.** ($p > 0.1$)

		Logistic regression model		
		Day 14	Day 35	Day 49
Parameter	α	4.82 (0.46)	10.08 (1.19)	8.97 (1.01)
	β	-1.96 (0.18)	-3.09 (0.37)	-3.00 (0.34)

Table 3.10 Estimated parameters α and β for levels of total BRSV-IgG on Days 14, 35 and 49 against levels of pre-existing Day 0 BRSV-IgG. Means (standard error) presented. ($p < 0.001$) for all regression coefficients. Logistic regression based on log transformed ROD data.

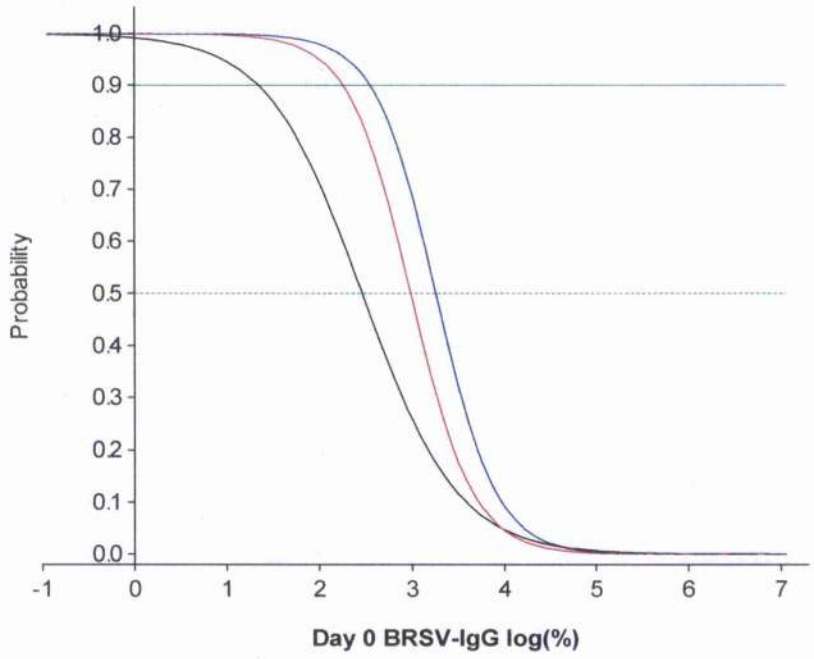


Figure 3.8 Probability of positive total BRSV-IgG antibody response on Days 14, 35 and 49 against level of Day 0-BRSV. Day 0 BRSV-IgG levels are log transformed. Lines shown for Δ*b*IgG(D0 to D14)~black, Δ*b*IgG(D0 to D35)~blue and Δ*b*IgG(D0 to D49)~red. p =0.5 (green dashed), p =0.9 (green solid). Logistic regression based on log transformed ROD data.

3.4.3 Analysis of genetic effects

3.4.3.1 Cross-breeding effects - Holstein

Holstein influenced levels of BRSV-IgG antibody (REML; $p < 0.001$) on pre-vaccination Days -28, -14 and Day 0 (the day of vaccination), but had no significant effect on BRSV-IgG levels, in the post-vaccination period. BH calves with greater proportions of Holstein genes (*Holstein* = 0.75) had the highest pre-vaccination levels of BRSV-IgG with mean ROD values of 19.7%, 12.2% and 7.6% on Days -28, -14 and Day 0, respectively (Figure 3.9). F2 calves with intermediate proportions of Holstein genes (*Holstein* = 0.50) had pre-vaccination levels of BRSV-IgG with mean ROD values of 13.1%, 8.4% and 5.2% on Days -28, -14 and Day 0, respectively. BCH calves with the lowest proportions of Holstein genes (*Holstein* = 0.25) had the lowest pre-vaccination levels of BRSV-IgG with mean ROD values of 8.6%, 5.9% and 4.0% on Days -28, -14 and Day 0, respectively. BRSV-IgG_2 antibody levels were much lower but a similar pattern existed. On Day 0, calves with proportions *Holstein* = 0.75, 0.50 and 0.25 had mean BRSV-IgG_2 ROD values of 2.7%, 1.7% and 1.5%, respectively.

Prevaccination, *breed-cross* BH calves had on average 1.5-fold (antilog) higher levels of BRSV-IgG than F2 calves, which in turn had 1.4-fold (antilog) higher levels than BCH calves. On Day 0, BRSV-IgG_2 levels in the BH calves were on average 1.4-fold (antilog) those of the BCH or F2 calves. Neither *breed-cross* nor *Holstein* influenced levels of BRSV-IgG or BRSV-IgG_2 in the post-vaccination period. This finding was confirmed by REML modelling (REML; $p > 0.1$), so that levels of BRSV-IgG and BRSV-IgG_2 were influenced by the factor *Holstein*, only pre-vaccination (Tables 3.11 and 3.12)

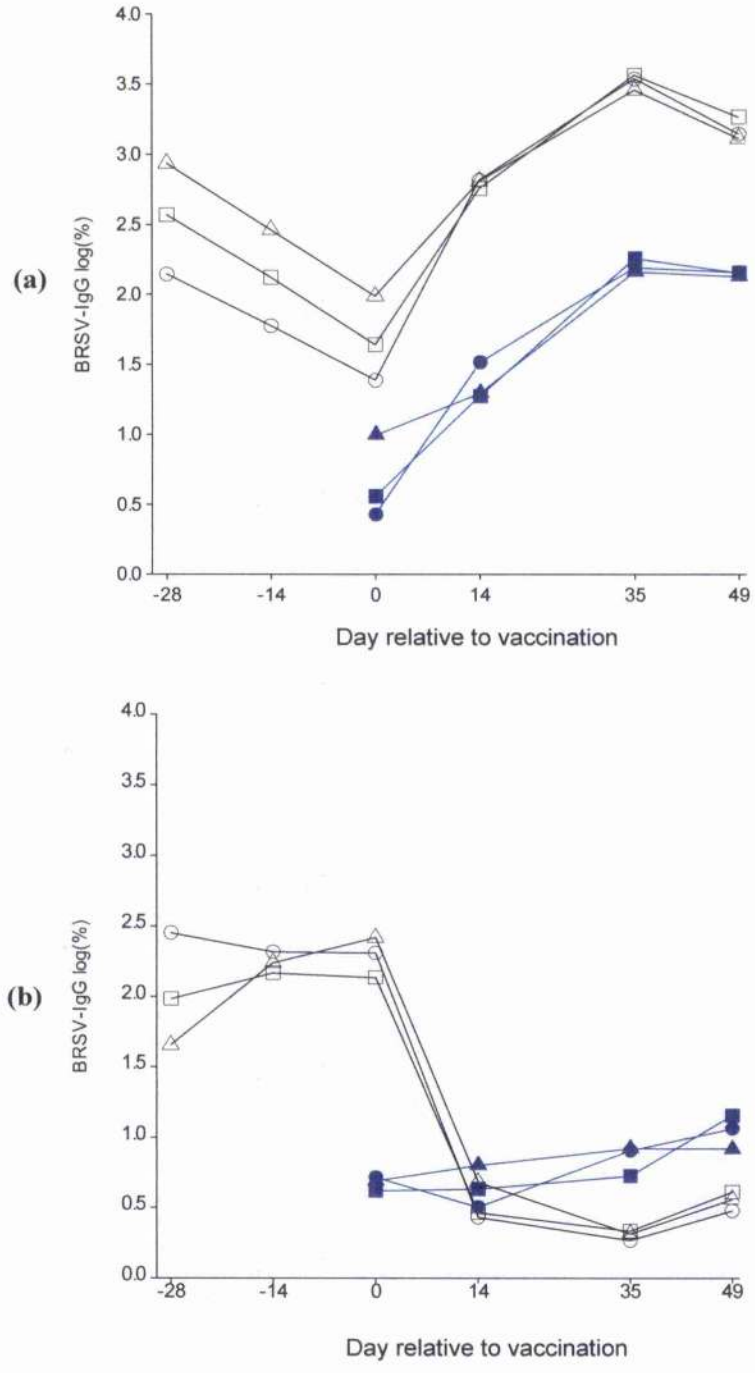


Figure 3.9 Levels of total BRSV-IgG (black) and BRSV-IgG_2 (blue) from Day -28 to Day 49, relative to vaccination. BRSV vaccine administered on Day 0 and Day 21. Means **(a)** and variances **(b)** presented. BRSV-IgG_2 levels not measured before Day 0. Values are log transformed relative optical densities (ROD) and presented grouped on **Holstein:** 0.25 (○), 0.50 (□) and 0.75 (△).

BRSV-IgG by Holstein

Day	REML term	Wald statistic	d.f.	*P* value
-28	Holstein.year-of-birth	10.25	3	0.017
-14	Holstein.year-of-birth	17.26	3	< 0.001
0	Holstein.year-of-birth	16.35	3	< 0.001
14	-	-	-	-
35	-	-	-	-
49	-	-	-	-

Table 3.11 Significant inclusions of the fixed effect **Holstein**, either as main effects or interactions for levels of total BRSV–IgG on Days -28 to 49, relative to vaccination. BRSV vaccine administered on Day 28. Values are log transformed relative optical density (ROD) data. Data analyses using REML models.

BRSV-IgG$_2$ by Holstein

Day	REML term	Wald statistic	d.f.	*P* value
0	Holstein	5.57	1	0.018
14	-	-	-	-
35	-	-	-	-
49	-	-	-	-

Table 3.12 Significant inclusions of the fixed effect **Holstein**, either as main effects or interactions for levels of BRSV IgG$_2$ on Days 0 to 49, relative to vaccination. BRSV vaccine administered on Day 28. Values are log transformed ROD data. Data analyses using REML models.

3.4.3.2 Cross-breeding effects – recombination loss

Figure 3.10 shows that there was no evidence in this study that *recombination-loss* significantly influenced levels of BRSV-IgG and BRSV-IgG_2 on any of the sampling days (REML; $p > 0.1$).

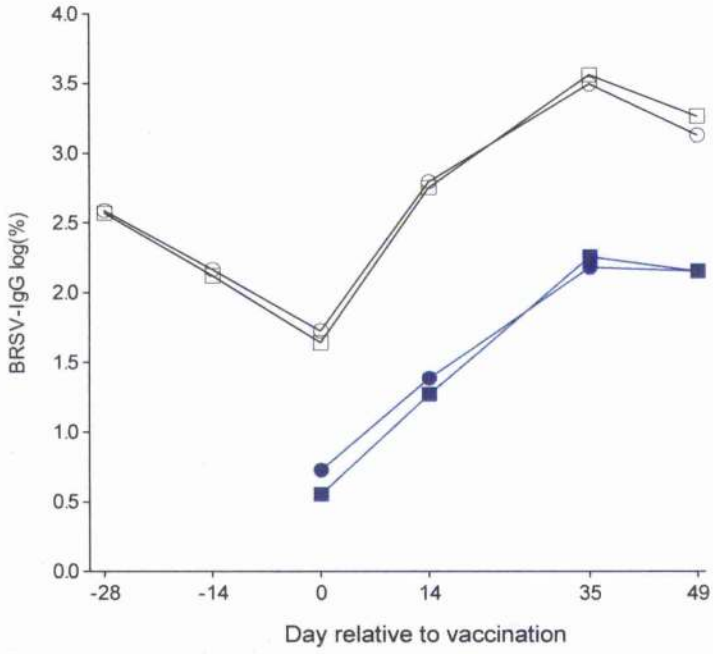


Figure 3.10 Levels of total BRSV-IgG (black) and BRSV-IgG_2 (blue) from Day -28 to Day 49, relative to vaccination. BRSV vaccine administered on Day 0 and Day 21. Means presented. BRSV-IgG_2 levels not measured before Day 0. Values are log transformed relative optical density (ROD) data and presented grouped on proportion **recombination-loss:** 0.5 (○) and 1.0 (□)

3.4.3.3 Sire effects

To maximise the potential power and experimental objectivity of the test, all available results were used, without stratification, to evaluate genetic effects (Table 3.13). Trait heritabilities and log-likelihood statistics for inclusion of *sire* on all sampling days up to and including the day of vaccination (Day 0) were relatively constant and of a lower order than those found on the later sampling days. Post-vaccination, the contribution of *sire* became much more significant on Days 14 and 35 before returning to lower levels on Day 49. Over this period, estimates of sire heritability, h^2 (±s.e.) peaked at 0.16 (±0.11) and 0.29 (±0.17) on Days 14 and 35, respectively. The Day 35 h^2 value is robust - restricting the analysis by removing the upper or lower ten per cent of the dataset causes the value to change only slightly to 0.32 (±0.19) and 0.26 (±0.16), respectively. The h^2 (±s.e.) values associated with the deviation in levels of antibody: Δ*b*IgG(D0 to D14), Δ*b*IgG(D0 to D35) and Δ*b*IgG(D0 to D49) were greater than those for total antibody levels, with values of 0.20 (±0.15), 0.52 (±0.26) and 0.17 (±0.14), respectively. Although heritable effects were detectable using area under the curve as an alternative phenotypic trait, there was no evidence that using these summary traits e.g. 3-day-area $bIgG_{(prevac)}$, provided any additional information. On a natural logarithm scale, BRSV-IgG phenotypic variance (σ_p^2) was much larger (4.2-fold; antilog) over the pre-vaccination period than the post-vaccination period. On the same scale, BRSV-IgG_2 σ_p^2 was approximately twice (antilog) that of BRSV-IgG σ_p^2 over the post-vaccination period.

On all three post-vaccination days, the progeny of one sire (R01) were consistently associated with the highest levels of BRSV-IgG, while the progeny of another sire (R11) were consistently associated with the lowest levels (Figure 3.11). The progeny of these two sires had very similar pre-vaccination levels of BRSV-IgG, with a mean 0.98-fold (antilog) difference over the three days. The ratios of the mean (antilog) levels of BRSV-IgG antibody (highest/lowest) between these two sires were 1.82, 1.67 and 1.75 on Days 14, 35 and 49, respectively. The sire with progeny with the highest (R05) pre-vaccination level of BRSV-IgG and the sire with progeny with the lowest (H27) pre-vaccination level of BRSV-IgG had intermediate responses to vaccination between the two extreme sires previously outlined.

trait	overall h^2	overall σ_p^2 on log scale	log-likelihood ratio for sire
BRSV-IgG			
Day -28 *b*IgG	0.10 (0.09)	1.46	n.s.
Day -14 *b*IgG	0.12 (0.09)	1.53	n.s.
Day 0 *b*IgG	0.10 (0.08)	1.44	n.s.
Day 14 *b*IgG	0.16 (0.11)	0.38	< 0.01
Day 35 *b*IgG	0.29 (0.17)	0.26	< 0.0001
Day 49 *b*IgG	0.11 (0.09)	0.41	0.06
3-day-area *b*IgG $_{(prevac)}$	0.10 (0.09)	-	< 0.05
3-day-area *b*IgG $_{(postvac)}$	0.23 (0.14)	-	< 0.001
BRSV-IgG$_2$			
Day 0 *b*IgG$_2$	0.14 (0.12)	0.67	n.s.
Day 14 *b*IgG$_2$	n.s.	0.76	n.s.
Day 35 *b*IgG$_2$	0.14 (0.11)	0.75	< 0.05
Day 49 *b*IgG$_2$	0.09 (0.09)	1.03	n.s.
3-day-area *b*IgG$_{2\ (postvac)}$	0.14 (0.11)	-	< 0.05
Δ BRSV-IgG			
Δ*b*IgG(D0 to D14)	0.20 (0.15)	0.80	< 0.05
Δ*b*IgG(D0 to D35)	0.52 (0.26)	0.49	< 0.0001
Δ*b*IgG(D0 to D49)	0.17 (0.14)	0.60	< 0.05
Δ*b*IgG$_2$(D0 to D14)	n.s.	1.23	n.s.
Δ*b*IgG$_2$(D0 to D35)	0.36 (0.20)	1.07	< 0.0001
Δ*b*IgG$_2$(D0 to D49)	0.21 (0.15)	1.04	< 0.01

Table 3.13 Trait heritabilities h^2(standard error), total phenotypic variances (σ_p^2) on natural logarithm scale and sire log-likelihood ratio shown. Statistics for absolute levels, area-under-curve and deviations (Δ) between Day 0 and Days 14, 35 and 49 post-vaccination, for both total BRSV-IgG and BRSV-IgG$_2$.
n.s. ($p > 0.1$). Estimates produced using REML.

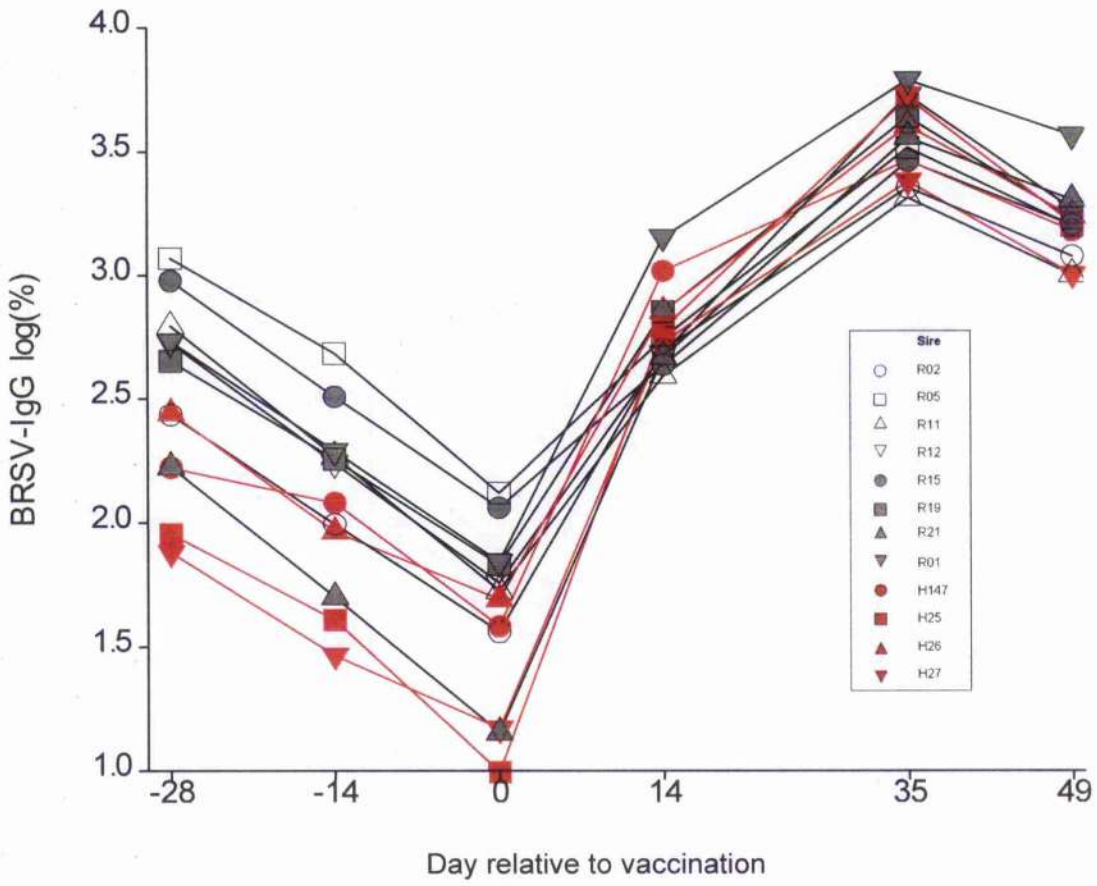


Figure 3.11 Levels of total BRSV-IgG from Day -28 to Day 49, relative to first vaccination. BRSV vaccine administered on Day 0 and Day 21. BRSV-IgG_2 not shown. Values are log transformed relative optical densities (ROD) and presented, grouped on **sire:** 4 F0 Charolais (red) and 8 F1 Holstein/Charolais (black). Note y-axis begins at 1.0.

No consistent pattern was apparent for mean levels of BRSV-IgG_2 antibody, when grouped on *sire*.

3.4.3.3 Maternal effects

3.4.3.3.1 Dam-age

Due to the availability of three pre-vaccination sampling points, an opportunity presented to study any persistent dam effects in the results. Dams aged four, five and six years old had calves with very similar levels of BRSV-IgG (ANOVA; $p > 0.1$). Together, dams aged 4-6 years old had offspring with significantly different levels of total BRSV-IgG than dams aged two, three and seven (ANOVA; $p < 0.05$) on all three pre-vaccination days (Figure 3.12). Older dams have calves with higher levels of BRSV-IgG, the effect appearing to stabilise for dams aged between 4 and 6 years old. (There are only 3 dams aged seven years or more which provides a very small sample). The age of the dam at calving influenced levels of BRSV-IgG pre-vaccination (REML; $p < 0.05$) present as an interaction with sex on Days -14 and 0. However, the overall effect of *dam-age* on BRSV-IgG appeared to be of a lower order than that for the factor *Holstein*.

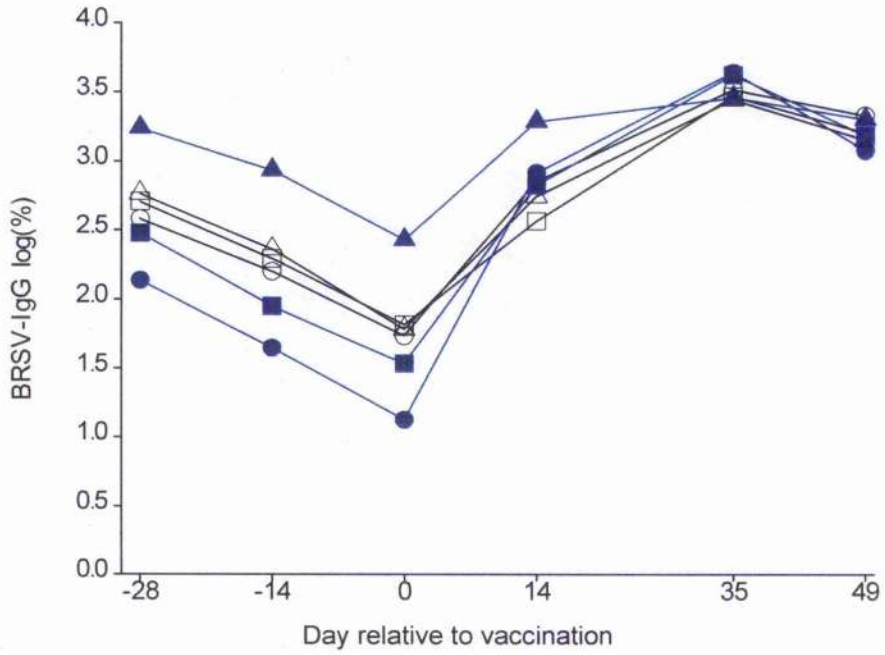


Figure 3.12 Levels of total BRSV-IgG from Day -28 to Day 49, relative to first vaccination. BRSV vaccine administered on Day 0 and Day 21. Mean values are log transformed relative optical densities (ROD) and presented grouped on **dam-age:** 2 (●), 3 (■), 4 (○), 5 (□), 6 (△) and 7 (▲) years.

Post-vaccination, *dam-age* influenced levels of BRSV-IgG on Day 35 only, as an interaction with *year-of-birth* (Table 3.14). *Dam-age* was significant for levels of BRSV-IgG_2 on post-vaccination Day 14 only (Table 3.15)

BRSV-IgG by dam-age

Day	REML term	Wald statistic	d.f.	*P* value
-28	-	-	-	-
-14	dam-age.sex	12.82	5	0.025
0	dam-age.sex	11.25	5	0.047
14	-	-	-	-
35	dam-age.year-of-birth	22.13	11	0.023
49	-	-	-	-

Table 3.14 Significant inclusions of the fixed effect **dam-age**, either as main effects or interactions for levels of total BRSV–IgG on Days -28 to 49, relative to vaccination. BRSV vaccine administered on Days 0 and 21. Values are log transformed relative optical density (ROD) data. Data analyses using REML models.

BRSV-IgG_2 by dam-age

Day	REML term	Wald statistic	d.f.	*P* value
0	-	-	-	-
14	dam-age	19.80	7	0.006
35	-	-	-	-
49	-	-	-	-

Table 3.15 Significant inclusions of the fixed effect **dam-age**, either as main effects or interactions for levels of BRSV–IgG_2 on Days 0 to 49, relative to vaccination. BRSV vaccine administered on Day 0 and 21. Values are log transformed ROD data. Data analyses using REML models.

3.4.3.3.2 Components of variation at individual time points

Other maternal effects were evaluated using ASREML procedures (ASREML, VSN International Ltd) instead of Genstat procedures (Genstat 7.0, VSN International Ltd) used previously for the *sire* effects. The ASREML software accounts for the Robogen pedigree structure differently than the Genstat 7.0 software and therefore slight differences between calculated coefficients, such as additive heritability, were expected and found.

As explored earlier (section 3.4.1), mean levels of BRSV-IgG (the phenotype) declined exponentially to low values pre-vaccination but then increased rapidly with a peak on Day 35 post-vaccination. In contrast, phenotypic variances were much larger post-vaccination than pre-vaccination (see section 3.4.3.3), an arrangement which appeared to be uniform across all levels of BRSV-IgG when segregated on *sex*, *year-of-birth* or *breed-cross* (sections 3.4.2.1, 3.4.2.2 and 3.4.3.1)

A clear pattern was observed for the components of the variation determined by the timing relative to BRSV vaccination (Table 3.16). Pre-vaccination, there was no evidence of additive (h^2) heritability ($p > 0.05$) but a strong and consistent maternal (m^2) transmissibility was observed, explaining 0.33 to 0.35 of the total phenotypic variance ($p < 0.001$). Post-vaccination, the partition pattern of phenotypic variance reversed. Over that period, the maternal variance was smaller than the additive variance and statistically significant ($p < 0.05$) on Day 35 only. In contrast, evidence of additive variation was found on all post-vaccination days ($p < 0.05$) and as previously established, additive heritability was greatest on Day 35 with a value of 0.285, compared to 0.164 and 0.171 for Days 35 and 49, respectively. However examination of the component heritabilities, in relation to the total phenotypic variances (Figure 3.13), suggests that for the BRSV antibody responses observed, total transmitted phenotypic variance is at similar levels post-vaccination to that pre-vaccination.

Trait statistic	Day relative to first vaccination					
	-28	-14	0	14	35	49
h^2	0.031 [0.000]	0.061 [0.000]	0.007 [0.000]	0.164 [0.018]	0.285 [0.069]	0.171 [0.018]
m^2	0.352 [0.229]	0.325 [0.204]	0.336 [0.211]	0.016 [0.000]	0.109 [0.000]	0.054 [0.000]
$\sigma_{P^2(f)}$	1.684	1.970	1.857	0.551	0.250	0.482
$\sigma_{P^2(m)}$	1.554	1.545	1.454	0.506	0.299	0.503
$\mu_{(f)}$	2.85 (0.181)	2.32 (0.209)	1.96 (0.180)	2.75 (0.130)	3.65 (0.099)	3.30 (0.122)
$\mu_{(m)}$	2.30 (0.169)	1.89 (0.180)	1.37 (0.154)	2.75 (0.122)	3.46 (0.106)	3.10 (0.122)

Table 3.16 Components of overall transmitted variance: additive heritability (h^2) and maternal transmissibility (m^2) for levels of total BRSV-IgG (log transformed) pre- and post-vaccination, together with phenotypic variances (σ_P^2) and means (μ) for female ($_f$) and male ($_m$) groups, respectively. BRSV vaccine administered on Day 0 and Day 21. Standard errors for the means are given in rounded parentheses, and the lower values of the 95% support intervals for h^2 and m^2 are given in square parentheses. Estimates produced using ASREML.

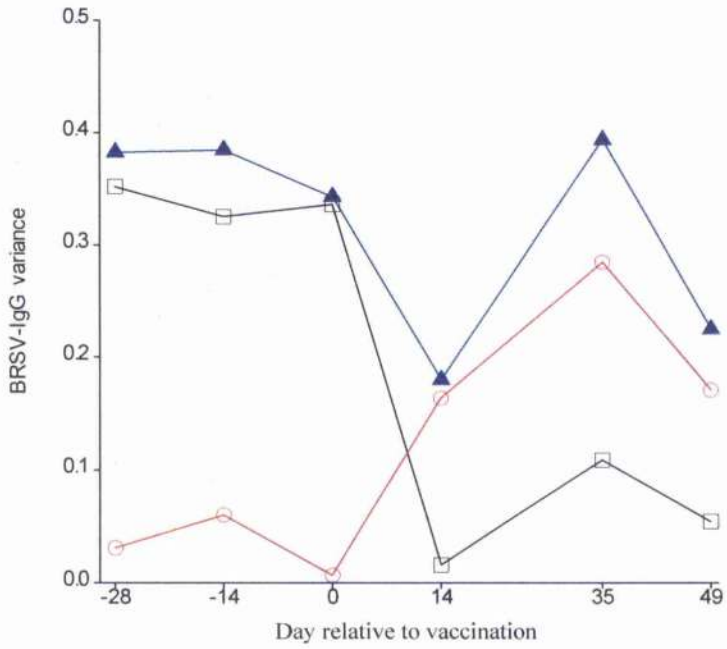


Figure 3.13 Relationships among variance components over time. Total transmitted variance (▲) and it's components: additive heritability fraction (○) and maternal transmissibility fraction (□) for levels of total BRSV-IgG (log transformed) on Days -28 to 49, relative to first vaccination. BRSV vaccine administered on Day 0 and Day 21. Estimates produced using ASREML.

3.4.3.3.3 Pre-vaccination traits

The correlations among the maternal components and the residual components over the three pre-vaccination observations are shown in Table 3.17. The results demonstrate the repeatable nature of the observations in this trial. The component attributable to the individual dam was consistent across all three occasions with correlations close to 1 (the formal test against zero correlations was $p < 0.001$, 3 d.f.). The residual variance, which includes variation in the dam effect between cohorts, environmental effects on the calf and laboratory technical errors, was also highly repeatable across occasions with correlations ranging from 0.79 to 0.88. It is this residual component that would have been poorly repeatable if wildtype BRSV infections were widespread in the pre-vaccination period. The high repeatability in the individual components of variation resulted in phenotypic correlations ranging from 0.86 to 0.93.

		Day relative to first vaccination		
		-28	**-14**	**0**
Day relative to first vaccination	**-28**	–	0.883 (0.012)	0.789 (0.020)
	-14	0.996*	–	0.800 (0.021)
	0	0.986*	0.992*	–

Table 3.17 The maternal and residual correlations for pre-vaccination levels of total BRSV-IgG on Days -28, -14 and 0 (log transformed). Maternal correlations are below the diagonal, residual correlations above. Results presented as means (standard error). Estimates produced using ASREML. *No standard errors available from ASREML due to proximity of estimates to positive definite boundary (1)

3.4.3.3.4 Post-vaccination traits

Post-vaccination, the patterns differed as shown in Table 3.18. Whilst there was evidence of positive genetic correlations (r_g) across Days 14, 35 and 49 ($p < 0.01$, all $r_g = 0$, 3 df), the residual correlations were more variable: in particular, Day 14 was poorly correlated with later observations (IgG production can take up to 18 days for peak production and in this study calves received a booster vaccination on Day 21), whilst the residual correlation of Day 35 to 49 was moderate to high. Day 14 would seem to represent a transitional antibody level whereas Day 35 and 49 seem to represent the more mature antibody response. This pattern was also reflected in a low phenotypic correlation of Day 14 with later days (0.21 and 0.17 for Days 35 and 49, respectively) but a high phenotypic correlation between Day 35 and Day 49.

		Day relative to first vaccination		
		14	**35**	**49**
Day relative to first vaccination	**14**	–	0.055 (0.061)	0.026 (0.056)
	35	0.760*	–	0.666 (0.039)
	49	0.896*	0.966*	–

Table 3.18 Genetic and residual correlations for post-vaccination levels of total BRSV-IgG (log transformed). Genetic correlations are below the diagonal, residual correlations above. Results presented as means (standard error). Estimates produced using ASREML.
* No standard errors available from ASREML due to proximity of estimates to positive definite boundary

3.4.3.3.5 Combined pre- and post-vaccination traits

In the light of the results obtained above, demonstrating a high repeatability (strong correlations) among observations pre-vaccination, and justified further in the Discussion, the observation for Days -28, -14 and 0 were averaged and treated as a single trait (PRE). Furthermore to overcome the convergence problems of estimating variance components on the limited numbers, the relationship of PRE with Day 14 and with Days 35 and 49 were tested in separate analyses.

The pre-vaccination variance identified included a maternal component but no additive genetic components, whereas by Day 14 the variance included additive genetic but no maternal component. Therefore the only relevant correlation (±se) which arose when Day 14 and PRE were tested is from the residual effects, which were 0.047 (±0.059; p >0.05), with the overall phenotypic correlation being smaller i.e. 0.034 (±0.043).

The relationships of Day 35 and Day 49 with PRE were clearer. The inclusion of PRE in the multi-trait analysis allowed better identification of maternal effects for levels of BRSV-IgG on Days 35 and 49. Correlations for the residual and maternal components are shown in Table 3.19. The results for the genetic components were very similar to those described above (i.e. the heritabilities as shown in Table 3.16 (p109), and the genetic correlation between Day 35 and Day 49 as shown in Table 3.18). The relationships between the maternal components for PRE and Days 35 and 49 were both negative and although standard errors were large, the formal test of each correlation was statistically significant (each p <0.05, 1 df).

		Day relative to first vaccination		
		PRE	**35**	**49**
Day relative to first vaccination	**PRE**	–	-0.258 (0.071)	-0.312 (0.064)
	35	-0.474 (0.236)	–	0.658 (0.054)
	49	-0.690 (0.453)	0.772 (0.287)	–

Table 3.19 Maternal and residual correlations for levels of total BRSV-IgG (log transformed) pre-vaccination mean (PRE) and post-vaccination Days 35 and 49. Maternal correlations are below the diagonal, residual correlations above. Estimates produced using ASREML. Results presented as means (standard error).

3.5 Discussion

This study demonstrated that serum levels of BRSV-IgG antibody are heritable. It appears to be the largest data-set available, to date, that provides evidence that antibody levels in cattle following vaccination against a specific viral agent are substantially influenced by both additive and maternal genetic factors, in addition to other environmental effects.

Respiratory disease caused by BRSV, is largely a condition of young cattle. Although it complicates the analysis, it is crucial that responses to BRSV vaccination should be studied in early life. This is particularly important over the period when levels of maternally-derived passive antibody subside in calves, which when young may be only partially immunocompetent. Epidemiologically, this window of vulnerability has very important implications for developing strategies to optimise BRSV vaccination and prevent clinical disease in young cattle.

The design of this experiment confounds management effects with those of physiological sex. Vertheyli (2001) reviews sex-based differences in humoral immunity in humans and mice but the majority of previous research in cattle suggests that management is the larger determinant of levels of IgG antibody. Management practices but not sex had significant effects on antibody titres to foreign antigens (human erythrocytes and ovalbumin) in dairy calves (Burton *et al.*, 1989a), while Muggli *et al.* (1987) found no difference between levels of antibody induced in male and female beef calves, vaccinated against the virus, BHV1. In the current study, sex/management exerted much stronger effects pre-vaccination than post-vaccination, probably due to a combination of group-specific pathogen exposure and the disparate calf-rearing systems used, effectively dairy and suckler regimes.

Many environmental factors such as housing, nutrition, weaning, transportation and field infections may also influence levels of IgG in juvenile cattle (Franklin *et al.*, 2003; Mackenzie *et al.*, 1997; Norheim and Simensen, 1985; Quigley *et al.*, 1995; Rajala and Castren, 1995) and may contribute to the variability between *year-of-birth* cohorts. Although the impact of physiological sex alone remains unknown, it is striking how, despite these external pressures, the post-vaccination BRSV-IgG antibody patterns of both sex/management groups were very similar in magnitude and frequency.

In younger calves, the half-life of maternally derived passive antibody and age are inversely linked. Based on the regular, predictable effect of damage and half antibody response patterns, it is probable that in the current study, the majority of pre-existing antibody is maternally derived. In this population of over 400 animals with ages ranging from 60 to 170 days at vaccination and using three data not sample points, the half-life of circulating BRSV-specific IgG was estimated as ~24 days. This value is consistent with most previous studies: 26 days (Ellenthal et al., 2000); 21-34 days (Larsen, 2000); 30 days (Kimman et al., 1987b) and within the margin for error of ~30(±5) days reported by others (Fulton et al., 2004).

The progressive maturation of the juvenile immune system to overcome declining levels of pre-existing maternal antibody is a function of age. Pre-existing BRSV IgG1 antibody from either previous natural infection or transfer of antibody via colostrum, suppresses humoral responses to vaccination (Belknap et al., 1991; Kimman and Westenbrink, 1990; Mahaney and Burrows, 2005; Lee et al., 1994). This study confirms that this suppressive effect of pre-existing IgG antibody operates even when serum levels are relatively low. Fulton et al. (2000) suggested that pre-existing antibody with titres of 1/32 or more curbed antibody responses to BRSV vaccination, while Kubota et al. (1992) found that suppression began at titres of 1/4 or higher. Maternally-derived antibody appeared to interfere proportionately with vaccination against influenza virus in foals (van Maanen et al., 1992). The nominal estimate of the inhibitory threshold due to pre-existing IgG antibody provided here is intended both as guidance for future vaccine research and a practical clinical recommendation. Even if an explicit antibody response is blocked due to maternally-derived antibody, immunological priming may occur so that vaccinated calves may have secondary-type antibody responses to BRSV challenge (Ellis et al., 1991; Larsen et al., 2001).

A physiological delay of 24-28 days between vaccination and maximal BRSV IgG1 synthesis has been reported previously (Larsen, 2000). This study supports this, with both absolute levels and deviations (Δ) in IgG levels peaking between Days 14 and 28 after first vaccination. In this study, calves, over 120 days old, reacted strongly to BRSV vaccination either because their immune systems were sufficiently mature or because pre-existing BRSV IgG had diminished sufficiently to minimise inhibition of the antibody response, or a combination of both. However, by Day 56 when the humoral response had peaked, overall age appeared to only marginally influence levels of BRSV-IgG. A study by Orell et al. (2000) found that circulating B-cell

levels were four-fold lower in calves aged less than 5 weeks old compared to those aged over 32 weeks old and also that older calves had higher circulating lymphocyte counts than younger calves. Those cellular findings may explain much higher levels of antibody responses found by those authors in older calves when compared to younger age groups. Age-associated responses have also been described previously for immunisation of cattle against antigens such as human serum albumin (Lie, 1979), keyhole limpet haemocyanin (Pollock *et al.*, 1994), BRSV and *Mycoplasma spp.* (Martin *et al.*, 1999). Schollum and Marshall (1985) reported that three times as many 6-month old calves as 3-month old calves, responded to vaccination against leptospiral bacterins. In that study, older groups had significantly higher antibody titres than younger ones. In Holstein calves from BVDV-immunised dams, the median ages by which BVDV-specific colostral antibodies declined to negligible levels were 141 and 114 days, respectively (Munoz-Zanzi *et al.*, 2002). In that study, subsequent antibody responses to BVDV vaccination were strongly influenced by peak levels of absorbed colostral antibody. Again in Holstein calves, Hodgins and Shewen (1998) reported an age-related unresponsiveness to immunisation against *Pasteurella* spp.

The relationship between age, level of maternally derived antibody and BRSV vaccine responses has been discussed previously (Kimman *et al.*, 1987b; Kimman *et al.*, 1988; Muggli-Cockett *et al.*, 1992). In human infants, a paradigm was proposed where immaturity of the immune system (*age*) negatively affected primarily antibodies directed against the hRSV F-glycoprotein, while pre-existing maternal antibody negatively affected primarily antibodies directed against the hRSV G-glycoprotein (Murphy *et al.*, 1986). The late decline in levels of BRSV-IgG despite a booster vaccination may reflect consumption, increased catabolism or a down-regulation of the synthesis of specific IgG. The summary trait, area under the curve, has been used previously by other authors (Grell *et al.*, 2005; Nielsen *et al.*, 2001) for evaluation of antibody response. In the current study, genetic effects were more apparent when evaluated for antibody levels on individual sampling days.

Cross-breeding influenced pre-vaccination levels of antibody; animals with a higher proportion of Holstein genes had greater levels of BRSV-specific IgG antibody than animals with higher proportions of Charolais genes. This dissimilarity may be due either to calf-breed or birth-dam factors. Breed-associated differences in antibody responses reported previously, such as higher antibody responses in Angus compared to Simmental calves (Engle *et al.*, 1999), focussed on broad contrasts between breeds.

Using the Robogen population there was no evidence for interbreed differences between Holstein and Charolais cattle breeds with regard to IgG antibody responses to BRSV vaccination. As only twelve different sires were used it is likely that a subset rather than the full repertoire of bovine alleles for humoral response may have been represented.

Whereas non-specific bovine respiratory disease was shown to have low heritabilities in housed and grazing cattle (Muggli-Cockett *et al.*, 1992), a variety of novel antigens such as human serum albumin, human erythrocytes and ovalbumin have induced antibody responses with heritabilities of 0.31 to 0.56 (Lie, 1979); 0.51 and 0.87 (Burton *et al.*, 1989a) and 0.50 (Wagter *et al.*, 2000) in cattle. Detilleux *et al.* (1994) described mean heritabilities of between 0.55 to 0.84, 0.24 to 0.63 and up to 0.43 for basal levels of IgM, IgG_2 and IgG_1, respectively. Newman *et al.* (1996) identified significant effects of both sire and MHC *BoLA* type on levels of IgG antibody, induced by immunisation against the bacterium *Brucella abortus* in mixed-breed beef heifers. Furthermore, García-Briones *et al.* (2000) described associations between allelic variability in specific *BoLA* genes and the humoral response of Hereford steers, to vaccination against foot-and-mouth disease. As in the latter two studies, by concentrating on antibody responses linked to pathogens, which are a present threat to cattle production systems, the current study is intended to demonstrate how farm animal genetics can influence individual animal immunity to infectious agents. If indicators of immunity such as BRSV-specific total IgG have h^2 of ~0.3 (obtained under pathogen and management conditions typical of UK commercial farms) it is a sufficiently heritable trait for practical incorporation into selective breeding programmes. This is within-breed variation based on sire variance, independent of other multi-breed effects such as heterosis. This report shows that such an approach is both robust and highly relevant to contemporary problems "in the field" such as BRD.

Calves are born agammaglobulinaemic. Until the maturation of the immune system at approximately three months old, the level of serum antibody depends entirely on absorption of passive antibody, from colostrum ingested at birth (Matte *et al.*, 1982). Initial post-absorption levels of maternally-derived passive antibody (MDPA) in neonatal calves are governed by the pathogen experience of the dam, colostrum quality, volume and efficiency of the intestinal absorptive process (Abel Francisco and Quigley, 1993). Thereafter, levels of serum MDPA decay exponentially due to increasing animal size and antibody catabolism. The effects of maternally inherited

immunity are accepted to be most important in neonatal animals and decline as animals mature (Grindstaff *et al.*, 2003). However, early bovine life is typically beset by infectious diseases, such as BRSV respiratory disease, so the impact of these maternal factors on long term productivity is significant (Belknap *et al.*, 1991; Wells *et al.*, 1996). Moreover, as passive immunity diminishes and is superseded by an endogenous immune response, the transitionary period marks a window of specific vulnerability and elevated susceptibility to infectious disease.

Extrinsic factors (season, climate, nutrition, etc) which control MDPA have been systematically investigated in cattle (Besser and Gay, 1994; Jaster, 2005). Genetic control however has been reported much more unevenly. Muggli *et al.* (1984) found associations between serum levels of non-specific MDPA and dystocia ($p<0.05$), calf-age ($p<0.05$), dam-age ($p<0.05$) and calf-breed ($p<0.01$). In that study, heritabilities (±se) for sire and maternal effects were estimated as 0.09 (±0.09) and 0.27 (±0.17), respectively. Gilbert *et al.* (1998) reported that line-of-sire and the degree of inbreeding of the dam influenced serum levels of IgG_1 in 36-hour old beef-breed calves. Those authors found calf-sex, calf-sire, sire-line or dam-age as non-significant determinants. In a study of crossbreeding between Angus and Brahma calves, Vann *et al.* (1995) reported that crossbred calves had higher levels of colostral antibody than purebred calves. Neither antibody-class or calf-breed appeared to have any effect on efficiency of absorption in that experiment. More recently, haplotypes expressing specific *Fc* antibody receptors (β-2-microglobulin), which mediate intestinal transmission of IgG, have been associated with failure of MDPA transfer in calves (Clawson *et al.*, 2004; Laegreid *et al.*, 2002).

An indirect heritability (i.e. maternal) is when the genotype of one individual influences the phenotype of another individual. In contrast, a direct heritability (i.e. additive) is when the genotype of one individual influences its own phenotype. Ideally, both indirect (maternal genes) and direct (progeny genes) heritable effects could be used jointly to beneficially modify immunity to infection, especially in younger cattle. These results show that the pattern of variation in antibody responses to BRSV vaccination is dynamic but clearly defined. Pre-vaccination, approximately 1/3 of the observed variation is maternal, attributable to the dam and repeatable across calvings without additive genetic variation. Post-vaccination, the dominant component of the heritable variance becomes additive rather than maternal. The evidence for repeatable maternal variation comes from utilising the Robogen herd

structure in which the same dams calved into the same farming environment over several calving seasons, the average number of progeny per dam being ~2.22 over the duration of the study.

Whether or not the maternal component is inherited is fundamental to understanding the genetics of vaccine response. The results show that the maternal transmissible component is substantial pre-vaccination and that antibody levels pre-vaccination are negatively correlated with antibody levels achieved post-vaccination. Different possibilities exist:

(i) The repeatable maternal component may be entirely due to the life history of the dam, i.e. previous exposure events. Then with no inheritance the opportunities for selection are limited and can be interpreted at their face value which emphasizes the role of farm management and husbandry.

(ii) They may be inherited so that the dam's maternal effect and the calf's additive genetic effect are positively correlated, in which case high maternal protection in early life leads to greater inhibition of vaccine response in her progeny. However the same progeny will benefit positively from additive immune function genes in mounting an endogenous antibody response, so the tendency is to reduce the overall genetic variation observed in the vaccine response, effectively masking the amount of genetic variation present.

(iii) They may be inherited so that the dam's maternal effect and the calf's additive genetic effect are negatively correlated. In this case, high maternal protection in neonatal life will suppress the potential response to vaccination. This is further compounded by the additive genetics inherited from the dam, also leading to a reduced vaccine response. This doubly negative outcome will tend to increase the overall genetic variation observed in the vaccine response, amplifying the amount of genetic variation apparent.

Exploration of the association between phenotypic responses to vaccination and animal genetics permits vaccine development to encompass vaccine responses in their entirety, to include host-vaccine interactions, in contrast to simply focussing on the attributes of the vaccine alone. Moreover, although management is important for controlling disease in domestic animals, an individual's genetic background also makes a sizeable contribution to vaccine response. Identification of animals with sub-optimal antibody responses should allow vaccine development to focus on lower responder groups, increasing overall herd efficacies for vaccines. In a wider sense,

with almost a third of variation accounted for by genetics, this study stresses the necessity for all vaccine studies to utilize as genetically diverse and genetically defined a test population as possible (Ovsyannikova *et al.*, 2004a). Although it may be presumed that the commercial vaccine has been optimised in terms of inoculate dose and content, genetic variability remains detectable, thus emphasizing its importance.

One of the most useful extensions to the current study would be to link the antibody phenotypic responses observed to protection from clinical disease. The size of the study (n=463), and the time and the resources available for this segment of the project, precluded the type of clinical assessments and bioassays required for a complete evaluation of disease protection. Such deficiencies affect all experiments based on indicator traits although the moderately high correlation (~0.7) between the SNT and ELISA results does help bridge this gap. Much greater resources are necessary in order to overcome the difficulties in objective assessment of challenge studies. More calves from a larger number of sires, using more cattle breeds and an experimental blocking design to permit the isolation of effects based on physiological sex alone, would have improved the clarity of the results. Altering the design of the study to facilitate the observation of the more transient antibody isotypes such as IgM and IgA and the inclusion of a respiratory cellular immune component would have broadened the overall scope of the conclusions. Another useful addition would be to enlarge the study to include both cellular and humoral type mucosal responses to BRSV vaccination.

Mammalian systemic antibody responses to vaccination are complex polygenic traits modified by environmental and host genetic factors. Recognising and understanding how host genotype controls such immunological responses will enhance the effectiveness of current and future generations of vaccines. This research verifies that immune responses to viral vaccines in cattle are heritable and makes progress towards the eventual identification of bovine genetic markers relevant to optimal antibody production following vaccination.

Chapter 4

Antibody to bovine parainfluenza-3 virus and bovine herpesvirus-1

4.1 Introduction

Bovine parainfluenza-3 virus (PIV3) and bovine herpesvirus 1 (BHV1) are common respiratory pathogens of cattle. In calves, PIV3 infection is usually mild or silent (Allan *et al.*, 1978), and often a component of mixed-infection respiratory disease (Adair, 1986; Bryson *et al.*, 1979a; Rosenquist and Dobson, 1974). In contrast, BHV1 infection can present as rhinotracheitis, meningoencephalitis, conjunctivitis, vulvovaginitis, abortion or neonatal systemic infection (Dungworth, 1992; Gibbs and Rweyemamu, 1977; Tikoo *et al.*, 1995). Parainfluenza-3 virus is almost ubiquitous among the British cattle population with average overall herd prevalences of ~97% (McCullough *et al.*, 1987) and with ~13% of all British calves being seropositive (Adair *et al.*, 2000). Parainfluenza-3 virus was detected in 49% of Northern Ireland BRD outbreaks with confirmed virus involvement (Graham *et al.*, 1998b). The prevalence of BHV1 in European cattle has been reported as between 26-35% in Italy, 20-38% in Poland and 16-64% in Hungary (Castrucci *et al.*, 2002). In the UK, between January and October 2004, 106 incidents of BHV1-associated disease were confirmed by the Veterinary Laboratory Agency, eighty of which presented primarily as respiratory disease (Holliman *et al.*, 2005).

Parainfluenza-3-virus-related disease is most common among calves aged two to twelve weeks old (Dungworth, 1992). Pyrexia, coughing, tachypnoea, nasal discharge, and depression are frequent clinical findings (Bryson *et al.*, 1979b). In comparison, BHV1 infection typically occurs in older animals (Babiuk *et al.*, 1996) with common clinical signs including pyrexia, coughing, conjunctivitis, lacrimation, rhinitis, nasal discharges, snoring, and inappetance (Edwards *et al.*, 1991). In common with other alphaherpesviruses, BHV1 causes life-long infection of the sensory nerve ganglia with the potential for periodic reactivation and virus shedding with associated epizootics (Pastoret *et al.*, 1982). Concurrent infections with BHV1 and PIV3 demonstrate delayed and lower specific antibody responses, higher serum cortisol levels and more exaggerated clinical signs than animals incubating either virus singly (Ghram *et al.*, 1989).

Vaccination is widely used for the control of both viruses with intranasal temperature-sensitive vaccines providing rapid, long-lasting immunity with useful mucosal action (Burroughs *et al.*, 1982; Martin *et al.*, 1983). Vaccination

against BHV1 reduces the period of virus shedding, and both peak and total amounts of virus shed (Kerkhofs *et al.*, 2003). Parainfluenza-3 virus (strain RLB 103) and BHV1 (strain RLB 106) vaccine strains are chemically-altered, genetically-stable mutants, incapable of replication above 39°C (Zygraich *et al.*, 1974a; Zygraich *et al.*, 1974b). Modified-live forms of these two viruses have been combined commercially for intranasal vaccination since 1976 (Todd, 1976). Specifically, immunisation with Imuresp RP (Pfizer Animal Health, Sandwich, UK) gave significant protection against PIV3 challenge (Bryson *et al.*, 1999), and both clinical signs and lung pathology were found to be reduced in challenged vaccinates. In addition, Imuresp RP induced BHV1 antibody (Lucas *et al.*, 1982), preventing clinical disease and significantly reducing BHV1 virus shedding (Fairbanks *et al.*, 2004). A PIV3-specific seroconversion rate of 82% was reported 10 weeks after a single intranasal PIV3/BHV1 vaccination (Durham *et al.*, 1991), while seroconversion rates of 87-94% were described for intranasal BHV1 monovalent vaccines. In another study, 93% of animals seroconverted to BHV1 and 100% of animals seroconverted to PIV3 following multivalent intranasal vaccination (Weiblen *et al.*, 1982).

No single laboratory test allows perfect assessment of immunocompetence due to the complexity of the mammalian immune system. However, it is accepted that specific antibody production is correlated with degrees of protection (Plotkin, 2001). Detection of serum antibody level is therefore an objective, reliable and useful means of determining the activation of the immune system, although it can never encompass the full extent and efficacy of the overall immune response (Perino and Hunsaker, 1997). Testing for antibody using commercial BHV1 and PIV3 ELISAs has undergone extensive validation (Graham *et al.*, 1997) and has been proved a robust and reliable method of detecting the major antibody isotype IgG_1. A BHV1 ELISA was found to be almost 100-fold more sensitive for diagnostic purposes than testing by virus neutralisation, the latter being specific only for antibodies which functionally neutralise BHV1 *in vitro* (Dubeski *et al.*, 1996).

To date, most research into BHV1 and PIV3 vaccine responses has either been small scale or alternatively deliberately designed to minimise or exclude fixed effects such as age, breed, sex and management (Ahl and Straub, 1985; van der Poel *et al.*, 1995; Zygraich *et al.*, 1975a). The host genetics behind immune

responses to the two viruses also remains relatively unexplored. Differences in allelic frequencies in host interferon genotype were linked to the severity of BHV1-induced disease (Ryan and Womack, 1997). Wimmers *et al.* (2004) found associations between levels of specific IgG antibody and *BF*, *DRB* and *DQB* porcine genotypes following vaccination against another important animal alphaherpesvirus, Aujeszkys Disease (AD) virus. Further, quantitative trait loci on chromosomes 9 and 11 have been demonstrated to correlate with clinical signs (neurological signs and pyrexia) in pigs which were experimentally infected with AD virus, thereby linking host genotype to disease phenotype (Reiner *et al.*, 2002).

The current study is one of the first to systematically address the many complex factors which control the humoral response to PIV3/BHV1 vaccination in cattle. A large juvenile crossbred bovine population of known parentage was immunised with a commercially available PIV3/BHV1 vaccine. Statistical models were developed, thoroughly investigating the effect of environmental factors such as year-of-birth, age and sex effects on vaccine response phenotypes. By integrating accurate pedigree records with these models, genetic effects should be detectable and discernible from the environmental effects and the relative contribution of the two sources of variation should be computable.

4.2 Methods

4.2.1 Vaccination and sampling

The study population was the Robogen herd. This experimental population and the vaccination protocol are described in detail in Chapter 2, section 2.2.3. Blood samples were collected by jugular venepuncture on Day 0, the day of vaccination, and Days 14, 28, 42, 63 and 77 post-vaccination, providing six samples per calf.

4.2.2 ELISA for detection of PIV3- and BHV1-IgG antibody

Sera were tested quantitatively by solid-phase antibody capture ELISA specific for total PIV3-IgG and total BHV1-IgG according to the manufacturer's guidelines (SVANOVA Biotech, Uppsala, Sweden). Briefly, samples were added at a dilution of 1/25 in PBS-Tween buffer (0.01 M pH 7.4 phosphate buffered saline (PBS) containing 0.05% Tween 20) to each of two wells, one coated with viral antigen and the other with control antigen, to a final volume of 100 µl. Positive and negative control sera were included on each 96-well microtitration plate at a dilution of 1/25. Each plate was shaken, sealed and incubated for one hour at 37°C, then washed three times with PBS-Tween buffer. After the third wash, each plate was tapped dry and 100 µl of horse-radish peroxidase-conjugated anti-bovine IgG added to each well. Each plate was then incubated for a further hour at 37°C and washed as previously described. One hundred µl of substrate solution (3',3',5',5'-tetramethylbenzidine and hydrogen peroxide) were added to each well and the plate was incubated at room temperature for ten minutes after which the reaction was stopped by adding 50 µl of 2 M H_2SO_4 per well. After shaking, the optical density (OD) of each well was measured immediately at 450 nm on a MRX microplate plate reader (Dynex Technologies, Chantilly, USA). The corrected optical density (COD) value for each sample and the assay control sera were calculated by subtraction of the OD value of each control antigen-coated well from that of the corresponding viral antigen-coated well, and the relative optical density (ROD) value for each sample calculated as a percentage of a highly positive control serum on a per-plate basis:

$$\text{ROD (\%)} = \frac{\text{COD serum sample} \times 100}{\text{COD positive control}} \qquad \text{[EQ 4.1]}$$

Samples were tested in duplicate and repeated if paired means were >10% discordant. The inter-plate repeatabilities for the PIV3-IgG ELISA and BHV1-IgG ELISA were 97.1% and 96.6%, respectively ($p < 0.001$).

4.2.3 Serum neutralisation test for detection of PIV3 and BHV1 antibody

The serum neutralisation test (SNT) is a well established *in-vitro* technique for determining specific antibody levels (Gillette, 1983). For assay validation and comparison, serum antibody levels specific to PIV3 and BHV1 were determined independently (Biobest Laboratories Ltd, Pentlands Science Park, UK) using the SNT, in addition to the ELISA. A panel of thirty sera, five selected from the six sampling days, were analysed, chosen to represent the range of PIV3-IgG and BHV1-IgG responses on each sampling day. Correlations between SNT results and the corresponding ELISA results were calculated.

4.3 Statistical Methods

4.3.1 Data preparation

Means, medians, variance and interquartile range for all traits were calculated using Genstat 7.0 (VSN International Ltd, Herts, UK). The same software was used to calculate skewness (a measure of distribution symmetry) for each trait, with normality set as a skewness of 0.0. The data collected on PIV3-IgG and BHV1-IgG antibody levels were normalised by log transformation.

Deviations (Δ) in antibody levels between days post-vaccination (Days 14, 28, 42, 63 and 77) and the day of vaccination (Day 0) were calculated for both viruses - so typically for Day 14:

$$\Delta p\text{IgG(D0 to D14)} = \text{Day 14 PIV3-IgG} - \text{Day 0 PIV3-IgG} \qquad \text{[EQ 4.2]}$$

and

Δ*I*IgG(D0 to D14) = Day 14 BHV1-IgG - Day 0 BHV1-IgG [EQ 4.3]

When overall antibody profiles had linear patterns, it was possible to calculate a summary trait, the **6-day-average**, taken as the arithmetic mean of the ROD values on all six sampling days.

For both PIV3-IgG and BHV1-IgG profiles, the total area under the antibody curve was calculated between Days 0 and 77, inclusive. The trapezoidal rule was used to provide a good working approximate of this second alternative summary trait (Abramowitz and Stegun, 1972).

So typically:

6-day-area *p*IgG = area under PIV3-IgG curve from Day 0 to Day 77 [EQ 4.4]

4.3.2 Non-regressive statistical methods

Depending on the number of factor levels, either unpaired Students t-test (2 factors) or one-way analysis of variance (ANOVA; ≥3 factors) were used to compare factor effects. Tests were confirmed as significant if $p < 0.05$ and highly significant if $p < 0.001$. Pearson product moment correlations between combinations of factors and variates were evaluated for levels of PIV3-IgG and BHV1-IgG on all sampling days and *r* values calculated. Excel 2003 (Microsoft Corp., Seattle, USA) was used for collation and preparation of data and Genstat 7.0 (VSN International Ltd, UK) was used throughout for the statistical analysis of results, unless otherwise stated.

4.3.3 Logistic regression

By introducing a binary coding system whereby Δ*p*IgG(D0 to D14), Δ*p*IgG(D0 to D28) and Δ*p*IgG(D0 to D42) were converted into either: 1 (positive Δ) or 0 (negative Δ) a variate suitable for analysis by logistic regression was generated. Linear binomial models with these binary variates as the response variable (y) and the level of Day 0 BRSV-IgG antibody as the explanatory variable (χ) were

fitted to the data using a logit link function, $\left(\log\left(\frac{p}{1-p}\right)\right)$ and the associated model parameters (α and β) estimated.

Using

$$y = \alpha + \beta\left(\chi - \bar{\chi}\right) \qquad \text{[EQ 4.5]}$$

where p is the probability of binary 1, (i.e. a positive Δ); $\bar{\chi}$ is the population mean level of Day 0 PIV3-IgG antibody. Assuming that when p =0.5, a positive or negative antibody response was equally likely, it was possible to provide an empirical estimate of the threshold at which pre-existing PIV3 antibody begins to block PIV3 vaccine response. If this first threshold is envisaged as the point at which 50% of the calves showed a positive response, a second useful threshold may be estimated at p =0.9 when a positive vaccine response may be expected in 90% of the population.

This analysis was then repeated exactly as above but using Δ*i*IgG(D0 to D28), Δ*i*IgG(D0 to D42) and Δ*i*IgG(D0 to D63) to obtain threshold values at which BHV1-IgG begins to exert a suppressive effect.

4.3.4 Univariate mixed linear regression

Mixed linear models were fitted to the data using Residual Maximum Likelihood (REML) methods, with Genstat procedures (Genstat 7.0, VSN International Ltd). The impact of a number of explanatory factors was evaluated and the remaining variation decomposed into variances of either direct heritable or environmental origin. The fixed effects in the model, with appropriate degrees of freedom (df), were *breed-cross* (BCH, BH, F2; 2df), *sex* (male, female; 1df), *year-of-birth* (1998, 1999, 2000, 2001; 3df), *dam-age* (2, 3, 4, 5, 6, 7; 5df) and *age* at first sampling (continuous variate), with calf *sire* included as a random effect. As male and female calves were reared separately and under different management, the effect *sex* incorporates management and environmental components, in addition to its physiological effect. To improve model clarity, the fixed effect *breed-cross* was further resolved into *Holstein* and *recombination-loss* fractions in accordance with: *breed-cross* **BCH** = (0.25 *Holstein*, 0.50 *recombination-loss*); *breed-cross* **F2** = (0.50 *Holstein*, 1.0 *recombination-loss)* and *breed-cross*

BH = (0.75 *Holstein*, 0.50 *recombination-loss)*. *Holstein* represents the expected genetic contribution of the Holstein cattle breed, while *recombination-loss* represents the expected fractional loss in phenotypic traits in progeny, arising from non-productive recombinations of genetic loci at fertilisation which previously interacted in either of the two parental breeds (Fries *et al.*, 2002).

Thus the linear model was:

$$Y_{ijklmnp} = \mu + \alpha_i + \beta_j + \delta_k + \pi_l + \gamma_m + \varepsilon(s_{ijklmnp} - \hat{s}) + u_{ijklmn} + e_{ijklmnp} \quad \text{[EQ 4.6]}$$

where

$Y_{ijklmnp}$ - observation of phenotypic trait,
μ - population mean,
α_i - effect (fixed) of sex i (i = 1,2),
β_j - effect (fixed) of Holstein fraction j (j = 1,2,3),
δ_k - effect (fixed) of recombination-loss fraction k (k = 1,2,3),
π_l - effect (fixed) of dam-age l (l = 2,3,4,5,6,7)
γ_m - effect (fixed) of year-of-birth m (m = 1,2,3,4),
$\varepsilon(s_{ijklmnp} - \hat{s})$ - effect (fixed) of sampling age $s_{ijklmnp}$ (age of calf *ijklmnp*) expressed as a deviation from the population mean age $\hat{s}$,
u_{ijklmn} - direct heritable effect (random) of sire $n \sim N(0, \mathbf{I}\sigma_s^2)$,
$e_{ijklmnp}$ - residual error (random) $p \sim N(0, \mathbf{I}\sigma_e^2)$.

Both u_{ijklmn} and $e_{ijklmnp}$ were assumed to have multivariate normal distributions with means 0 and (co)variances $\mathbf{I}\sigma_s^2$, and $\mathbf{I}\sigma_e^2$, respectively. Estimates of direct heritability ($\mathbf{h}^2$) were calculated using:

$$h^2 = 4\frac{\sigma_s^2}{\sigma_p^2} \quad \text{[EQ 4.7]}$$

where phenotypic variance σ_p^2 was estimated as $\sigma_p^2 = \sigma_s^2 + \sigma_e^2$, as defined above.

The basic model was extended for post-vaccination Days 14, 28, 42, 63 and 77 by adding the appropriate level of pre-existing PIV3-IgG or BHV1-IgG antibody on the day of vaccination (Day 0) as covariates (q_1 and q_2).

The significances of the fixed effects and their interactions were estimated by the generalised Wald test. Only those interactions achieving statistical significance ($p < 0.05$) were retained in the final best-fit model. The significance of random effects in the model was determined using log-likelihood ratio tests. The robustness of each model produced was verified by examining the linearity of standardised deviance residuals against normal order statistics (Belsley *et al.*, 1980).

4.4 Results

4.4.1 Descriptive analysis

Due to the large numbers of calves with low levels of specific antibody, the distributions of both the PIV3-IgG and BHV1-IgG raw data are right-skewed (Table 4.1(a+b)). Both datasets better approximated normality after log transformation. The multivalent BHV1/PIV3 vaccination occurred in the presence of high levels of pre-existing antibody to both viruses. Overall Day 0 IgG mean levels against BHV1 and PIV3, were quite similar (ROD =35.7%) and (ROD =39.6%), respectively (Table 4.1(a+b)). But at individual calf level, there was strong evidence that the pre-vaccination Day 0 level of PIV3-IgG was consistently higher than the equivalent level of BHV1-IgG on Day 0 (t-test; $p < 0.001$) and the subsequent pathogen-specific IgG response profiles showed many dissimilarities between responses to BHV1 and PIV3.

Generally, overall mean levels of PIV3-IgG were high throughout the study yet these tended to show a moderate and progressive increase, to Day 77 (Figure 4.1(a)). Post-vaccination, overall mean PIV3-IgG levels declined 1.07-fold (antilog) between Day 0 and Day 14 then increased between Days 28 and 77 (Table 4.1(a)). Day 63 and Day 77 PIV3-IgG levels (antilogs) were 1.66-fold (t-test; $p<0.001$) and 1.62-fold (t-test; $p<0.001$) those of Day 0, respectively. Differences (Δ) between consecutive sampling days were most significant for ΔpIgG(D14 to D28) and ΔpIgG(D28 to D42) (t-test; $p < 0.001$) and dropped in magnitude by ΔpIgG(D63 to D77) (Table 4.1(a)). Overall Day 28 had the minimum PIV3-IgG variance being 2.24-fold smaller than that on Day 0, the day with the maximum value. The PIV3-IgG variance was 1.58-fold smaller on Day 77 compared to that of Day 0. However, the interquartile range (dQ) decreased to Day 28 before increasing steadily to Day 77. The summary trait, 6-day-area *p*IgG, had a much more normal distribution than most of the other traits examined.

(a) PIV3-IgG

Trait	(ROD) mean	variance	(ROD) median	(ROD) dQ	skewness
Day 0 *p*IgG	39.6	1082.0	29.9	46.2	0.9
Day 14 *p*IgG	37.0	988.8	26.0	37.0	1.3
Day 28 *p*IgG	42.7	677.7	36.8	27.7	1.4
Day 42 *p*IgG	47.0	700.7	41.1	34.0	0.9
Day 63 *p*IgG	52.0	1088.2	42.9	46.6	0.8
Day 77 *p*IgG	54.4	1568.6	40.9	54.8	0.8
Δ*p*IgG(D0 to D14)	-2.6	228.8	-3.8	14.2	1.0
Δ*p*IgG(D14 to D28)	5.8	284.4	5.1	19.5	0.0
Δ*p*IgG(D28 to D42)	4.1	386.9	3.1	20.1	0.4
Δ*p*IgG(D42 to D63)	4.7	809.4	0.0	22.1	1.5
Δ*p*IgG(D63 to D77)	1.9	569.6	-2.3	15.7	2.0
6-day-area *p*IgG	446.0	4360353	572.0	2920.0	0.0

(b) BHV1-IgG

Trait	(ROD) mean	variance	(ROD) median	(ROD) dQ	skewness
Day 0 *i*IgG	35.7	1096.6	26.6	55.6	0.7
Day 14 *i*IgG	28.0	850.2	17.0	44.3	1.0
Day 28 *i*IgG	23.6	474.3	17.0	29.9	1.3
Day 42 *i*IgG	19.2	326.6	14.7	23.3	1.5
Day 63 *i*IgG	15.0	264.3	9.2	18.7	1.6
Day 77 *i*IgG	12.3	243.3	6.3	15.5	2.2
Δ*i*IgG(D0 to D14)	-7.7	147.3	-5.6	14.5	0.1
Δ*i*IgG(D14 to D28)	-4.2	239.7	-3.6	14.9	-0.2
Δ*i*IgG(D28 to D42)	-4.5	146.4	-3.0	12.5	-0.3
Δ*i*IgG(D42 to D63)	-4.2	118.8	-2.8	10.4	-0.6
Δ*i*IgG(D63 to D77)	-4.2	65.3	-1.5	6.1	1.1
6-day-area *i*IgG	-1087.0	2897664	-1161.0	2380.0	0.0
6-day-average *i*IgG	2.4	2.4	1.0	1.4	-0.2

Table 4.1 Means, variance, medians, interquartile ranges and skewness for levels of PIV3-IgG (**a**) and BHV1-IgG (**b**) on Days 0 to 77, relative to vaccination. Also descriptive statistics for **Δ*p*IgG** and **Δ*i*IgG, 6-day-area *i*IgG, 6-day-area *p*IgG and 6-day-average *i*IgG** on Days 0 to 77. BHV1/PIV3 vaccine administered on Day 0. Values based on non-transformed relative optical density data. **dQ** (interquartile range), **ROD** (relative optical density).

Overall mean levels of BHV1-IgG tended to decline gradually and continuously from Day 0 to Day 77, with the mean Day 77 BHV1-IgG level 2.7-fold (antilog) lower than that of Day 0 (Figure 4.1 (b)). The rate of decrease remained relatively constant at (~1.2-fold) between consecutive days. The variance also steadily decreased from Day 0 to Day 77 as more calves reached undetectably low levels of BHV1-IgG. The BHV1-IgG variance was 2.60-fold smaller on Day 77 compared to Day 0, the day with the maximum variance. In line with the decreasing mean and variance, the interquartile range (dQ) decreased for later sampling days. Once again, the summary trait, 6-day-area *i*IgG, was much more normally distributed than most of the other traits examined.

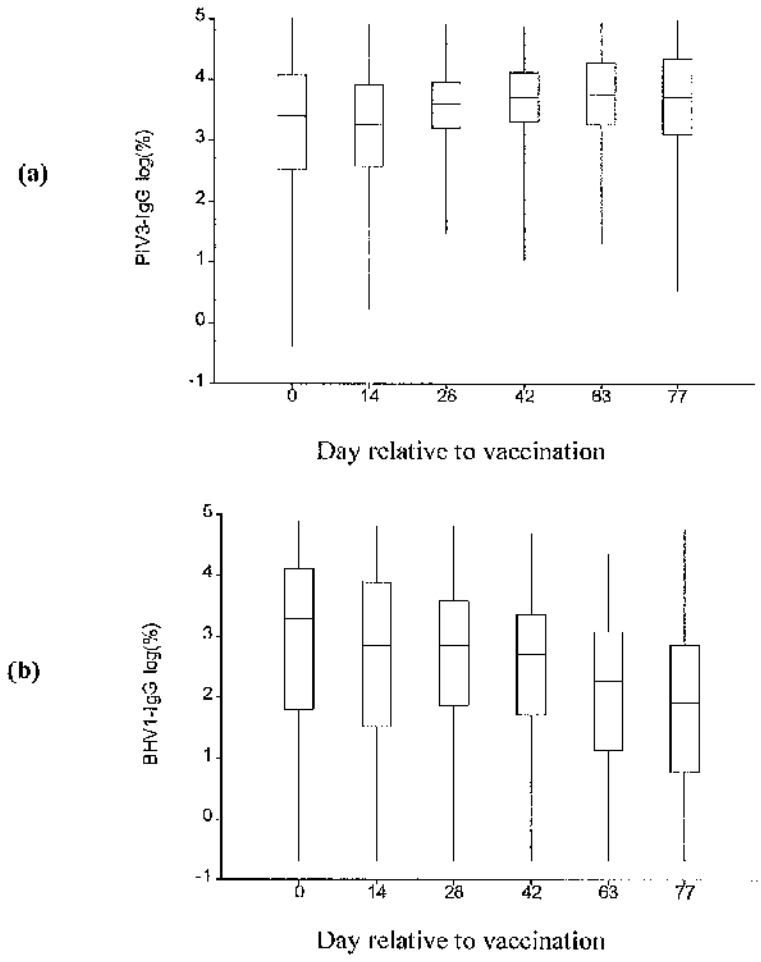


Figure 4.1 Levels of **(a)** PIV3-IgG and **(b)** BHV1-IgG on Days 0, 14, 28, 42, 63 and 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Median (central horizontal line), quartiles (outer horizontal lines), and range (outer vertical lines) shown. Values are log transformed relative optical density data.

4.4.1.1 PIV3- and BHV1-IgG antibody half-lives

Based on the regression coefficients from REML analysis on Day 0, the half-life of PIV3-IgG was estimated as 32.2 days (95% confidence interval: 29.4 to 35.6 days). Using a similar method for BHV1-IgG, the antibody half-life was estimated as 25.8 days (95% confidence interval: 19.8 to 37.2 days).

4.4.1.2 PIV3- and BHV1-IgG antibody correlations

Correlations between levels of PIV3-IgG and BHV1-IgG are shown graphically in Figure 4.2. Table 4.2 shows that intra-virus correlations between the first three samplings days (Day 0, 14 and 28) were high (r >0.600). Inter-virus (PIV3 and BHV1) correlations on these three days tended to be lower but those between Day 0 PIV3-IgG and Day 0 and Day 14 BHV1-IgG were still moderately high (r >0.500). All other inter-virus correlations between days were low (r <0.250) except those between Day 14 PIV3-IgG and Day 14 BHV1-IgG (r =0.520), between Day 14 PIV3-IgG and Day 28 BHV1-IgG (r =0.410) and between Day 28 PIV3-IgG and Day 14 BHV1-IgG (r =0.346). All reported correlation significances were $p < 0.001$.

The raw data for levels (untransformed) of total PIV3-IgG and BHV1-IgG on Days 0 to 77, relative to vaccination are shown in Appendices B.1 and B.2, respectively.

4.4.1.3 PIV3- and BHV1-specific serum neutralisation tests

The Pearson correlation coefficient between results from the BHV1-IgG ELISA and those obtained using BHV1-specific serum neutralisation test (SNT) was high, calculated as 0.72 (p <0.001). The equivalent Pearson correlation coefficient between results from the PIV3-IgG ELISA and those obtained using PIV3-specific SNT was even higher, calculated as 0.78 (p <0.001).

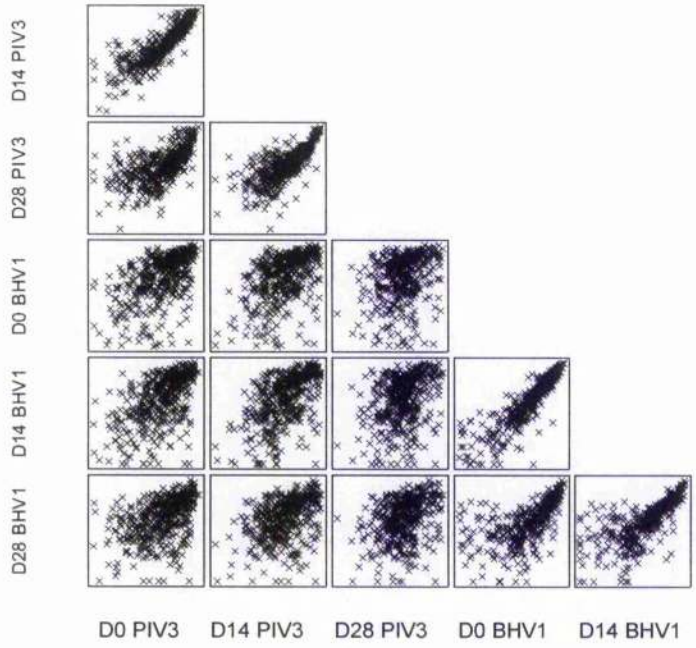


Figure 4.2 Matrix scatter plots showing correlations between levels of PIV3-IgG and BHV1-IgG on Days 0 and 28, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. All values are log transformed relative optical density data.

	Day 0 PIV3	Day 14 PIV3	Day 28 PIV3	Day 0 BHV1	Day 14 BHV1	Day 28 BHV1
Day 0 PIV3	1					
Day 14 PIV3	0.855	1				
Day 28 PIV3	0.609	0.728	1			
Day 0 BHV1	0.542	0.481	0.302	1		
Day 14 BHV1	0.560	0.520	0.346	0.895	1	
Day 28 BHV1	0.440	0.410	0.335	0.652	0.743	1

Table 4.2 Correlations between levels of PIV3-IgG and BHV1-IgG on Days 0 and 28 ($p < 0.001$). BHV1/PIV3 vaccine administered on Day 0. Values were calculated using log transformed relative optical density data.

4.4.2 Analysis of fixed effects

4.4.2.1 Sex / Management

Mean levels of PIV3-IgG in female calves were 1.69-fold, 1.69-fold, 1.35-fold and 1.24-fold (all back-transformed) higher than those of male calves (t-test; $p < 0.001$) on Days 0, 14, 28 and 42, respectively (Figure 4.3(a)). By Day 63, there was no difference in levels of PIV3-IgG between sexes (t-test; $p > 0.05$) and on Day 77, the male calves that had 1.34-fold higher levels of PIV3-IgG than the female calves (t-test; $p < 0.001$). For both sexes, the PIV3-IgG variance followed a very similar pattern between Day 0 and Day 28, a sharp ~2.2-fold decline. Female PIV3-IgG variance remained virtually constant between Days 42 and 77 while the male PIV3-IgG variance increased to 1.18-fold and 1.43-fold (antilog) that of the female group on Days 63 and 77, respectively (t-test; $p < 0.001$). Table 4.3 shows the significance attached when the effect, *sex*, is included in the REML models developed for PIV3-IgG on Days 0 to 77. *Sex* is highly significant across all sampling days either as a main effect or as a two-way interaction, the effect apparently strongest on Day 77.

In general, BHV1-IgG responses to vaccination were weak. In contrast to PIV3-IgG, the ratios (back-transformed) of mean levels of BHV1-IgG in the female calves to those of the male calves remained relatively constant (see Figure 4.3(b)) being 1.34, 1.43, 1.29, 1.29 and 1.35 on Days 0, 14, 28, 42 and 63, respectively (t-test; $p < 0.05$). Although on Day 77, female calves continued to have higher mean levels of BHV1-IgG compared to the male calves, the difference was not significant (t-test; $p > 0.05$). Bovine herpesvirus 1 vaccination appeared to only marginally affect mean levels of BHV1-IgG in either sex; overall mean Day 28 BHV1-IgG levels are only 1.17-fold mean Day 14 BHV1-IgG levels. However despite the small change in means between Day 14 and Day 28, the overall BHV1-IgG variance declined 3.09-fold over the same period. The male BHV1-IgG variance was lower, on average ~0.8-fold than the female BHV1-IgG variance up to and including Day 42 but on Days 63 and 77, it was the male BHV1-IgG variance which was ~1.5-fold higher than the female group. The variances of both sexes rose steadily from the low levels attained on Day 28. Table 4.4 shows the significance attached when the effect,

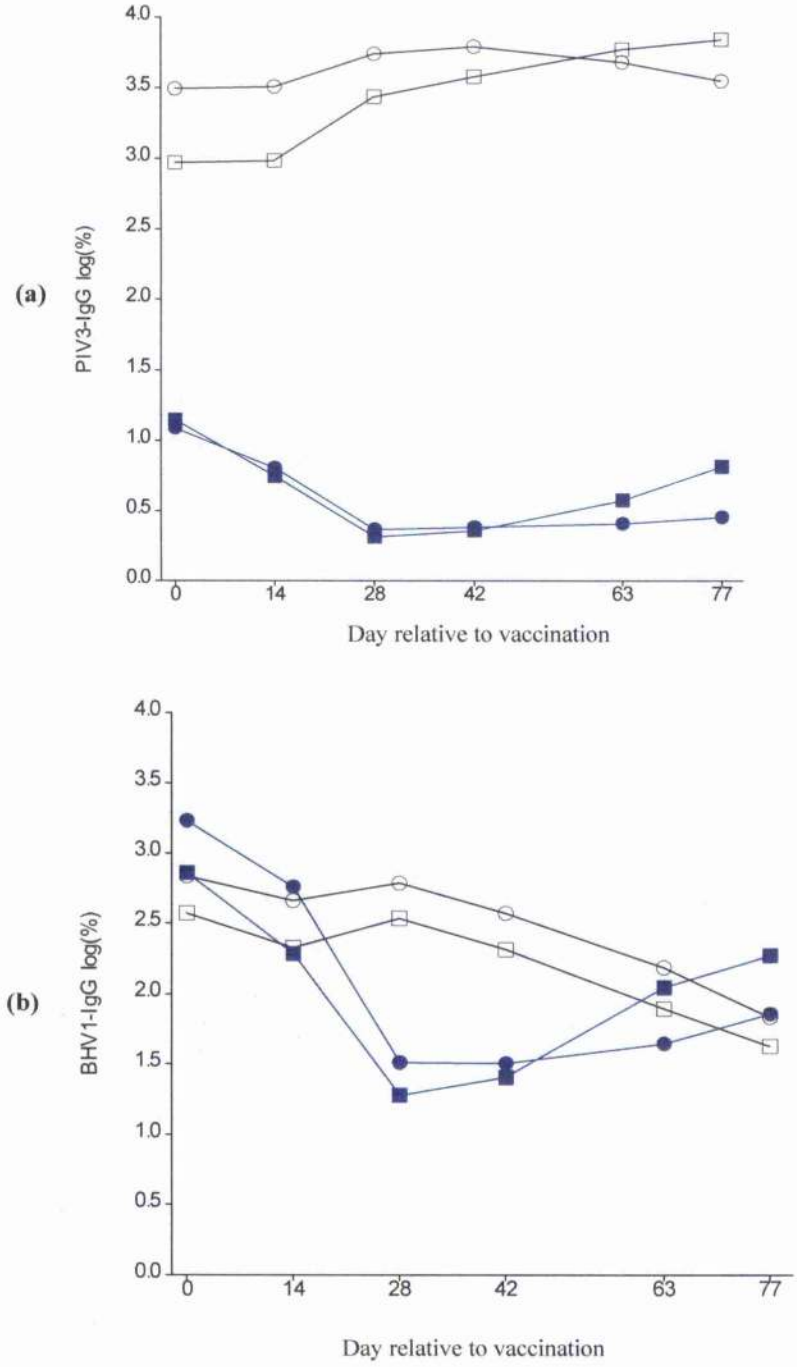


Figure 4.3 Levels of PIV3-IgG (**a**) and BHV1-IgG (**b**) from Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data and presented grouped on **sex:** female(○), male (□). Mean **(black)** and variance **(blue)** of data presented.

PIV3-IgG by sex

Day	REML term	Wald statistic	d.f.	*P* value
0	sex.year-of-birth	31.3	3	< 0.001
14	sex	17.4	1	< 0.001
	sex.Holstein	8.5	1	0.003
28	sex.year-of-birth	27.0	3	< 0.001
	sex.age	9.1	1	0.003
42	sex	10.5	1	0.001
	sex.Day 0-PIV3	7.9	1	0.005
	sex.Holstein	7.3	1	0.007
63	sex.year-of-birth	65.6	3	< 0.001
77	sex.year-of-birth	111.1	3	< 0.001

Table 4.3 Significant inclusions of the fixed effect **sex**, either as main effects or interactions for levels of PIV3–IgG on Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data. Day 0-PIV3 (level of PIV3-IgG on Day 0). Data analyses using REML models.

BHV1-IgG by sex

Day	REML term	Wald statistic	d.f.	*P* value
0	sex	5.0	1	0.026
14	sex.Day 0-BHV1	4.0	1	0.045
28	sex.Day 0-BHV1	7.8	1	0.004
42	sex.Day 0-BHV1	11.0	1	< 0.001
	sex. year-of-birth	7.9	3	0.048
	sex.recombination-loss	7.5	1	0.006
63	sex. Day 0-BHV1	21.3	1	< 0.001
	sex.year-of-birth	18.8	3	< 0.001
	sex. recombination-loss	8.3	1	0.004
77	sex. Day 0-BHV1	15.9	1	< 0.001
	sex.year-of-birth	14.4	3	0.002

Table 4.4 Significant inclusions of the fixed effect **sex**, either as main effects or interactions for levels of BHV1–IgG on Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data. Day 0-BHV1 (level of BHV1-IgG on Day 0). Data analyses using REML models.

sex, is included in the REML models developed for Days 0 to 77 BHV1-IgG. *Sex* is less significant early post-vaccination than on later sampling days, commonly present as two-way interaction with either *year-of-birth* or *Day 0-BHV1*. Altogether, *sex* seems to be of a lower order of significance for BHV1-IgG than for PIV3-IgG.

4.4.2.2 Year-of-birth

There are clear differences in mean levels of PIV3-IgG, based on *year-of-birth* (Figure 4.4(a)). In cohort 1999, the mean level of PIV3-IgG remains relatively constant over the six sampling days with a highest/lowest ratio of only 1.43 (back-transformed) whereas mean levels of PIV3-IgG in cohort 2001 show a relatively linear increase over the six sampling days with a highest/lowest ratio of 2.78. The mean PIV3-IgG profiles for cohorts 1998 and 2000 show features intermediate between the former two. Despite the disparities in the mean levels of PIV3-IgG between cohorts, PIV3-IgG variances follow very similar patterns over 1998, 1999, 2000 and 2001, generally falling to Day 28 and remaining at that level until Day 77. Table 4.5 shows the significance attached when the effect, *year-of-birth*, is included in the REML models developed for PIV3-IgG on Days 0 to 77. *Year-of-birth* is highly significant either as a main effect or a two-way interaction with *sex* or *age* for all sampling days except Day 42 and appears to be more important for the later sampling days.

In general, BHV1-IgG responses to vaccination were weak. Apart from Day 0 (ANOVA; $p < 0.01$), the day of vaccination, there were no significant differences (ANOVA; $p > 0.05$) between mean levels of BHV1-IgG based on *year-of-birth* (Figure 4.4(b)). Mean BHV1-IgG levels based on *year-of-birth* are minimal on Day 28 as all four cohorts tend to converge to ROD ~14%. Although only small changes are detectable in the mean BHV1-IgG levels based on *year-of-birth* over the six sampling days, vaccination is associated with a sharp decline in BHV1-IgG variance in all four years until Day 28 or Day 42, followed by a more gradual increase to Day 77. BHV1-IgG variance profiles, grouped on *year-of-birth*, converged on Day 28 with a highest/lowest ratio of only 1.24 (antilog) compared to an average ratio of 1.71 for the other five sampling days. Table 4.6 shows the significance attached when the effect, *year-of-birth*, is

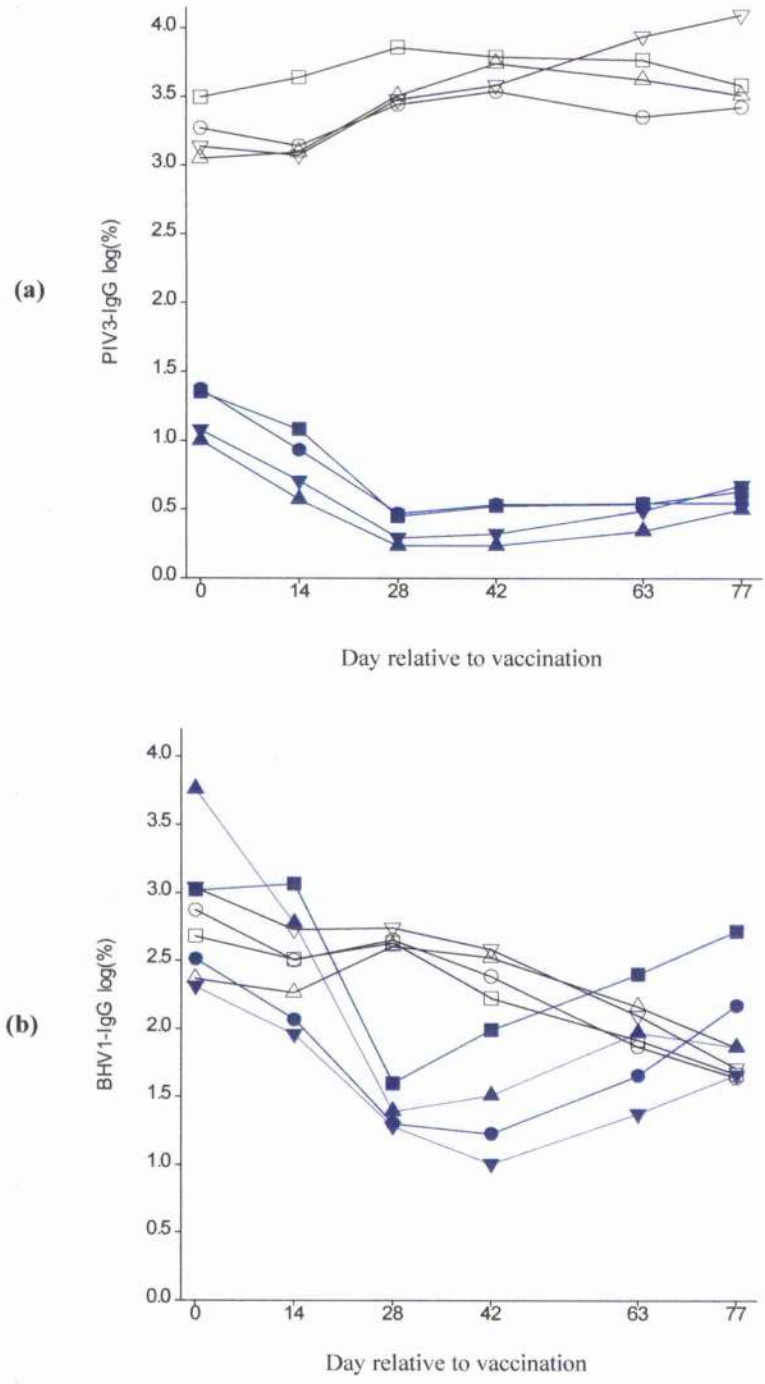


Figure 4.4 Levels of PIV3-IgG (**a**) and BHV1-IgG (**b**) from Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data and presented grouped on **year-of-birth:** 1998 (○), 1999(□), 2000(△) and 2001(▽). Mean **(black)** and variance **(blue)** of data presented.

PIV3-IgG by year-of-birth

Day	REML term	Wald statistic	d.f.	*P* value
0	year-of-birth.sex	31.3	3	< 0.001
14	year-of-birth	38.2	3	< 0.001
28	year-of-birth.sex	27.0	3	< 0.001
	year-of-birth.age	15.4	3	< 0.001
42	year-of-birth	7.6	3	0.05
63	year-of-birth.sex	65.6	3	< 0.001
	year-of-birth.age	20.4	3	< 0.001
77	year-of-birth.sex	111.1	3	< 0.001
	year-of-birth.age	22.5	3	< 0.001

Table 4.5 Significant inclusions of the fixed effect **year-of-birth**, either as main effect or interactions for levels of PIV3–IgG on Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data. Data analyses using REML models.

BHV1-IgG by year-of-birth

Day	REML term	Wald statistic	d.f.	*P* value
0	year-of-birth.age	14.9	3	0.002
	year-of-birth	11.5	3	0.009
14	year-of-birth	7.8	3	0.050
28	year-of-birth.Day 0-BHV1	15.3	3	0.002
42	year-of-birth.Day 0-BHV1	13.6	3	0.004
	year-of-birth.sex	7.9	3	0.048
63	year-of-birth.Day 0-BHV1	10.3	3	0.016
	year-of-birth.sex	18.8	3	< 0.001
	year-of-birth.recombination-loss	8.1	3	0.044
77	year-of-birth.sex	14.4	3	0.002

Table 4.6 Significant inclusions of the fixed effect **year-of-birth**, either as main effect or interactions for levels of BHV1–IgG on Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data. Day 0-BHV1 (level of BHV1-IgG on Day 0). Data analyses using REML models.

included in the REML models developed for Days 0 to 77 BHV1-IgG. Overall, although the effect of *year-of-birth* seems to be constant across sampling days, it has a much lower order of significance than that shown for PIV3-IgG.

4.4.2.3 Pre-existing PIV3-IgG antibody

In general, levels of pre-existing PIV3-IgG antibody on Day 0 (*Day 0-PIV3)* suppressed subsequent humoral responses to vaccination. These effects were most apparent early post-vaccination. Figure 4.5 shows the PIV3-IgG deviation induced by Day 14 (ΔpIgG(D0 to D14)). Smaller *Day 0-PIV3* values tend to have positive ΔpIgG while larger *Day 0-PIV3* values tend to have negative ΔpIgG.

Regression coefficients when levels of pre-existing PIV3-IgG antibody (q) were included in REML models for induced PIV3-IgG on Days 14 to 77 are shown in Table 4.7. Levels of PIV3-IgG on Days 14, 28, 42 were positively associated with pre-existing *Day 0-PIV3*, the effect decreasing with later samples. By Day 63, the effect was still significant but negative and became still more strongly negative by Day 49. Table 4.8 shows the significance attached when the effect, *Day 0-PIV3*, is included in the REML models developed for Days 14 to 77. The main effect is most strongly significant early post-vaccination on Days 14 and 28, declining on Days 42, 63 and 77.

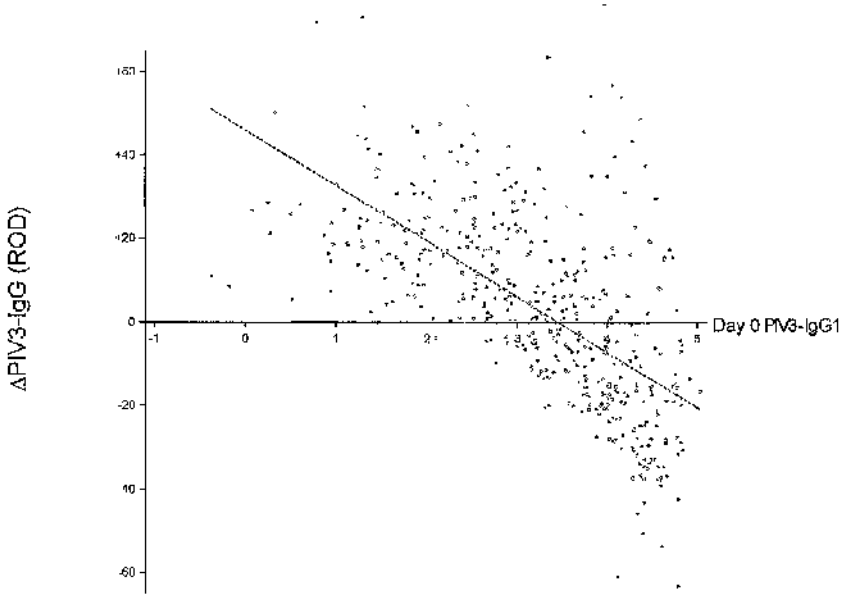


Figure 4.5 Deviations in PIV3-IgG level ΔpIgG(D0 to D14) against Day 0 level of PIV3-IgG. Best-fit regression line shown (**dashed**). ΔpIgG(D0 to D14) values are untransformed ROD data, Day 0 PIV3-IgG are log transformed ROD data.

		induced PIV3-IgG antibody				
		D14	D28	D42	D63	D77
REML model coefficents	*q* (s.e.)	0.65 (0.02)	0.26 (0.02)	0.14 (0.04)	-0.09 (0.03)	-0.29 (0.08)

Table 4.7 Regression coefficients when Day 0 level of pre-existing PIV3-IgG (*q*) is included in models for PIV3-IgG level on Days 14 to 77. Mean values (standard errors) are presented on log scale. Data analysis using REML models (EQ 4.6).

		PIV3-IgG by *Day 0 PIV3-IgG*		
Day	**REML term**	**Wald statistic**	**d.f.**	***P* value**
0	-	-	-	-
14	Day 0-PIV3	798.0	1	< 0.001
	Day 0-PIV3.age	12.4	1	< 0.001
28	Day 0-PIV3	128.0	1	< 0.001
	Day 0-PIV3.age	13.4	1	< 0.001
42	Day 0-PIV3.sex	7.9	1	0.005
63	Day 0-PIV3	8.3	1	0.004
77	Day 0-PIV3.year-of-birth	14.1	3	0.003

Table 4.8 Significant inclusions of the fixed effect pre-existing *p*IgG, either as main effect or interactions, for levels of PIV3–IgG on Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data. Data analyses using REML models (EQ 4.6). Day 0-PIV3 (level of PIV3-IgG on Day 0).

Using logistic regression, it was possible to develop binomial models (with parameters α and β, as detailed in Table 4.9 and therefore calculate and graph the probability (p) that *Day 0-PIV3* antibody were associated with a positive deviation in PIV3-IgG. This process was performed for ΔpIgG(D0 to D14), ΔpIgG(D0 to D28) and ΔpIgG(D0 to D42). Consequently estimates were obtained of the 50% (p =0.5) and 90% (p =0.9) threshold levels for *Day 0-PIV3* antibody for each of Days 14, 28 and 42 (Figure 4.6).

For Day 0 PIV3-IgG, the 50% inhibitory threshold was 14.3% for Day 14 responses, 35.2% for Day 28 responses, and 35.3% for Day 42 responses. For Day 0 PIV3-IgG, the 90% inhibitory thresholds on Day 0 were 1.5%, 11.6% and 14.3% for responses on Days 14, 28 and 42, respectively. Days 28 and 42 have similar 50% and 90% thresholds, while those for Day 14 were much lower, perhaps reflecting the transitionary state of antibody production at this point.

		induced ΔPIV3-IgG		
		(D0 to D14)	**(D0 to D28)**	**(D0 to D42)**
Logistic model parameters	**α**	2.59 (0.36)	7.04 (0.66)	8.66 (0.81)
	β	-0.98 (0.11)	-1.97 (0.18)	-2.43 (0.22)

Table 4.9 Estimated parameters (**α** and **β**) for deviation ΔpIgG$_i$ on Days 14, 28 and 42 against level of pre-existing PIV3-IgG on Day 0. Mean values (standard errors) are presented. p <0.001 for all regression coefficients. Data analysis using logistic regression models (EQ 4.5).

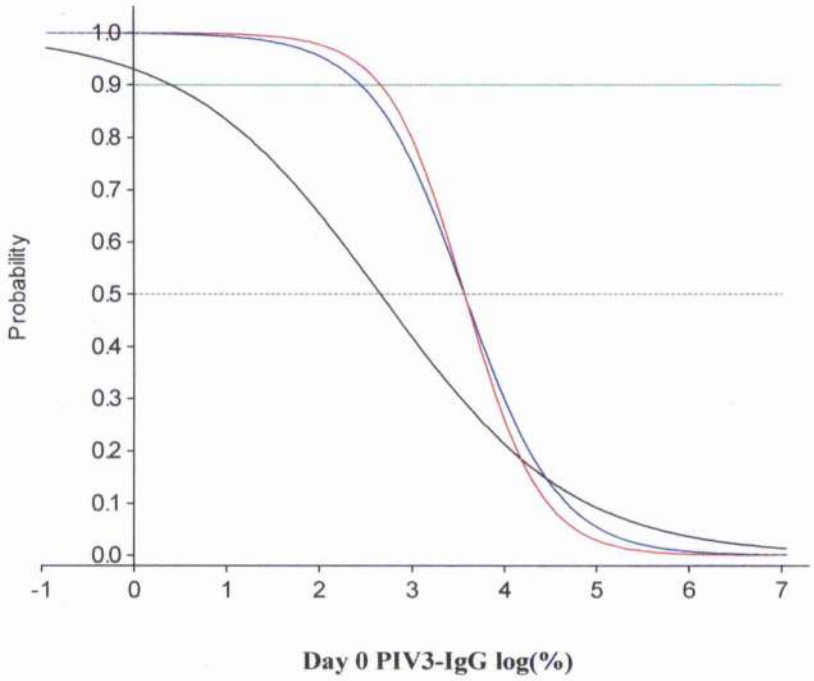


Figure 4.6 Data analysis using logistic regression models (EQ 4.5).
Probability of positive PIV3-IgG antibody response on Days 28, 42 and 63: (ΔpIgG(D0 to D28: **black),** ΔpIgG(D0 to D42: **blue)**, ΔpIgG(D0 to D63: **red**) against Day 0 PIV3-IgG level. ΔpIgG values are untransformed, Day 0 PIV3-IgG level is log transformed. p =0.5 (**green dashed**); p =0.9 (**green solid**).

There was evidence that post-vaccination levels of BHV1-IgG on Days 0, 14, and 28, were influenced by *Day 0-PIV3* (REML; p <0.001), with correlations r = 0.541, 0.558 and 0.439, respectively (all p <0.001).

4.4.2.4 Pre-existing BHV1-IgG antibody

During the study, changes in levels of BHV1-IgG following vaccination were generally much lower than those for PIV3-IgG but it was still possible to use a logistic regression approach to estimate the threshold level at which suppression of antibody responses occurs. Figure 4.7 shows the deviation in levels of BHV1-IgG induced between Day 0 and Day 63, (ΔiIgG(D0 to D63)), relative to the Day 0 level of pre-existing BHV1-IgG *(Day 0-BHV1)*. Again, lower *Day 0-BHV1* tends to result in positive ΔiIgG while higher *Day 0-BHV1* tends to result in negative ΔiIgG.

Regression coefficients when levels of pre-existing BHV1-IgG antibody (q) were included in REML models for induced BHV1-IgG on Days 14 to 77 are shown in Table 4.10. Levels of BHV1-IgG on Days 14, 28 and 42 were positively associated with pre-existing Day 0 levels of BHV1-IgG. BHV1-IgG levels on Days 63 and 77 were negatively associated with *Day 0-BHV1*. Table 4.11 shows the attached significance when the effect *Day 0-BHV1* is included in the REML models developed for Days 14 to 77. There is a two-way interaction of *Day 0-BHV1* with *age* which features on every sampling day and another two-way interaction of *Day 0-BHV1* with *sex* on four out of five of the post-vaccination sampling days.

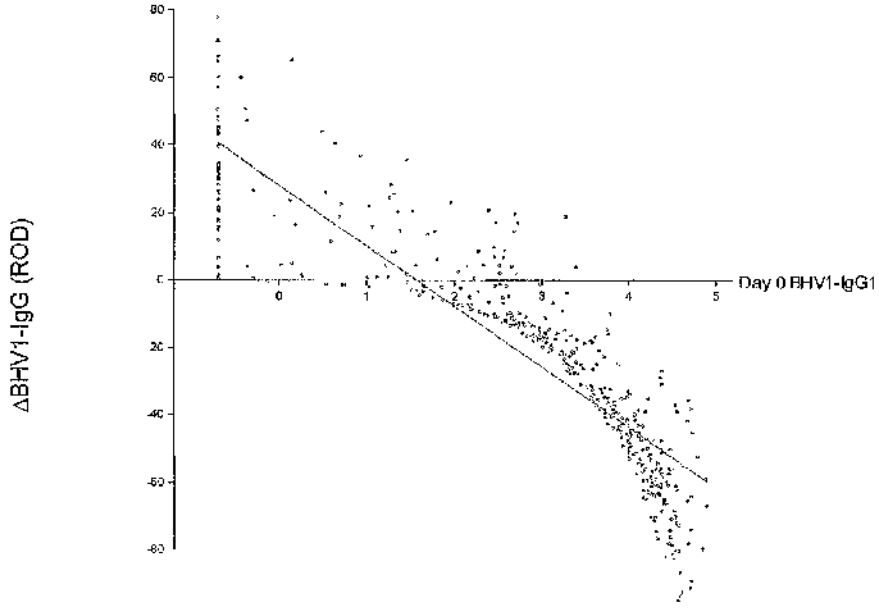


Figure 4.7 Deviations in levels of BHV1-IgG – ((ΔiIgG(D0 to D63)) against level of Day 0 BHV1-IgG. Best-fit regression line shown **(dashed)**. ΔiIgG values are untransformed, Day 0 BHV1-IgG are log transformed ROD data.

		induced BHV1-IgG antibody				
		D14	D28	D42	D63	D77
REML model coefficients	q (s.e.)	0.84 (0.03)	0.49 (0.09)	0.30 (0.11)	-0.16 (0.07)	-0.83 (0.14)

Table 4.10 Regression coefficients when Day 0 level of pre-existing BHV1-IgG (*q*) is included in models for BHV1-IgG level on Days 14 to 77. Mean values (standard errors) are presented on log scale. Data analysis using REML models (EQ4.6)

		BHV1-IgG by Day 0 IIgG antibody		
Day	**REML term**	**Wald statistic**	**d.f.**	***P* value**
0	-	-	-	-
14	Day 0-BHV1.age	8.8	1	0.003
28	Day 0-BHV1.age	21.7	1	< 0.001
	Day 0-BHV1.year-of-birth	15.3	3	0.002
	Day 0-BHV1.sex	7.9	1	0.004
42	Day 0-BHV1.age	42.6	1	< 0.001
	Day 0-BHV1.year-of-birth	13.6	3	0.004
	Day 0-BHV1.sex	11.0	1	< 0.001
	Day 0-BHV1.Holstein	5.7	1	0.017
63	Day 0-BHV1.age	35.9	1	< 0.001
	Day 0-BHV1.sex	21.3	1	< 0.001
	Day 0-BHV1.recombination-loss	14.1	1	< 0.001
	Day 0-BHV1.year-of-birth	10.3	3	0.016
	Day 0-BHV1.Holstein	6.9	1	0.009
77	Day 0-BHV1.age	31.4	1	< 0.001
	Day 0-BHV1.sex	15.9	1	< 0.001
	Day 0-BHV1.Holstein	12.2	1	< 0.001
	Day 0-BHV1.recombination-loss	5.5	1	0.019

Table 4.11 Significant inclusions of the fixed effect **pre-existing *i*IgG$_1$**, either as main effect or interaction, for levels of BHV1–IgG on Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed ROD data. **Day 0-BHV1** (level of BHV1-IgG on Day 0). Data analyses using REML models (EQ 4.6).

Parameters (α and β) for the binomial models developed using logistic regression, are detailed in Table 4.12. Threshold levels of pre-existing *Day 0-BHV1* at which positive or negative effects were equally likely (p =0.5) are shown in Figure 4.8. For BHV1, the threshold was 4.1% for Day 14 responses, 5.6% for Day 28 responses and 5.3% for Day 42 responses. Threshold levels of pre-existing antibody were estimated at (p =0.9) at which 90% of calves were expected to respond with an increased antibody response. For BHV1 antibody responses on Days 14, 28 and 42, these values were 0.9%, 1.4% and 1.3%, respectively.

		induced ΔIgG		
		(D0 to D28)	(D0 to D42)	(D0 to D63)
Logistic model parameters	α	2.67 (0.44)	2.05 (0.41)	2.64 (0.37)
	β	-1.55 (0.17)	-1.46 (0.16)	-1.59 (0.15)

Table 4.12 Estimated parameters (α and β) for deviation ΔIgG on Days 14, 28 and 42 against level of pre-existing BHV1-IgG on Day 0. Mean values (standard errors) are presented. p<0.001 for all regression coefficients. Data analysis using logistic regression models (EQ 4.5).

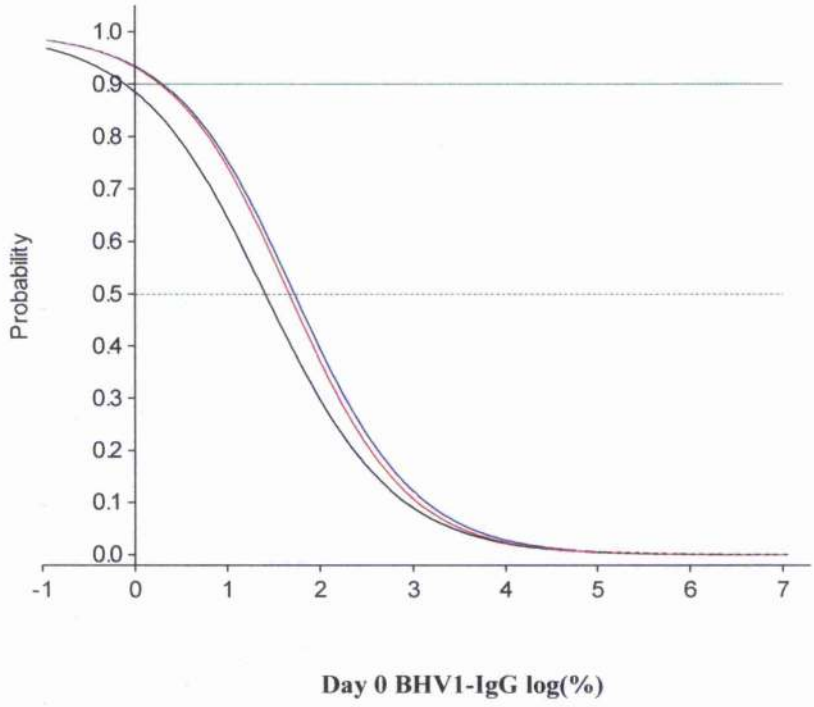


Figure 4.8 Probability of positive BHV1-IgG antibody response on Days 28, 42 and 63 (ΔpIgG(D0 to D28: **black),** ΔpIgG(D0 to D42: **blue**), ΔpIgG(D0 to D63: red) against Day 0 BHV1-IgG level. ΔiIgG values are untransformed, Day 0 BHV1-IgG level is log transformed. $p = 0.5$ (**green dashed**); $p = 0.9$ (**green solid**). Data analysis using logistic regression models (EQ 4.5).

There was no evidence that pre-existing levels of BHV1-IgG had a significant effect on post-vaccination levels of PIV3-IgG (REML; $p > 0.1$).

4.4.2.5 Age at first vaccination

There is a clear relationship between calf *age* and the level of pre-existing PIV3 antibody on Day 0. Older animals have lower levels of PIV3-IgG, with an almost constant ~1.4-fold (antilog) increment between consecutive age-groups (Figure 4.9(a)). Although all age-groups showed some increase in mean levels of PIV3-IgG between Days 14 and 28, for age-groups I, II and III, this increase was both small and transient, peaking at only ~1.2-fold. However in older calves (age-groups IV and V), levels of PIV3-IgG increased 1.54-fold and 1.58-fold, respectively between Days 14 and 28, and continued to increase until Day 77, post-vaccination. Mean PIV3-IgG levels in age-groups IV and V were different on Day 0 (t-test; $p < 0.05$) only, with no detectable differences between the two age-groups on the other five sampling days (t-test; $p > 0.1$). Table 4.13 shows the significance attached when *age* is included in the REML models developed for PIV3- IgG Days 0 to 77. The strongest effect of *age* is on Day 0 but *age* persists as part of two-way interactions for all sampling days except Day 42.

In general, BHV1-IgG responses to vaccination in this study were weak. A pattern similar to *Day 0 PIV3-IgG* is apparent for *Day 0 BHV1-IgG* levels but with more variation in increments between age-groups and age-group IV, not age-group V, having the lowest levels of *Day 0 BHV1-IgG* (Figure 4.9(b)). There were no detectable increases in mean levels of BHV1-IgG in the youngest calves (age-groups I and II), post-vaccination. Between Days 14 and 28, age-groups III and V did show small positive deviations in levels of BHV1-IgG but it was age-group IV, the second oldest set of calves, which had lowest levels of Day 0 BHV1-IgG and correspondingly the largest increase in levels of BHV1-IgG between Days 14 and 28 post-vaccination. Age-group V had higher levels of Day 0 BHV1-IgG and only intermediate increases in levels of BHV1-IgG post-vaccination. Table 4.14 shows the significance attached when *age* is included in the REML models developed for BHV1 Days 0 to 77. The strongest effect of *age* is on Days 42, 63 and 77 as an interaction with *Day 0-BHV1* but *age* is present as a main effect or two-way interaction on all sampling days.

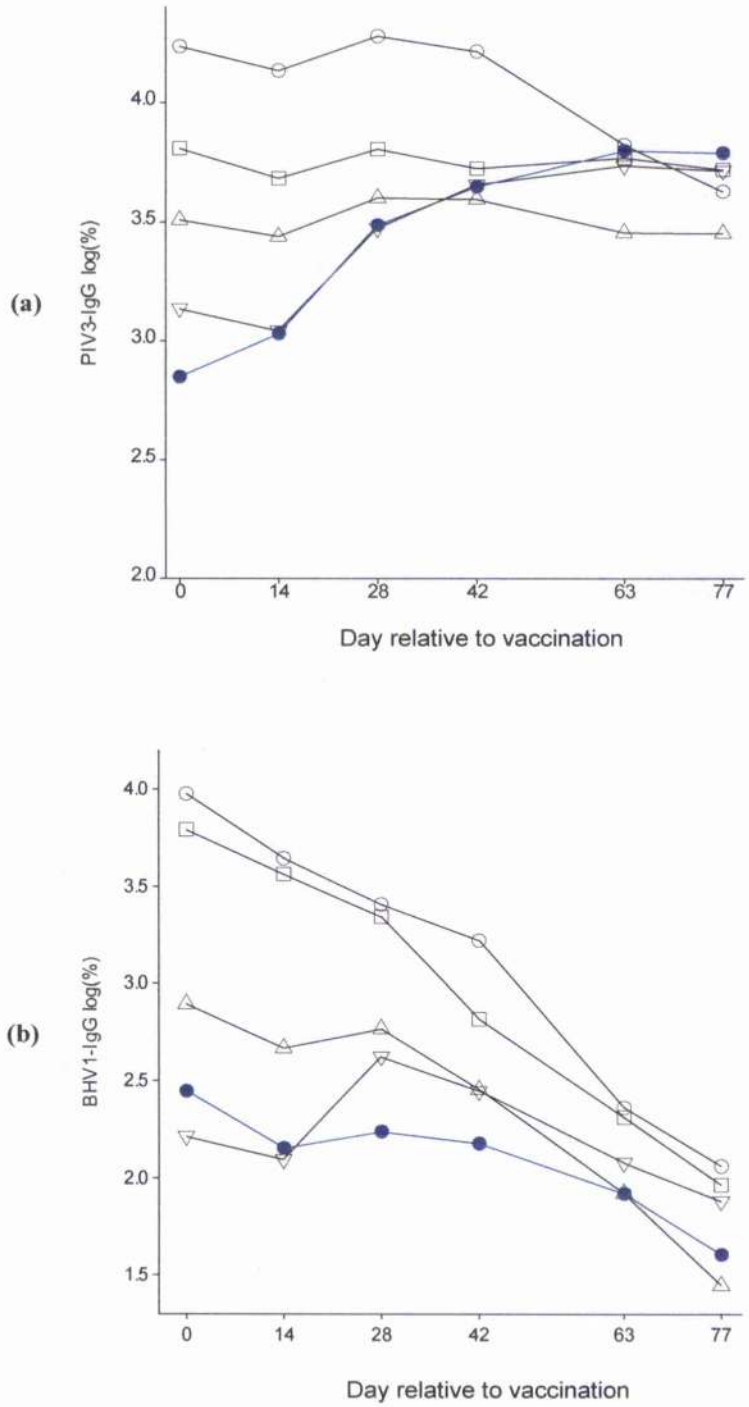


Figure 4.9 Levels of PIV3-IgG (**a**) and BHV1-IgG (**b**) from Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Results are log transformed ROD data and presented grouped on **age**: group I ~ 60-81 days (○), group II ~ 82-103 days (□), group III ~ 104-125 days (△), group IV ~ 126-147 days (▽) and group V ~ 148-169 days (●).

PIV3-IgG by age

Day	Model term	Wald statistic	d.f.	*P* value
0	age	74.7	1	< 0.001
14	age.Day 0-PIV3	12.4	1	< 0.001
28	age.Day 0-PIV3	13.4	1	< 0.001
	age.year-of-birth	15.4	1	0.002
	age.sex	9.1	1	0.003
42	-	-	-	-
63	age.year-of-birth	20.4	3	< 0.001
77	age.year-of-birth	22.5	3	< 0.001

Table 4.13 Significant inclusions of the fixed effect **age**, either as main effect or interactions, for levels of PIV3–IgG on Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Values are log transformed ROD data. **Day 0-PIV3** (level of PIV3-IgG on Day 0). Data analyses using REML models (EQ 4.6).

BHV1-IgG by age

Day	Model term	Wald statistic	d.f.	*P* value
0	age.year-of-birth	14.9	3	0.002
14	age	9.0	1	0.003
	age.Day 0-BHV1	8.8	1	0.003
28	age.Day 0-BHV1	21.7	1	< 0.001
	age	11.7	1	< 0.001
42	age.Day 0-BHV1	42.6	1	< 0.001
63	age.Day 0-BHV1	35.9	1	< 0.001
77	age.Day 0-BHV1	31.4	1	< 0.001

Table 4.14 Significant inclusions of the fixed effect **age**, either as main effect or interactions for levels of BHV1–IgG on Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Values are log transformed ROD data. **Day 0-BHV1** (level of BHV1-IgG on Day 0). Data analyses using REML models (EQ 4.6).

4.4.2.6 Dam-age

Dams of all ages had calves with very similar levels of PIV3-IgG (ANOVA; p >0.1) on all sampling days except Day 77 (ANOVA; p <0.01). On Day 77, there were small incremental rises in levels of PIV3-IgG associated with *dam-age* (Figure 4.10). On Day 63, the order was the same although the actual differences were even lower. Note that as there are only three dams aged seven years old, this group is small and statistically unreliable. Table 4.15 shows the significance attached when *dam-age* is included in the REML models developed for PIV3 Days 0 to 77. The effect is only significant on Day 42 post-vaccination, present as two-way interaction with *year-of-birth.*

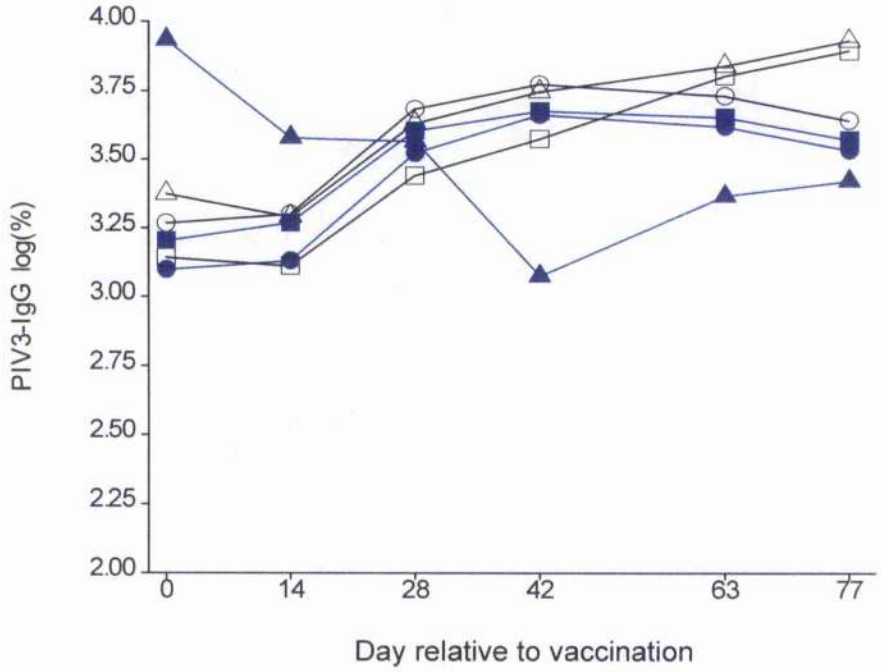


Figure 4.10 Levels of PIV3-IgG from Day 0 to Day 77, relative to vaccination. PIV3/BHV1 vaccine administered on Day 0. Mean values are log transformed relative optical density (ROD)and presented grouped on **dam-age:** 2 (●), 3 (■), 4 (○), 5(□), 6 (△) and 7 (▲) years. Note y-axis begins at 2.00.

PIV3-IgG by dam-age

Day	REML term	Wald statistic	d.f.	*P* value
0	-	-	-	-
14	-	-	-	-
28	-	-	-	-
42	dam-age.year-of-birth	30.29	10	< 0.001
	dam-age.recombination-loss.	14.29	4	0.006
63	-	-	-	-
77	-	-	-	-

Table 4.15 Significant inclusions of the fixed effect **dam-age**, either as main effects or interactions, for levels of PIV3–IgG on Days 0 to 77, relative to vaccination. PIV3/BHV1 vaccine administered on Day 0. Values are log transformed relative optical density (ROD) data. Data analyses using REML models.

Dams of all ages had calves with very similar levels of BHV1-IgG (ANOVA; $p>0.1$) on all sampling days except Day 42 (ANOVA; $p<0.05$). There was no obvious pattern in levels of BHV1-IgG when grouped on *dam-age* (Figure 4.11). Table 4.16 shows the significance attached when *dam-age* is included in the REML models developed for BHV1 Days 0 to 77. The effect is only significant early post-vaccination on Days 14 and 28, present as two-way interactions with *year-of-birth* and *Day 0-BHV1*, respectively.

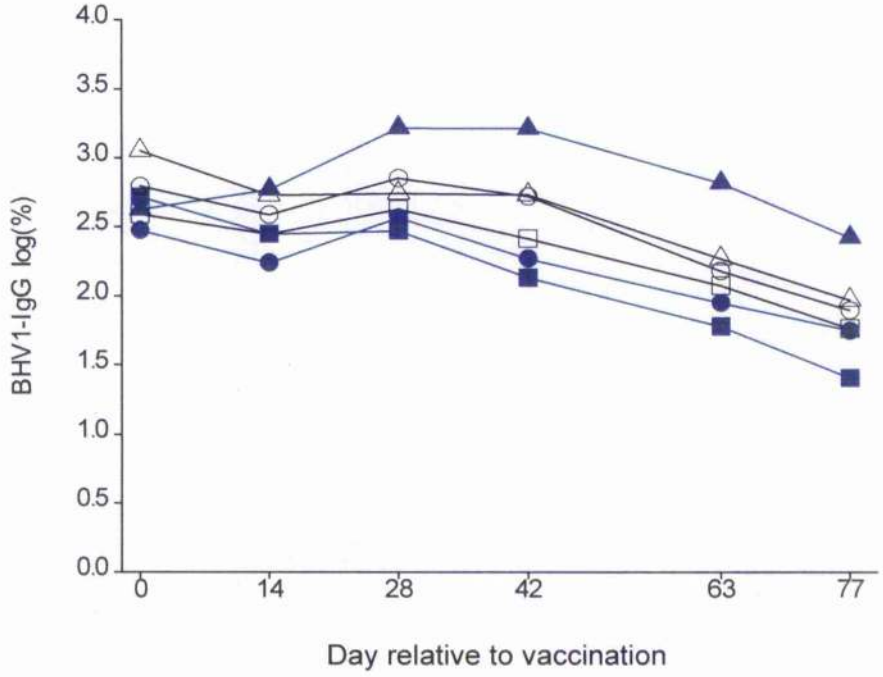


Figure 4.11 Levels of BHV1-IgG from Day 0 to Day 77, relative to vaccination. PIV3/BHV1 vaccine administered on Day 0. Mean values are log transformed relative optical density (ROD) data and presented grouped on **dam-age:** 2 (●), 3 (■), 4 (○), 5(□), 6 (△) and 7 (▲) years.

BHV1-IgG by dam-age

Day	REML term	Wald statistic	d.f.	*P* value
0	dam-age.year-of-birth	21.34	10	0.019
14	dam-age.Day 0-BHV1	22.55	5	< 0.001
28	-	-	-	-
42	-	-	-	-
63	-	-	-	-
77	-	-	-	-

Table 4.16 Data analyses using REML models. Significant inclusions of the fixed effect **dam-age**, either as main effects or interactions for levels of BHV1–IgG on Days 0 to 77, relative to vaccination. PIV3/BHV1 vaccine administered on Day 0. Values are log transformed ROD data. Day 0-BHV1 (level of BHV1-IgG on Day 0)

4.4.3 Analysis of genetic effects

4.4.3.1 Crossbreeding effect - Holstein

On Days 0 and 14, calves with greater proportions of Holstein genes (*Holstein* = 0.75) have 1.19-fold higher levels of PIV3-IgG than calves with a lower proportion of Holstein genes, (*Holstein* = 0.5) which in turn had 1.21-fold higher levels of PIV3-IgG than calves with *Holstein* = 0.25. Figure 4.12(a) shows that between Day 28 and Day 77, mean levels of PIV3-IgG based on *Holstein* were not significantly different (ANOVA; $p > 0.05$). All three profiles for PIV3-IgG variance followed very similar patterns when grouped on *Holstein* = 0.25, 0.50 and 0.75, however, ranked in the opposite order (0.75 > 0.50 > 0.25) to that found with the PIV3-IgG mean profiles. Table 4.17 shows the significance attached when *Holstein* is included in the REML models developed for PIV3 Days 0 to 77. *Holstein* is significant only on Days 14 and 42 as a two-way interaction with *sex*.

In contrast to PIV3, calves with greater proportions of Holstein genes (*Holstein* = 0.75) had lower levels of BHV1-IgG than calves with a lower proportion of Holstein genes (*Holstein* = 0.25 or 0.50) on Days 0, 14, 28 and 42, relative to vaccination ($p < 0.05$). There were negligible differences (t-test; $p > 0.1$) between levels of BHV1-IgG in calves with either *Holstein* = 0.25 or 0.50 on any of the six sampling days (Figure 4.12(b)). Mean BHV1-IgG levels grouped on *Holstein* (0.25, 0.50 and 0.75) tend to converge on Day 63 and Day 77 post-vaccination. The BHV1-IgG variance profiles of all three Holstein groups broadly follow similar patterns although for *Holstein* = 0.25 calves, the Day 77 variance is 3.60-fold (antilog) that of Day 28. For *Holstein* = 0.50 and = 0.75 calves, the Day 77 BHV1-IgG variances are only 1.92-fold (antilog) and 1.35-fold (antilog) the respective Day 28 BHV1-IgG values. Table 4.18 shows the significance attached when *Holstein* is included in the REML models developed for BHV1 Days 0 to 77. *Holstein* is significant on all sampling days except Day 14, either as a main effect or as an two-way interaction with *Day 0-BHV1*. Its influence seems to be highest on Day 0 and Day 77, declining on the intervening sampling days, Day 14 to Day 63. The three profiles for BHV1-IgG variance followed very similar patterns when grouped on *Holstein* = 0.25, 0.50 and 0.75, however again, ranked

in the opposite order (0.75 > 0.50 > 0.25) to that found with the BHV1-IgG mean profiles.

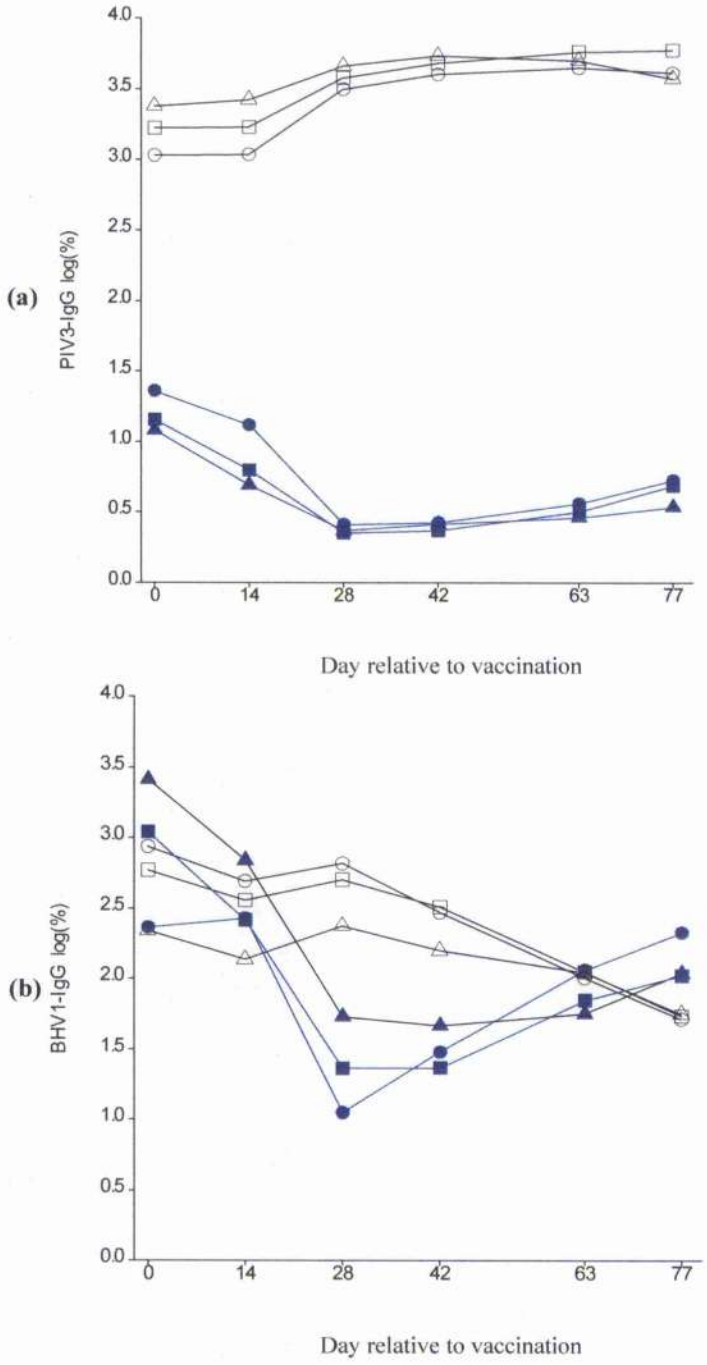


Figure 4.12 Levels of PIV3-IgG (**a**) and BHV1-IgG (**b**) from Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Values are log transformed and presented grouped on **Holstein:** 0.25 (○), 0.50 (□) and 0.75(△). Mean **(black)** and variance **(blue)** of data presented.

PIV3-IgG by Holstein

Day	REML term	Wald statistic	d.f.	*P* value
0	-	-	-	-
14	Holstein.sex	8.5	1	0.003
28	-	-	-	-
42	Holstein.sex	7.3	1	0.007
63	-	-	-	-
77	-	-	-	-

Table 4.17 Significant inclusions of the fixed effect **Holstein**, either as main effect or interactions for levels of PIV3–IgG on Days 0 to 77, relative to vaccination. BHV1/PIV3 vaccine administered on Day 0. Values are log transformed relative optical density (ROD) data. Data analyses using REML models (EQ 4.6).

BHV1-IgG by Holstein

Day	REML term	Wald statistic	d.f.	*P* value
0	Holstein	12.3	1	< 0.001
14	-	-	-	-
28	Holstein	6.2	1	0.013
42	Holstein.Day 0-BHV1	5.7	1	0.017
63	Holstein.Day 0-BHV1	6.9	1	0.009
77	Holstein.Day 0-BHV1	12.2	1	< 0.001

Table 4.18 Significant inclusions of the fixed effect **Holstein**, either as main effects or interactions for levels of BHV1-IgG on Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Values are log transformed ROD data. Day 0-BHV1 (level of BHV1-IgG on Day 0). Data analyses using REML models (EQ 4.6).

4.4.3.2 Crossbreeding effect - recombination-loss

As Figure 4.13 (a,b) shows, there were no significant differences (REML; p >0.1) between mean levels of PIV3-IgG and BHV1-IgG when grouped on *recombination-loss* on any of the six sampling days tested.

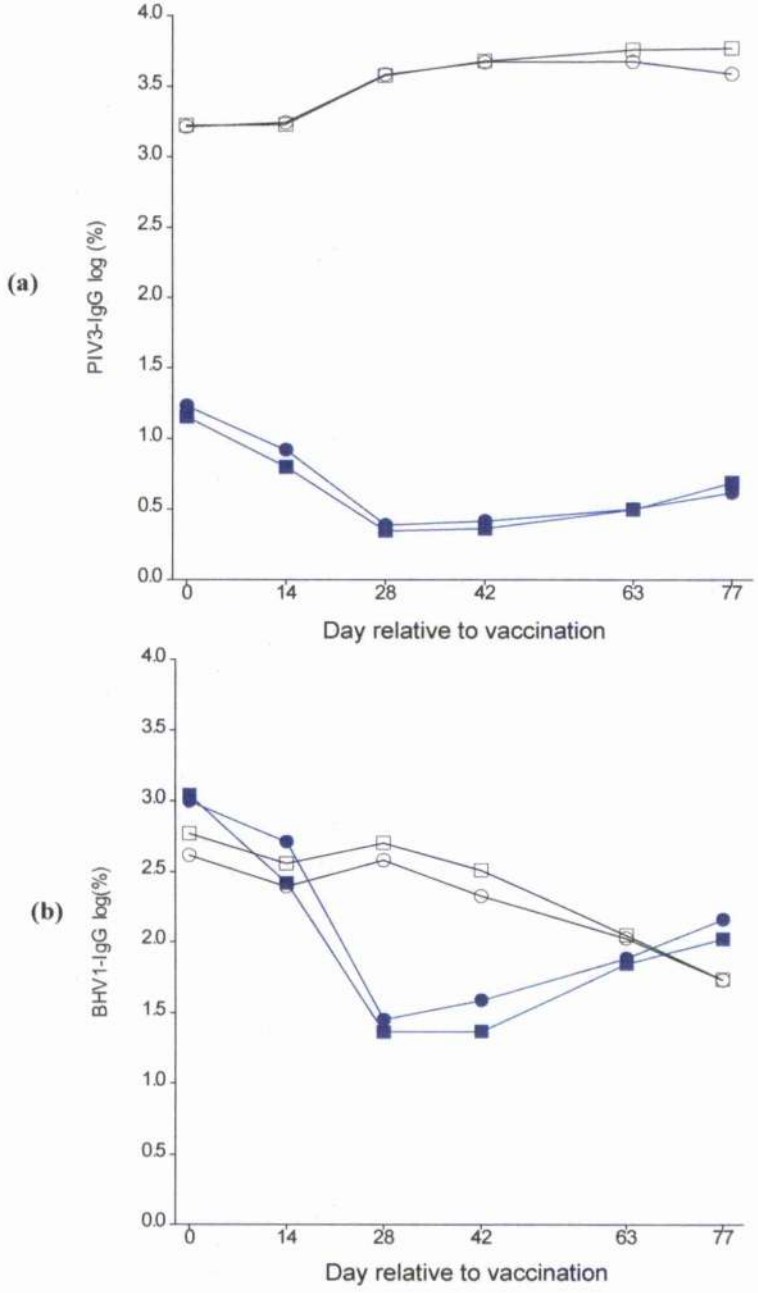


Figure 4.13 Levels of PIV3-IgG (**a**) and BHV1-IgG (**b**) from Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Values are log transformed relative optical density (ROD) data and presented, grouped on **recombination-loss:** 0.5 (○) and 1.0 (□). Mean (black) and variance (blue) of data presented.

4.4.3.3 Sire effects

Generally mean levels of PIV3-IgG, based on *sire*, increased from Day 0 to between Days 14 and 42 and then remained at that higher level until Day 77 (Figure 4.14). There was a 3.36-fold difference between the half-sib group with the highest (sire = R05) and lowest (sire = H147) Day 0 PIV3-IgG level but only a 1.95-fold difference between the half-sib group with the highest (sire = R19) and lowest (sire = H25) Day 77 PIV3-IgG level. Half-sib group R02 had the largest Day 0 to Day 77 deviation, (ΔpIgG(D0 to D77)), a 2.52-fold increase, while half-sib group R05 had the smallest ΔpIgG(D0 to D77), a 1.29-fold decrease. Half-sib group R12 had the smallest increase (1.04-fold) between Days 14 and 28, while over the same period half-sib group R11 has the largest increase (1.43-fold). Half-sib group R21 had the smallest deviation: an actual 1.03-fold decrease between Days 28 and 42, while over the same period half-sib group R01 had the largest increase (1.24-fold). Although there is only a 1.05-fold difference between PIV3-IgG levels of half-sib groups R02 and H25 on Day 14, this had increased to 1.56-fold by Day 77.

Overall heritability (h^2) for PIV3-IgG level decreased initially post-vaccination to negligible levels on Day 14, then rose to 0.30 by Day 28, peaking at 0.31 on Day 42 then falling to lower levels on Day 63 and 77 (Table 4.19). The Day 28 PIV3-IgG h^2 value was robust, after removing either the upper or lower ten per cent of the data - the h^2 value moved to 0.38 (±0.22) and 0.24 (±0.15), respectively. Similarly, after removing either the upper or lower ten percent of the data - the Day 42 PIV3-IgG h^2 value changed to 0.40 (±0.22) and 0.29 (±0.17), respectively. The pattern of PIV3-IgG heritability also differed between sexes. In the female calves, PIV3-IgG h^2 started high on Day 0, fell on Day 14 but increased quickly by Day 28 and remained at a high level until Day 77. In contrast in the male calves, PIV3-IgG h^2 started low, increased more slowly to a slightly lower peak on Day 42 and Day 63, then declined back to low levels by Day 77.

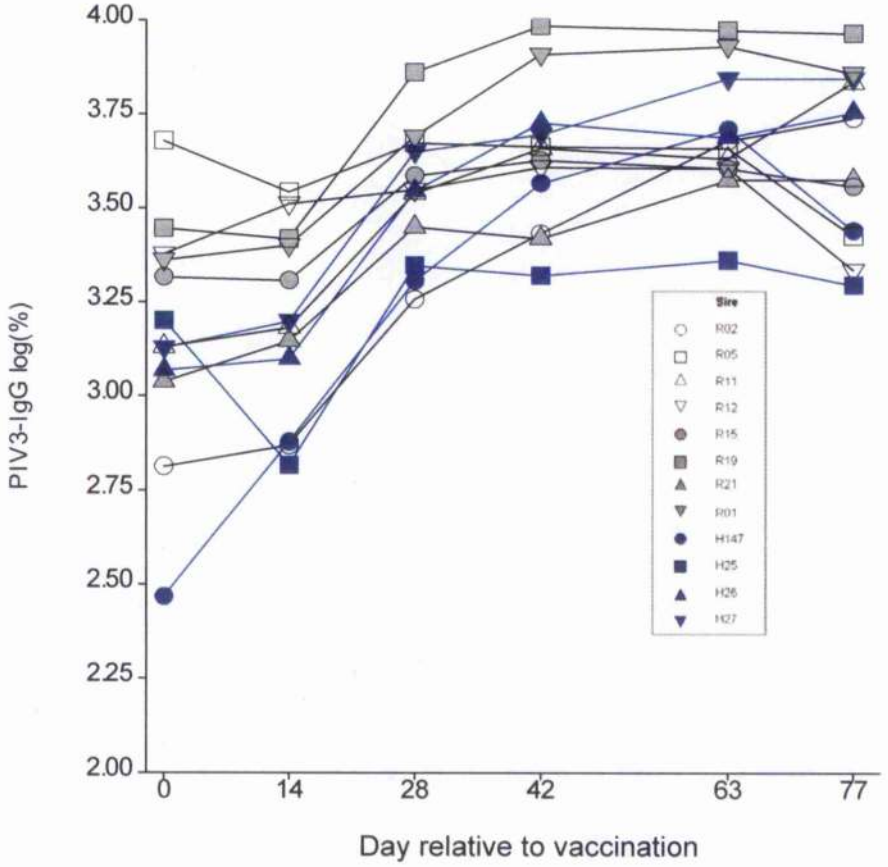


Figure 4.14 Levels of PIV3-IgG from Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Values are log transformed ROD data and means are presented, grouped on **sire:** 8 F1 Holstein/Charolais (**black**) and 4 F0 Charolais (**blue**). Note y-axis begins at 2.00.

The summary trait 6-day-area *p*IgG proved useful and representative of the individual sampling days, providing representative h^2 values for overall PIV3-IgG and PIV3-IgG when grouped on *sex*.

trait	overall h^2	overall σ_p^2 on log scale	log-likelihood ratio for sire
PIV3-IgG (both sexes)			
Day 0 *p*IgG	0.25 (0.16)	0.31 (0.21)	< 0.002
Day 14 *p*IgG	0.00 (0.05)	0.00 (0.01)	n.s.
Day 28 *p*IgG	0.30 (0.17)	0.12 (0.07)	< 0.0001
Day 42 *p*IgG	0.31 (0.17)	0.24 (0.14)	< 0.0001
Day 63 *p*IgG	0.24 (0.14)	0.24 (0.15)	< 0.0001
Day 77 *p*IgG	0.17 (0.12)	0.16 (0.12)	< 0.005
Δ*p*IgG(D14 to D28)	0.10 (0.09)	-	0.03
6-day-area *p*IgG	0.37 (0.20)	-	< 0.0001
PIV3-IgG (males only)			
Day 0 *p*IgG	0.10 (0.15)	0.11 (0.16)	n.s.
Day 14 *p*IgG	0.23 (0.21)	0.05 (0.05)	n.s.
Day 28 *p*IgG	0.15 (0.15)	0.05 (0.05)	n.s.
Day 42 *p*IgG	0.47 (0.28)	0.32 (0.21)	< 0.0005
Day 63 *p*IgG	0.23 (0.19)	0.24 (0.21)	0.025
Day 77 *p*IgG	0.01 (0.09)	0.01 (0.08)	n.s.
6-day-area *p*IgG	0.26 (0.20)	-	< 0.05
PIV3-IgG (females only)			
Day 0 *p*IgG	0.32 (0.23)	0.36 (0.24)	0.013
Day 14 *p*IgG	0.15 (0.18)	0.04 (0.05)	n.s.
Day 28 *p*IgG	0.44 (0.30)	0.17 (0.12)	0.009
Day 42 *p*IgG	0.51 (0.31)	0.35 (0.24)	0.002
Day 63 *p*IgG	0.47 (0.28)	0.38 (0.26)	0.0008
Day 77 *p*IgG	0.54 (0.31)	0.49 (0.32)	0.0008
6-day-area *p*IgG	0.65 (0.35)	-	< 0.001

Table 4.19 Additive trait heritabilities h^2 (standard error) and total phenotypic variances (σ_p^2) on natural logarithm scale and sire log-likelihood ratios. Statistics for levels of PIV3-IgG antibody, and 6-day-area for PIV3-IgG antibody levels between Days 0 and 77, post-vaccination. Results presented overall and grouped on *sex*. **n.s.** ($p > 0.1$). Estimates produced using REML.

Generally, mean levels of BHV1-IgG based on *sire* decreased progressively between Days 0 and 77 with small, transient increases between Days 14 and 28 (Figure 4.15). There was a 3.56-fold difference between the half-sib groups R05 (the highest) and R02 (the lowest) Day 0 BHV1-IgG levels. By Day 77 this had increased to a 6.03-fold difference between the highest (sire = H147) and lowest (sire = H25) half-sib groups. Between Days 0 and 77, half-sib group R05 had the largest decrease (ΔROD = -38.2%) while half-sib group R02 had the smallest decrease (ΔROD = -38.2%).

Between Days 0 and 14, half-sib groups H25 and H27 had very similar levels of BHV1-IgG but between Days 14 and 28, half-sib group H27 had a positive (1.46-fold) BHV1-IgG deviation while half-sib group H25 showed an actual (1.20-fold) decrease. Mean Day 0 BHV1-IgG levels for half-sib group H147 were high but not the highest (sire = R05) and although they did show a small increase post-vaccination, they remained relatively constant compared to the other half-sib groups tested.

Half-sib group R02 had the smallest decrease (Δ ROD = -0.7%) between Days 14 and 28 while half-sib group R12 had the greatest decrease (Δ ROD = -10.9%). Half-sib group R02 also had the lowest mean BHV1-IgG level on Day 0 and the most prolonged BHV1-IgG response, remaining above Day 0 levels until after Day 63, post-vaccination. Half-sib group R19 had both the highest 6-day-area *i*lgG and the highest 6-day-area *p*lgG in the study. The 6-day-average *i*lgG does appear to act as a useful summary trait, overall representative of the individual sampling days involved.

As shown in Table 4.20, all heritabilities (h^2) for the BHV1-IgG traits (including summary traits) analysed were low (≤ 0.16). In fact, over Days 14, 28 and 42, the h^2 levels dropped to non-significant levels, even below the basal levels on Day 0. They apparently returned to those basal levels by Day 77. Such a pattern suggests little impact of additive genetics on post-vaccination levels of BHV1-IgG, as detected in the phenotype of the current study.

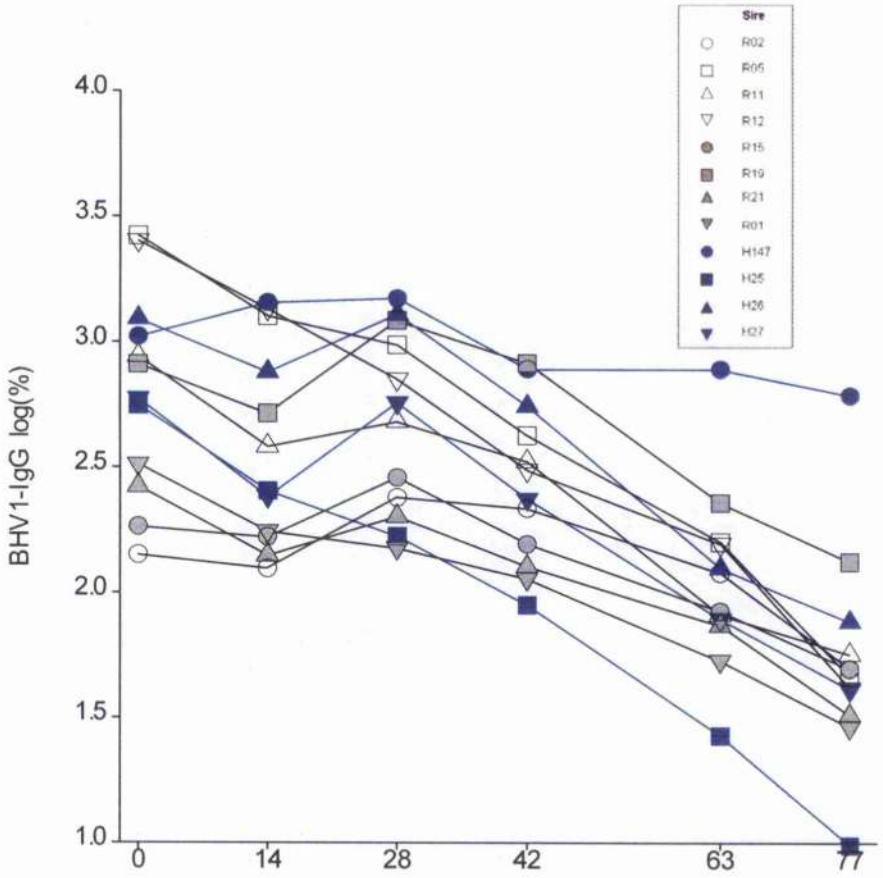


Figure 4.15 Levels of BHV1-IgG from Days 0 to 77, relative to vaccination. Vaccine administered on Day 0. Values are log transformed ROD data and means are presented, grouped on **sire:** 8 F1 Holstein/Charolais (**black**) and 4 F0 Charolais (**blue**). Note the y-axis begins at 1.0.

BHV1-IgG (both sexes)

trait	overall h^2	overall σ_p^2 on log scale	log-likelihood ratio for sire
Day 0 *i*IgG	0.14 (0.11)	0.09 (0.07)	0.03
Day 14 *i*IgG	n.s.	-	n.s.
Day 28 *i*IgG	0.04 (0.07)	0.01 (0.02)	n.s.
Day 42 *i*IgG	0.08 (0.09)	0.02 (0.02)	n.s.
Day 63 *i*IgG	0.11 (0.10)	0.04 (0.04)	n.s.
Day 77 *i*IgG	0.16 (0.13)	0.07 (0.06)	0.04
Δ*i*IgG(D14 to D28)	0.00 (0.00)	-	n.s.
6-day-average *i*IgG	0.12 (0.10)	0.02 (0.02)	0.06
6-day-area *i*IgG	0.09 (0.09)	0.02 (0.02)	n.s.

Table 4.20 Additive trait heritabilities h^2 (standard error) and total phenotypic variances (σ_p^2) on natural logarithm scale. Sire log-likelihood ratio statistics for levels of BHV1-IgG antibody, ΔBHV1-IgG deviation between Days 14 and 28, overall 6-day-average and 6-day-area for BHV1-IgG antibody levels between Days 0 and 77, post-vaccination. **n.s.** ($p > 0.1$). Estimates produced using REML.

4.5 Discussion

Substantial differences in host immunity between the responses to PIV3 and BHV1 vaccination have been highlighted by the current study of pathogen specific IgG antibody levels in a large group of young cattle. The study was also able to demonstrate some of the differences in the epidemiology of the two viruses. The antibody profiles observed for PIV3 post-vaccination are characteristic of secondary-type immune responses, suggesting previous exposure to the virus. This is in contrast with the antibody profiles observed following BHV1 vaccination, a pattern of low response to this vaccine commented on previously by Patel *et al.* (2005). Those authors also described extremely low rates of seroconversion to this BHV1 vaccine although it still retained protective efficacy as determined by virus shedding following challenge.

Initial levels of pre-existing antibody to both viruses prior to vaccination were very similar and it is impossible to distinguish passive (originating from colostrum) from active (induced by naturally occurring infection) IgG antibody by ELISA alone. However over the 77-day course, the mean PIV3-IgG profile was consistent with an anamnestic antibody response occurring against a background of high levels of PIV3 antibody. This effect was most pronounced in the male calves, which had freely mixed with the adult bovine population from birth. Anamestic antibody responses tend to be higher, more rapid and more sustained than primary antibody responses (Graham *et al.*, 1999). In contrast, the mean BHV1-IgG profile obtained in the current study is typical of the largely inhibitory effect of high levels of pre-existing BHV1 antibody on responses to BHV1 vaccination (Menanteau-Horta *et al.*, 1985).

Bovine herpesvirus 1, in common with many other herpesvirus, causes true latent infections producing high levels of antibody in the adult herd and consequently high levels of maternally-derived passive antibody in the calf population (Brar *et al.*, 1978). Although calves do not always seroconvert after BHV1 vaccination (Le Jan and Asso, 1981), once infection has occurred antibodies to BHV1 remain readily detectable for at least three years (Zygraich *et al.*, 1975a). Passive BHV1 antibody from colostrum can persist for at least nine months (Brar *et al.*, 1978).

Lemaire *et al.* (2000b) found that using ELISA to detect maternally-derived BHV1 antibody levels extended the period of detection to between 6 and 10 months instead of between 5 and 8 months using the SNT. In the current study, both the overall antibody profile and apparently short half-life of the BHV1 antibody imply that most of this antibody may indeed be colostral in origin. The dynamics of PIV3 antibody observed were very different. High pre-vaccination antibody levels in the calf population in the current study may well be maintained by field PIV3 infections. In this study, vaccination against PIV3 may therefore be considered to induce secondary immune responses rather than the primary immune responses observed against BRSV vaccination.

Based on the results of this study, there appears to be little difference between levels of BHV1-IgG based on *sex/management*, apart from determining the initial level of pre-existing antibody on the day of vaccination. In contrast, the antibody profile for PIV3-IgG in the male calves is distinctly different from that of the female calves. The disparate responses to these two respective viral antigens may be explained thus 1) environment/management (and/or *sex*) may have a specific antigen-dependent effect on antibody response to vaccination; 2) environment/management (and/or *sex*) may play an indirect role in determining antibody responses to vaccination, due to challenge of the calves by naturally circulating field virus infections; 3) a combination of 1) and 2). The divergent antibody responses between specific vaccine components are notable but as *sex* and environment/management are confounded in this experiment, discriminating which variable has the greatest significance is not possible. These results highlight the importance of farming practices in disease management and demonstrate how pathogen-specific microenvironments may co-exist on a single farm.

In addition to the inherent variables of location, weather, nutrition and overall herd health, many management factors such as weaning, transport, feedlot acclimatisation, overcrowding, and marketing can produce stress, trauma and malnutrition in young cattle. Buffalo calves maintained with outdoor space allowances had higher antibody responses to keyhole limpet hemocyanin (KLH) than other calves with more restricted indoor accommodation (Grasso *et al.*, 1999). Antibody responses specific to KLH were lower in calves maintained in open pens compared to hutch-housing, with increased stress proposed as the

underlying negative cause (Cummins and Brunner, 1991). In contrast, van Reenen *et al.* (2000) found no differences in antibody responses between group-housed and individually-housed 12-week old male Holstein Friesian calves, vaccinated against BHV1. A Dutch bulk-milk tank survey found that farm epidemiological characteristics, such as herd size, herd density and stock purchase rate were important determinants of levels of seropositivity to field BHV1 (van Wuijckhuise *et al.*, 1998).

Post-vaccination BHV1 antibody levels were higher in vaccinated crossbred beef cattle treated with the antibiotic, florfenicol, than in cattle which did not receive florfenicol medication (Hunsaker *et al.*, 1999). Bovine herpesvirus 1 antibody responses to vaccination improved when levamisole was administered one week after the vaccine (Babiuk and Misra, 1982), while concurrent BVDV infection caused increased virus shedding but no difference in antibody response to BHV1 vaccination (Edwards *et al.*, 1986). In the current study, the factors *year-of-birth* and *sex* contain much of the non-genetic and environmental variation as outlined above, as they include seasonal variation in herd disease, climate, nutrition and farm management.

Conflicting results have been recorded for the effect of *age* on vaccine response. An inverse correlation was described previously between calf *age* and levels of maternally-derived passive BHV1 antibody (Menanteau-Horta *et al.*, 1985; Stott *et al.*, 1987). Lapierre *et al.* (1975) found no evidence of age-related effects in eight to seventeen week-old calves vaccinated against BHV1, and in another study, calf *age* was found not to be an important determinant of antibody response following PIV3 vaccination (Burroughs *et al.*, 1982). Although clinical disease was more severe and longer lasting in younger calves, *age* was again found not to be important for seroconversion following BHV1 infection (Msolla *et al.*, 1983). However, severe disease occurred in very young (3 day-old) calves following intramuscular administration of a combined PIV3/BHV1 vaccine (Bryan *et al.*, 1994). Non-specific reactions lowered the specificity of the indirect BHV1 ELISA between 160 days and 240 days, post-vaccination (Cho *et al.*, 2002) but this is largely beyond the age range of calves in the current study.

Temperature-sensitive PIV3 and BHV1 viruses have been combined in intranasal live vaccines for decades (Zygraich *et al.*, 1975b). However, interferon induced by BHV1 intranasal vaccination may interfere with the immune responses

induced by simultaneous exposure to PIV3 virus (Cummins and Rosenquist, 1982). Furthermore, four month old calves with mixed BHV1/PIV3 infections had delayed and depressed antibody responses compared to those with single BHV1 and PIV3 virus infections (Ghram *et al.*, 1989). In addition, wild-type BHV1 infection has been reported to depress CMI functions such as chemotaxis, cytotoxicity and mitogenic responses (Bielefeldt and Babiuk, 1985) and multivalent bacterial polysaccharide vaccines showed immunological interference between antigens (Fattom *et al.*, 1999). Although early levels of IgG antibody to both viruses were positively associated, most probably due to some common colostral origin, the current study found no evidence of immunologic interference between the two viruses based on levels of specific IgG antibody. These results are consistent with previous research (Fulton *et al.*, 1995; Talens *et al.*, 1989; Tollis *et al.*, 1996).

Although BHV1 and PIV3 were components of the same multivalent vaccine, the contrasting patterns of specific-IgG responses induced also stresses how the immune system deals with each antigen independently. Calves between 126 and 169 days old (Group IV) had unusually high IgG responses to BHV1. Possible explanations include: 1) the pattern may be stochastic in origin but with age-group IV containing 142 calves compared to 76 and 159 in age-groups III and V, respectively, this would seem unlikely or 2) the pattern may relate to levels of pre-existing antibody. Pre-vaccination levels of BHV1 antibody were lowest in age-group IV, not age-group V as would be expected if all antibody was of colostral origin. The reasons for this particular deviance remain unclear and 3) there may be a temporary age-associated shift in BHV1-IgG vaccine responsiveness between 126 to 147 days. This transient receptiveness is perhaps due to the development of the immune system, disease epidemiology or other considerations. The effect was not prolonged, and when age-group IV itself grew older (on later sampling days), the pronounced BHV1-IgG response subsided and resumed a pattern of constant antibody decay, comparable to all other age-groups.

Previous research has reported that BHV1 antibody has a half-life of ~21 days while PIV3 antibody has a half-life of ~30 days (Brar *et al.*, 1978; Fulton *et al.*, 1995). The values obtained from the current study are very similar. The pre-vaccination level of IgG antibody is accepted to influence the subsequent

antibody response to vaccination. Pre-existing levels of maternal antibody interfered with the BHV1 antibody response induced by both infection (Bradshaw and Edwards, 1996) and vaccination (Brar *et al.*, 1978; Lucas *et al.*, 1982; Menanteau-Horta *et al.*, 1985) but had little effect on cell-mediated immune responses (Lemaire *et al.*, 2000a). Stott *et al.* (1987) found that younger calves, with higher levels of maternally derived antibody, had lower responses to PIV3 vaccination, a finding confirmed by Cummins *et al.* (1982). Lemaire *et al.* (2000b) demonstrated that intranasal BHV1 vaccination slowed the decay of maternally derived antibody, with the actual response to vaccination inversely dependent on the level of BHV1 maternally derived antibody. In human infants, an inverse correlation was reported between pre-vaccination levels of PIV3 antibody and post-vaccination PIV3 antibody levels (Karron *et al.*, 1996).

Suppression of antibody tends to be immunologically specific and it was reported that, although pre-existing passive BHV1 antibody completely prevented seroconversion following vaccination (Brar *et al.*, 1978), the calves were sensitized to BHV1 virus. Maternally derived antibody inhibited or prevented seroconversion following experimental exposure to BHV1 (Bradshaw and Edwards, 1996; Brar *et al.*, 1978; Le Jan and Asso, 1981). It is unlikely that this suppression is absolute however and in the current study nominal threshold values were estimated for suppression of PIV3 and BHV1 vaccine responses. Threshold antibody titres above which inhibition of antibody responses occurs have been previously described are 1:4 for BHV1 and 1:128 for PIV3 (Zygraich *et al.*, 1975b). Fulton *et al.* (1995) also estimated threshold titres of 1:20 for BHV1 and 1:32 for PIV3 before seroconversion was inhibited. In contrast Ellis *et al.* (1996) suggested that some low level BHV1 antibody production did occur following live vaccine immunisation, despite high levels of colostral antibody. The results from the current study confirm just such a weak response. Antibody half-lives were extended for both BHV1 and PIV3 in 3-month old beef calves following vaccination (Fulton *et al.*, 1995). Furthermore, despite immunological immaturity and the blocking effect of passive antibody, intranasal BHV1 vaccination at four days old extended the detectable period as measured by both blocking and indirect ELISA (Lemaire *et al.*, 2000a). It is probable that stronger responses would be evident if mucosal levels of BHV1 antibody had been investigated in the current study.

The design of practical vaccination programmes must account for the variety of responses occurring which in turn depends on the prevailing type of immunological preconditioning: either passive or active. In the present study pre-vaccination PIV3 antibody levels were higher than BHV1 antibody levels. However vaccination stimulated strong PIV3 responses but weak BHV1 responses. In another study, maternally-derived BHV1 antibody declined from a peak titre of 1:32 to undetectable levels by day 170 in mixed-sex beef calves (Menanteau-Horta *et al.*, 1985). The same authors found that calves vaccinated at 84 days of age appeared not to seroconvert, with antibody levels continuing to decline. Calves given BVDV vaccine concurrently, however, did seroconvert despite BHV1 antibody levels being lower than those for BVDV. The effect of pre-existing antibody level on subsequent antibody response is therefore compelling and has clinical importance in disease management schemes for young cattle.

Few researchers have investigated genetic control of BHV1/PIV3 infection or vaccination. Despite this, some genetic effect on responses to vaccination should be anticipated. Major histocompatability complex (B) haplotypes influence vaccinal immunity against the chicken alphaherpesvirus: Marek's disease virus, as reviewed by Bacon *et al.* (2001). Also in poultry, Sacco *et al.* (1994b) described both higher antibody responses to Newcastle Disease vaccine (caused by a paramyxovirus like PIV3) in turkey lines selected for increased body weight and elevated antibody levels in female rather than male birds. A peak heritability of 0.47 was described for sire-based genetic effects controlling homologous antibody titres against strains of foot-and-mouth virus in adult bulls (Samina *et al.* 1998.). In addition, cattle-breed significantly affected both rapidity and magnitude of antibody (IgG) responses in seven-month-old Angus and Simmental heifers following intramuscular administration of pig erythrocytes (Engle *et al.*, 1999).

More specifically, using 111 Holstein-Friesian adult cows selected for milk fat production, Sinclair *et al.* (1999) found no evidence of genetic control of antibody responses to intranasal BHV1 vaccination. In that study, 26% of cows were recorded as non-responders to the vaccine but animal age, exposure history and limited genetic variability may also have impacted upon the results. Differences based on breed-cross were recorded for responses to the BHV1

component of a live multivalent vaccine but not the PIV3 component by Burton *et al.* (1994). That study was small and primarily designed to demonstrate the immunomodulatory effects of chromium. Breed-cross did however significantly determine post-vaccination antibody levels to BHV1 (killed vaccine) in seven-month-old crossbred calves (Cole *et al.*, 2001). Genetically predetermined variability in immune responses to BHV1 vaccination was suggested by Zhu *et al.* (1996) and following experimental observation, the Guernsey breed was postulated as having increased resistance to BHV1 infection (Bielefeldt and Babiuk, 1985).

Immune responses do not occur in isolation from other metabolic processes and genetic variation may reflect individual ability to absorb and metabolise nutrients. Vitamin-B supplementation boosted antibody responses to BHV1 vaccination in stressed calves (Dubeski *et al.*, 1996), while cattle augmented with zinc had higher antibody responses to BHV1 vaccination than control animals (Spears *et al.*, 1991). A complex of minerals, including zinc, cobalt and copper, increased responses to vaccination against both PIV3 and BHV1 (George *et al.*, 1997). Selenium-deficient calves had lower secondary responses to BHV1 than selenium-adequate animals (Reffett *et al.*, 1988). Copper supplementation increased antibody production to erythrocytes in stressed steers but decreased the level of antibody in non-stressed animals (Ward and Spears, 1999). In vaccinated 120 day-old Holstein calves, plasma pH was negatively correlated with levels of both PIV3 and BIIV1 antibody while increased levels of plasma B-hydroxybutyrate were associated with lower antibody levels to BHV1 (Donovan *et al.*, 2003).

Unfortunately, due to the large number of non-responders and weak responders to BHV1 vaccination, the phenotype generated by the current study was not suitable to allow meaningful analysis for heritable effects. The corresponding antibody responses to PIV3 did however indicate strong sire-based genetic effects. Due to the complicating effect of colostral immunity antibody, present for both viruses, it is difficult to determine if this difference in responses is pathogen (virus) specific or distorted due to stronger immunological suppression by BHV1 passive antibody.

The PIV3-IgG heritability estimates (h^2) were obtained against high background levels of PIV3 antibody and are consistent with a secondary-type antibody

response. A distinction therefore can be drawn between this phenotype and those phenotypic responses obtained when calves were vaccinated against BRSV (primary-type antibody response - Chapter 3). The peak h^2 for PIV3-IgG is appreciably higher than that obtained for the primary immune response registered against BRSV vaccination. It is however striking that progeny of sire R01 which had the highest levels of BRSV antibody (Chapter 3) were found to have the second highest levels of PIV3 antibody. Further evaluation is required to determine if these secondary-type PIV3 immune responses are amplified versions of primary responses or if they operate via (partially or completely) independent genetic mechanisms. With ~50% of variation in PIV3-IgG responses heritable, even in the face of circulating field virus infections, effective disease control programmes should now take account of immunogenetic effects. These PIV3 results substantiate those obtained for BRSV, suggesting that selection for increased vaccine response to these two viruses in cattle is likely to yield real welfare and production benefits in the future.

Chapter 5

Linking antibody phenotype to cattle genotype

5.1 Introduction

Immunological traits such as antibody and cellular responses to viral infection are under complex regulation, controlled by both patent environmental factors and concealed genetic components (Lofthouse and Kemp, 2002). It is these types of immune traits that mediate resistance to BRD but have proved difficult to decipher and improve for several reasons (Soller and Andersson, 1998). Such functional traits tend to respond poorly to selective breeding using conventional techniques as they often have low heritabilities. There are many practical difficulties in obtaining firm data to substantiate selection for relevant immune traits and protection against clinical disease. In addition, modern farming practices to date, have tended to concentrate exclusively on production traits. However, recently there has been an adjustment towards more balanced breeding goals, with immune function traits explicitly included in total merit indices (Mark, 2004).

The emergence of precise genetic linkage maps composed of many microsatellite markers has facilitated quantitative trait loci-mapping in cattle. Quantitative trait loci are chromosomal segments, obeying Mendelian inheritance, which exert predictable effects on quantitative phenotypic traits (Hui Liu, 1998). Their detection uses phenotypic, pedigree and genetic marker information to dissect those allelic mutations underlying the particular phenotypic trait. Quantitative trait loci are most beneficial when traits are difficult or expensive to measure, when traits are poorly heritable, when each individual phenotype has limited informativeness and when it is difficult to clearly assign polygenic cause. Searches for QTL may even be performed without prior knowledge of the physiological or biochemical functions of the loci involved. Once QTL have been recognised, additional markers can be selected to saturate the prospective segment of chromosome to finely localize the position, the eventual aim being identification of single gene function through bioinformatic and other technologies.

Mapping of QTL is a well established, effective technique in cattle populations, with most research emphasis on production traits. To date, attributes successfully linked to QTL include milk yield (Casas and Cundiff, 2003), milk composition (Plante *et al.*, 2001), reproduction (Casas *et al.*, 2004; Cruickshank *et al.*, 2004), birthweight (Grosz and MacNeil, 2001), and growth and carcase traits (Stone *et*

al., 1999). Selective breeding aims at providing animals with the most favourable sets of genes for production, conformation and functional traits. Marker assisted selection directed by QTL should enhance the rate of genetic progress towards cattle optimised for modern farming requirements.

To date, QTL searches for immune function have tended to concentrate on those species which have highly structured breeding programmes, large litter sizes, short generation intervals and particularly intensive farming practices. In pigs, the heritability (±se) of serum antibody levels following vaccination against AD was estimated as 0.42 (±0.13) and differences based on pig-breed have also been described (Rothschild *et al.*, 1984). Swine Leukocyte Antigen (MHC equivalent) loci have been associated with this antibody response against AD virus (Mallard *et al.*, 1989; Wimmers *et al.*, 2004), and QTL have been identified for AD resistance (Reiner *et al.*, 2002). In addition, QTL for antibody responses were detected in pigs against *Escherichia coli* antigens (Edfors-Lilja *et al.*, 1998). Similarly in chickens, the heritability of antibody response has been established against *Escherichia coli* and Newcastle Disease virus (Leitner *et al.*, 1994; Yunis *et al.*, 2002b), and in turkeys against *Pasteurella multocida* and Newcastle Disease virus (Sacco *et al.*, 1994a). Quantitative trait loci for antibody responses were detected in chickens against exogenous antigens such as sheep erythrocytes (Siwek *et al.*, 2003b) and keyhole limpet hemocyanin (Siwek *et al.*, 2003a). Polygenic quantitative control of antibody responses has been also confirmed by other studies in mice (De Souza *et al.*, 2004; Puel *et al.*, 1995), chickens (Siwek *et al.*, 2003a) and turkeys (Sacco *et al.*, 1994a).

In cattle, however, most immunogenetic studies have concentrated on dairy health traits. There have been many QTL studies in dairy cattle almost universally using somatic cell count as the indicator trait (Ashwell *et al.*, 1996; Heyen *et al.*, 1999; Klungland *et al.*, 2001; Kuhn *et al.*, 2003; Schulman *et al.*, 2004). The inflammatory protein conglutinin, envisaged as protective against both viral and bacterial infections is under heritable control in cattle, although some sex-based variation may also exist (Holmskov *et al.*, 1998). Quantitative trait loci for the phenotypic traits anaemia, bodyweight and parasitaemia, relevant to host resistance against Trypanosomosis, have been described in breeds of African cattle (Hanotte *et al.*, 2003). However, a QTL search for resistance against the nematode *Trichostrongylus* spp. in another ruminant species, sheep, indicated only limited

success (Beh *et al.*, 2002). Quantitative trait loci have been identified for resistance and susceptibility to bovine spongiform encephalopathy in Holstein cattle (Zhang *et al.*, 2004).

To produce cattle with improved health, specific criteria for selection of disease resistance must be established. Standardised vaccination, a method of stimulation of immune responses allows all study individuals to be equally exposed to the same antigenic stimulus. The results provide a good approximation of the underlying immunogenetic variation, much more clearly than by studying wild-type clinical respiratory disease and associated immune responses (Snowder *et al.*, 2005). Using indirect ELISA, an established, repeatable, sensitive and specific assay, the antibody responses induced by viral vaccination have been assessed (Chapters 3 and 4). Models were developed to partition the phenotypic performances of individual animals into additive genetic components plus intrinsic (*sex*, *age*, etc) and extrinsic (*year-of-birth*, *dam-age*, etc) factor contributions.

The objectives of the current study were to exploit the genetic diversity between Charolais and Holstein cattle breeds to detect associations between microsatellite markers and QTL controlling immune function traits in cattle for antibody response to respiratory vaccination and antigen-stimulated cellular responses. Using thoroughly analysed phenotypic data, putative QTL were mapped over the entire bovine genome and their effects quantified. This experiment was unique due to the structured breeding programme employed, the relatively large number of progeny involved, the type of immunological phenotype observed, and the extent of the bovine genome covered.

5.2 Materials

5.2.1. Cattle

The current study used the RoboGen experimental cattle herd, described in detail in Chapter 2, with the entire pedigree population comprised of 954 genotyped animals (281 males and 673 females). This overall number included 587 F2 animals and 152 F1 animals. Phenotypic data was collected from 463 second-generation Holstein-Charolais calves (BH, BCH and F2 crosses), in four cohorts between 1998 and 2001, as described earlier in Chapters 2, 3 and 4.

5.2.2. Markers and Maps

Deoxyribonucleic acid (DNA) was prepared from semen samples collected from F0 and F1 bulls and from blood collected from the F0 and F1 dams and all the F2 progeny. DNA extraction was performed using a lysis and phenol/chloroform standard protocol (Maniatis *et al.*, 1982). For the genome scan, 139 microsatellite markers were selected at ~20 cM (Kosambi) intervals on all 29 *Bos taurus* autosomes (BTA) using published cattle linkage maps as a guide for intermarker distances (Barendse *et al.*, 1997; Kappes *et al.*, 1997). Markers were tested for position, technical clarity and potential informativeness (minimum target informativity ~0.50) and substitutions made as required (Table 5.1). Microsatellite genotypes were determined initially by an independent laboratory (Geneseek Inc, Nebraska, USA) then verified in-house (ARK-Genomics, Midlothian, UK).

The microsatellite markers used in this study are shown in Appendix C.1 on a per-chromosome basis. Up to eight markers were assigned per chromosome, the maximum and minimum intervals between markers being 63.2 cM (BTA7) and 0.2 cM (BTA13) respectively, with the mean interval being 20.5 cM to make a total mapped length of 2202.3 cM (Table 5.1). Estimated total BTA length and the number of linkage groups are based on the bovine genome map reported by Everts van der Wind *et al.* (2004).

Total genome coverage was approximately 80% of current published chromosome lengths (Band *et al.*, 2000). The mean marker interval per chromosome, ranged between 6.8 cM (BTA25) and 26.5 cM (BTA12) with a genomewide average of

BTA	No of markers	Map Length (cM)	Average Interval (cM)	BTA length[a] (cR_{5000})	Heterozygosity		
					min	max	average
1	7	126.8	18.1	256(3)	0.5721	0.8320	0.6643
2	6	108.7	18.1	502(5)	0.7363	0.8085	0.7672
3	8	123.0	15.4	303(2)	0.5141	0.8189	0.6798
4	6	93.6	15.6	431(5)	0.4455	0.8689	0.6701
5	5	111.6	22.3	508(1)	0.6357	0.7822	0.6909
6	6	85.2	14.2	521(3)	0.5042	0.7933	0.6295
7	5	109.6	21.9	547(3)	0.4833	0.7476	0.6096
8	6	101.7	17.0	513(5)	0.4544	0.8870	0.6825
9	5	76.2	15.2	345(2)	0.4800	0.7944	0.6580
10	6	73.9	12.3	466(1)	0.5512	0.8226	0.7122
11	6	95.8	16.0	557(3)	0.5851	0.7984	0.6838
12	4	106.0	26.5	226(5)	0.5536	0.8239	0.6747
13	4	44.2	11.1	384(2)	0.6093	0.7403	0.6972
14	5	63.4	12.7	361(6)	0.6268	0.8113	0.7354
15	5	92.4	18.5	452(3)	0.7071	0.8378	0.7672
16	5	65.1	13.0	563(4)	0.4742	0.8601	0.6787
17	6	94.6	15.8	440(3)	0.5400	0.7900	0.6709
18	5	70.5	14.1	549(4)	0.5980	0.7443	0.7069
19	4	65.6	16.4	513(5)	0.6652	0.7009	0.6824
20	4	64.3	16.1	150(3)	0.6874	0.8241	0.7478
21	3	62.1	20.7	316(2)	0.5370	0.7030	0.6201
22	4	44.2	11.1	291(3)	0.6742	0.8006	0.7321
***23**	3	64.3	21.4	462(1)	0.7077	0.8148	0.7619
24	4	44.6	11.2	219(1)	0.4985	0.7435	0.6248
25	3	20.5	6.8	286(2)	0.7068	0.8540	0.7755
26	4	60.0	15.0	333(1)	0.5380	0.7972	0.7144
27	3	64.1	21.4	175(2)	0.6397	0.7038	0.6802
28	4	37.4	9.4	170(1)	0.5830	0.8310	0.7075
29	3	32.9	11.0	469(1)	0.6937	0.8504	0.7906
	139[b]	2202.3[b]	15.8[c]	11308(82)[b]	-	-	0.6971[c]

Table 5.1 *Bos taurus* autosomes (BTA1 to BTA29): number of autosomal markers; total mapped length (centiMorgans; cM); average marker interval (cM); estimated total BTA length (number of linkage groups); minimum, maximum and average chromosomal heterozygosity; [a]measured in centiRay cR_{5000} where 4cR ≈ 1cM; [b]overall genome total; [c]overall genome average; *BoLA-containing autosome.

15.8 cM. Sex-specific maps were used when analysis was based on subsets of either male or female animals. The distribution of markers across the genome is presented graphically in Figure 5.1.

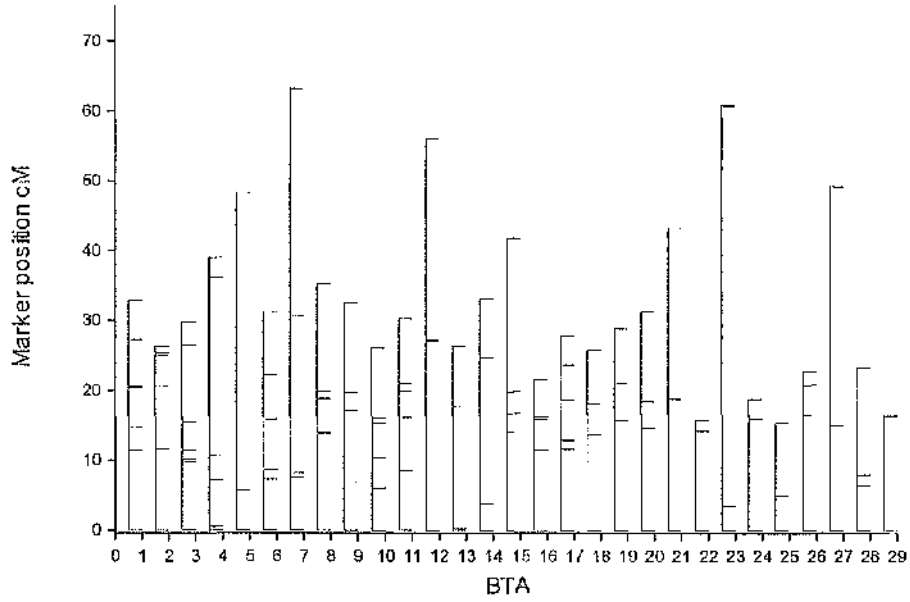


Fig 5.1 Graphical representation of marker distribution and marker density for all 29 *Bos taurus* autosomes (BTA1 to BTA29). Autosome number on the horizontal axis; position on the autosomes on vertical axis, provided in centiMorgans (cM). Each marker is indicated by a small horizontal bar on the vertical line representing the chromosome. The y-axis = 0 represents the terminal marker and not the actual end of the autosome.

It should be noted that, in genomic terms, there are large gaps in the marker maps for BTA7, BTA12, BTA21, BTA23 and BTA27.

Most markers were relatively informative. The maximum number of alleles per marker was nineteen (BTA10; TGLA272) and three chromosomes had markers with only three alleles (BTA6; BP7), (BTA11; HUJV174) and (BTA12; INRA5). Heterozygosity is the expected probability that an individual will have different alleles at a given single or multiple loci. For individual chromosomes, heterozygosity ranges between 0.000 and 0.900. Heterozygosity values based on the microsatellite genotyping were calculated per-autosome using the Windows-based TFPGA software package, Version 1.3 (Miller, 1997). Average heterozygosity per chromosome ranged from 0.610 (BTA7) to 0.791 (BTA29). The lowest marker heterozygosity was 0.446 found on (BTA4; IDVGA51) and the

highest was 0.887 found on (BTA8; DIK106). Average genome-wide heterozygosity across all chromosomes was 0.697 (Table 5.1).

Linkage between sets of markers was analysed using the Unix-based software package CRI-MAP Version 2.4 (Green *et al.*, 1990) and both sex-averaged and sex-specific maps constructed using the BUILD function to give most likely orders of and recombination rates among markers. The resulting framework linkage maps complied with global and interval support levels outlined by Keats *et al.* (1991).

5.2.3. Phenotypic data

In previous chapters, phenotypic data based on the IgG antibody responses folowing vaccination against the viruses BRSV, PIV3 and BHV1 were extensively assessed. Fixed effects and covariates for those datasets were evaluated using REML models and overall pre- and post-vaccination patterns of antibody kinetics considered biometrically. Phenotypic data for BCV IgG levels, presumably due to field infections (Chapter 2) was also tested for QTL, for comparative purposes.

A further set of immunological data (Young, 2002) for the same Robogen population of calves was available, collected from 445 calves at approximately five months old. In this case, the phenotypic traits were lymphocyte proliferation responses induced by formalin-killed *Staphylococcus aureus* ANTIGEN, phytohemagglutinin (PHA) MITOGEN and a matched blank CONTROL. The bacterium *Staphylococcus aureus* is a common mastitis pathogen of cattle (Sutra and Poutrel, 1994) and PHA, a potent T-lymphocyte-specific mitogen (Pearson *et al.*, 1979). Lymphocyte proliferation data were generated by incorporation of [^{3}H]thymidine, expressed as counts-per-minute readings on Days 2, 3, 4, 5, 9 and 10 of *in vitro* culture and were log transformed before use. This dataset has also been analysed thoroughly using REML and the heritable nature of specific traits established (Young *et al.*, 2005). An ideal opportunity presented to further characterise and analyze this related dataset for immune-related QTL, in conjunction with the antibody response data from the same set of calves.

5.3 Methods

5.3.1 Background

All phenotypic variance is a product of environment and genetic variation. Genetic variance can be partitioned into additive, dominance and epistatic variances (Falconer and Mackay, 1995). Additive effects of genes are cumulative over generations and are the main source of variation exploited by most animal breeding programmes. Interactions between alleles at any single locus are termed dominance, while interactions between alleles of different loci are termed epistasis (Lynch and Walsh, 1997).

Estimates of the additive and dominance effects of each prospective QTL were obtained from the all animals. Additive effects are the average genotypic effects at any locus and depend on the related breeding value. Additive effects (**a**) were defined as half of the deviation in phenotypic expression (fitness) between animals homozygous for locus-specific alleles (Figure 5.2).

$$\mathbf{a} = \tfrac{1}{2}(A_2A_2 - A_1A_1)$$

Dominance effects (**d**) were calculated as the deviation in phenotypic expression of locus-specific heterozygous animals from the phenotypic mean of animals homozygous for the same locus (Figure 5.2).

$$\mathbf{d} = (A_2A_1) - \tfrac{1}{2}(A_2A_2 + A_1A_1)$$

Genotype	A_2A_2	A_2A_1	A_1A_1
Genotypic value	**-a**	**d**	**a**

Figure 5.2 Graphical representation of allelic relationships at a single locus. Alleles A_1 and A_2 shown. Additive genetic effect (**a**) and dominance genetic effect (**d**)

A third situation also requires definition - overdominance (or heterosis) is when the phenotypic expression of the heterozygote eg. A_2A_1 is greater than either of the homozygotes (A_2A_2 or A_1A_1). Overdominance permits the stable coexistence of both alleles within a population and so maintains genetic variability.

In Chapters 3 and 4, the peak heritability h^2(±sc) of the vaccination-induced antibody response traits involved was established as 0.32(±0.17) for BRSV, 0.54(±0.31) for PIV3 and 0.16(±0.13) for BHV1. The h^2 values estimated for

antibody responsiveness to vaccination against BRSV and PIV3 are therefore medium high and that for BHV1 low. The results for BRSV and PIV3 antibody confirm that genetic control exists for these traits.

5.3.2 QTL Analysis

Linkage between genetic markers and immunological traits were tested on 29 autosomes for 34 antibody response traits (BRSV, PIV3, BHV1 and BCV). The traits comprised of antibody levels on the six sampling days for each vaccine, with deviations in antibody levels between days and area-under-curve also tested. Linkage between genetic markers and phenotypic traits were also tested for 18 lymphocyte proliferation traits (ANTIGEN, MITOGEN and CONTROL) using a multiple marker regression approach (Knott *et al.*, 1996). The lymphocyte proliferation traits comprised of counts-per-minute readings for six days of *in vitro* culture (ranging Day 2 to Day 10) for each agent (ANTIGEN, MITOGEN and CONTROL), with Day 0, the commencement of *in vitro* incubation. Both data sets were log transformed before analysis.

F-statistic values were obtained by regression interval mapping analysis using the web-based Java software QTL Express (http://qtl.cap.ed.ac.uk) as described by Seaton *et al.* (2002) which uses the multi-marker linear regression method. Relevant fixed effects and covariates were fitted as determined in Chapters 3 and 4 for the BRSV, PIV3 and BHV1 antibody datasets. Relevant fixed effects and covariates were fitted as pre-determined in Young *et al.* (2005) for the ANTIGEN, MITOGEN and CONTROL lymphocyte proliferation datasets. Due to the large sex-based disparity identified in the BRSV and PIV3 antibody kinetic patterns, QTL analysis for the BRSV and PIV3 sets of traits was performed in data grouped on sex, in addition to the population of animals as a whole.

The mapped genome was queried to locate regions where the markers explain a large proportion of the phenotypic variation, by regressing the offspring phenotypes onto the additive and dominance coefficients on every cM between markers. Briefly, a conditional probability of inheriting a specific haplotype from the sire was derived using the marker genotypes in all half-sib offspring. Then the

phenotypic trait value was regressed onto the probability that a particular QTL allele was inherited from the sire. To evaluate the resulting putative QTL, a test-statistic comparing a model with a QTL at that position against a model without the putative QTL was calculated. This test statistic is the ratio of the difference in residual sums of squares under the null hypothesis (no QTL) and the residual sums of squares under the QTL model **over** the residual sum of squares under the QTL model.

Under the null hypothesis of no QTL, the ratio of the mean squares of the effect of the allele to the residual mean squares should have a central F distribution. The maximum test statistic (F-statistic) was regarded as the most likely position of a QTL. F-statistic testing is robust against small deviations from normality and all segregating QTL should increase the F-statistic. F-statistics were calculated at intervals of 1 cM on all 29 autosomes. Quantitative trait loci searches across 29 chromosomes involve a huge number of statistical tests for marker-trait associations. In order to keep the number of false positives to an acceptable level, appropriate significance thresholds must be applied to each test. Due to the complex multiple test procedures involved in QTL detection, precise calculation of threshold F-statistics are inappropriate. Empirical threshold values of the F-statistic were estimated for each chromosome using permutation testing (n =1000; Knott *et al.*, 1998).

Permutation-based testing is based on non-parametric methods and better accommodates population-specific characteristics such as missing genotypes, differences in marker density and non-normal phenotypic distributions. Briefly, the permutation method of Churchill and Doerge (1994) involves random reassignment of trait values among individuals within the population. The resulting new data set was reanalysed and a new F-statistic calculated. This procedure was repeated 1000-fold to derive reliable empirical significance thresholds (Lander and Kruglyak, 1995). Thresholds against the null model at the 5% and 1% chromosome-wide level were derived for each QTL model for all traits. When significant QTL were detected, confidence intervals were estimated using bootstrapping (Visscher *et al.*, 1996). The 95% confidence interval was established as the chromosomal region remaining when the top and bottom 2.5%

of estimated positions were dropped. Further permutation work on the Robogen population has identified a 5% genome-wide significance level of ~9.0 (Weiner, 2005).

Detection of QTL depends on population size, the genomic distance between markers, the actual QTL, other QTL and the size of the effect of the QTL (Weller *et al.*, 1990). Rejection of QTL at too stringent a level defeats the purpose of the preliminary genome scan as neither a QTL position or its effect are known *a priori* (Fernando *et al.*, 2004). According to Lander and Kruglyak (1995), all significant results at $p < 0.05$ should be reported. As this was an exploratory analysis, to prevent missing QTL because of conservative test thresholds, all QTL were reported above the 5% chromosome-wide threshold level.

It is possible that two linked QTL with smaller effects can mimic the presence of one QTL with a large effect. A two-dimensional search was performed for 2-QTL models, with all combinations of positions for the 2-QTL evaluated. By computing and comparing the F-statistic generated by the 2-QTL model against that for the best 1-QTL model, the null hypothesis of a 1-QTL could be evaluated. An informative empty marker interval is necessary between two QTL to make discrimination possible and with limited marker maps, this may not always be possible.

5.4. Results

5.4.1 All QTL findings

Inclusion of both antibody and cellular traits for all 29 autosomes resulted in 1508 trait/chromosome combinations being tested for QTL. All QTL satisfying the 5% chromosome-wide F-statistic threshold were reported. The frequency distribution of F-statistics for the 1508 trait/chromosome combinations tested is listed in Table 5.2. Also shown in Table 5.2 are the number of QTL reported at, or greater than, the 5% significance level (unshaded) and the number of QTL expected at the 5% significance level due to random variation alone (shaded).

F-statistic	Percentage (frequency)													
	BRSV		PIV3		BHV1		ANTIGEN		MITOGEN		CONTROL		BCV	
0-1	28.3	(115)	24.4	(85)	19.2	(39)	20.6	(36)	22.9	(40)	15.4	(40)	34.5	(10)
1-2	36.0	(146)	33.9	(118)	41.4	(84)	30.9	(54)	31.4	(55)	34.9	(55)	31	(9)
2-3	19.5	(79)	22.7	(79)	18.7	(38)	28.0	(49)	26.3	(46)	22.9	(46)	17.2	(5)
3-4	9.1	(37)	12.4	(43)	11.3	(23)	10.9	(19)	11.4	(20)	13.1	(20)	10.3	(3)
4-5	3.9	(16)	3.7	(13)	3.4	(7)	5.1	(9)	5.7	(10)	8.0	(10)	0	(0)
5+	3.2	(13)	2.9	(10)	5.9	(12)	4.6	(8)	2.3	(4)	5.7	(4)	6.9	(2)
Total	100.0	(406)	100.0	(348)	100.0	(203)	100.0	(175)	100.0	(175)	100.0	(175)	100.0	(29)
Reported (5%)	8.8	(36)	7.7	(27)	6.9	(14)	8.0	(14)	3.0	(6)	9.0	(15)	5.9	(2)
Expected (5%)	5.0	(20)	5.0	(17)	5.0	(10)	5.0	(9)	5.0	(9)	5.0	(9)	5.0	(2)

Table 5.2 Percentage (frequency) of F-statistics generated by fitting QTL models to each of the immunological traits: **BRSV, PIV3, BHV1** and **BCV** represent levels of virus-specific IgG. Total lymphocyte proliferation data induced by staphylococcal **ANTIGEN**, phytohaemagglutinin **MITOGEN** and **CONTROL**.

All six types of phenotypic traits; BRSV, PIV3, BHV1, ANTIGEN, MITOGEN and CONTROL, even those previously assigned low heritability, were associated with putative QTL, with 119 significant trait/chromosome combinations tabulated for 28 different chromosomes. There were 82 significant trait/chromosome combinations reported for antibody responses (Table 5.3a-e) and 37 for lymphocyte proliferation responses (Table 5.4a-c). As some phenotypic traits were highly correlated, this reduced the number to 41 distinct QTL for vaccine-induced antibody responses and 16 distinct QTL for lymphocyte proliferation responses. Twelve of the antibody response QTL were for pre-vaccination sampling days. Only one chromosome (BTA15) contained no prospective QTL while eight chromosomes contained QTL related to one trait only (BTA4, BTA5, BTA13, BTA14, BTA22, BTA23, BTA25, and BTA29). Some chromosomes appeared to contain genes for a number of traits; BTA7, BTA18 and BTA21 each had four different types of trait, with four, three and five different QTL detected, respectively. Taking all chromosomes together, there were 21 QTL for BRSV IgG antibody, 13 QTL for PIV3 IgG antibody and 9 QTL for BHV1 IgG antibody. Only two prospective QTL were detected for the BCV dataset (BTA1 and BTA3) and both had relatively low F-statistics. Summarized for all chromosomes, six QTL for ANTIGEN lymphocyte proliferation reached the 5% chromosome-wide significance level, with two QTL reported for MITOGEN lymphocyte proliferation and nine QTL for CONTROL lymphocyte proliferation results.

As expected, some traits were highly correlated, especially antibody levels on successive sampling days and this is reflected in the series of QTL with the same or *near same* chromosomal position such as BHV1 (BTA1; 54-58 cM) and BRSV (BTA6; 63-65 cM). There were two overlapping QTL for antibody traits against more than one type of virus: PIV3 and BHV1 (BTA24; 44 cM) and BRSV and PIV3 (BTA28; 0-2 cM). However other QTL were identified, which were clearly specific to particular times in particular antibody response profiles. There were also two overlapping QTL for lymphocyte proliferation traits on BTA7 (0-1 cM) and BTA18 (63-70 cM), both associated with ANTIGEN and CONTROL.

No evidence was found to favour the 2-QTL model over the 1-QTL model on any chromosome in the current study and testing for such provided little additional information to the analysis.

Trait	Analysis Subset	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
BHV1-(Day 28)		1	54	INRA128	6.92	4.906/6.746	3.017	0.315	-0.211	0.088	0.355	0.126
BHV1-(Day 42)		1	59	INRA128	5.62	5.011/6.566	3.009	0.359	-0.200	0.098	0.375	0.139
BHV1-(Day 63)		1	58	INRA128	7.03	4.868/6.414	3.087	0.422	-0.331	0.117	0.411	0.165
PIV3-(Day 28)	M	1	87	BM884	6.26	4.464/6.774	4.090	0.186	0.207	0.060	-0.070	0.084
6-day area BHV1		1	59	INRA128	5.84	5.580/7.498	8.183	0.272	-0.202	0.083	0.289	0.117
BCV-(Day 77)		1	126	BMS4044	5.47	4.749/6.778	4.964	0.074	-0.053	0.021	0.066	0.028
BRSV-(D0 to D14)		2	54	*BM4440-TGLA226*	5.94	5.232/7.521	-22.290	3.903	0.834	1.211	-6.243	1.820
BRSV-(D0 to D35)		2	49	BM4440	5.61	4.834/6.493	-33.508	7.304	2.126	2.112	-10.183	3.109
PIV3-(Day 63)		2	108	IDVGA2	8.02	5.001/7.103	3.112	0.253	0.139	0.057	0.239	0.075
PIV3-(Day 77)		2	108	IDVGA2	6.19	5.002/7.144	3.437	0.281	0.152	0.063	0.214	0.083
PIV3-(D0 to D28)		2	107	IDVGA2	7.12	4.797/7.462	-45.931	6.147	6.700	1.832	2.211	2.451
PIV3-(D0 to D42)		2	108	IDVGA2	4.98	4.800/7.393	-55.228	9.142	7.628	2.692	5.007	3.574
PIV3-(D0 to D63)		2	108	IDVGA2	5.76	4.643/6.344	-84.766	11.217	9.023	3.302	8.898	4.385
BRSV-(D0 to D49)		2	40	*CSSM42–BM4440*	5.51	4.569/6.531	-48.451	7.594	2.950	2.247	-10.417	3.301
BHV1-(Day 0)		2	31	*CSSM42–BM4440*	7.41	4.743/6.123	4.331	0.467	-0.111	0.144	0.788	0.206
BRSV-(D14 to D35)	F	3	25	ILSTS96	6.19	4.818/6.689	-17.449	9.812	-6.837	2.707	-9.742	3.860
BCV-(Day 77)		3	47	*ILSTS96-TGLA263*	5.19	5.121/6.588	4.928	0.075	-0.030	0.023	0.106	0.035
BRSV-(Day -28)	F	4	39	MAF50	5.31	4.872/6.872	3.559	0.535	-0.480	0.172	-0.406	0.224
BRSV-(D14 to D35)		5	0	BM6026	4.84	4.136/6.138	-10.027	5.884	-5.483	1.779	-0.493	2.671

Table 5.3(a) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL. If analysis subset is used: Male (M) and Female (F).

Trait	Analysis Subset	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
BRSV-(Day -28)		6	65	*BMS739-BM2320*	6.57	4.716/6.691	3.570	0.403	0.340	0.125	-0.428	0.185
BRSV-(Day -14)		6	66	*BMS739-BM2320*	6.99	4.685/6.189	3.584	0.421	0.333	0.131	-0.513	0.194
BRSV-(Day 0)		6	63	*BMS739-BM2320*	8.41	4.859/6.152	2.956	0.423	0.315	0.131	-0.626	0.193
BHV1-(Day 77)		6	4	BM1329	4.73	4.486/5.844	3.207	0.436	0.418	0.138	-0.068	0.200
BRSV-(D0 to D49)	F	7	31	*BM1853-ILSTS6*	5.81	4.601/7.026	-82.938	13.005	-18.307	5.450	5.866	9.368
BRSV-(Day 42)	F	7	35	*BM1853-ILSTS6*	5.00	4.682/6.272	3.4194	0.344	0.2933	0.141	-0.587	0.243
BRSV-(Day 35)-G2	F	7	42	*BM1853-ILSTS6*	6.97	5.040/7.437	1.122	0.415	-0.615	0.168	-0.205	0.295
BRSV-(Day 49)-G2	F	7	29	*BM1853-ILSTS6*	11.51	4.528/5.836	0.412	0.474	-0.865	0.180	0.066	0.308
BHV1-(Day 0)		7	100	INRA53	4.74	4.470/5.817	4.845	0.461	-0.365	0.136	0.299	0.192
BRSV-(Day 49)		8	42	*DIK106-HUJ174*	6.12	4.623/7.227	2.344	0.194	0.211	0.062	-0.066	0.090
BRSV-(Day 35)	M	8	41	*DIK106-HUJ174*	6.49	5.093/6.950	3.257	0.208	0.193	0.069	-0.231	0.103
BRSV-(Day 49)	M	8	41	*DIK106-HUJ174*	8.96	4.426/6.376	2.664	0.257	0.336	0.086	-0.195	0.128
BRSV-(D14 to D35)	M	8	47	*DIK106-HUJ174*	4.38	4.730/5.962	-1.753	6.286	4.722	2.023	-5.306	2.877
BRSV-(Day 0)	M	9	76	INRA84	4.52	4.406/6.022	3.022	0.521	0.566	0.188	-0.129	0.259
BRSV-(Day 14)	M	9	76	INRA84	3.51	4.755/6.912	3.021	0.542	0.519	0.196	-0.137	0.269
BRSV-(Day 28)	M	9	76	INRA84	3.75	4.302/6.056	2.402	0.557	0.551	0.201	-0.123	0.276
BHV1-(Day 14)		9	61	*UWCA9-MM12E6*	5.05	4.400/6.879	0.764	0.215	0.105	0.069	-0.287	0.099
PIV3-(Day 63)	M	10	52	*BM888-CSRM60*	6.55	4.691/7.443	3.356	0.244	-0.132	0.075	-0.337	0.107
PIV3-(Day 77)	M	10	63	*CSRM60-TGLA272*	6.75	4.681/6.511	3.190	0.260	-0.080	0.080	-0.410	0.114
PIV3-(D0 to D63)	M	10	51	*BM888-CSRM60*	5.37	4.710/7.249	-56.877	15.237	-8.786	4.768	-18.377	6.828
PIV3-(D28 to D63)	M	10	62	*CSRM60-TGLA272*	5.38	4.566/6.591	-31.274	11.153	-4.151	3.378	-15.161	4.849

Table 5.3(b) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL. If analysis subset is used: Male (**M**) and Female (**F**).

Trait	Analysis Subset	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
PIV3-(Day 63)		11	95	BMS607	4.97	4.645/7.343	3.207	0.254	-0.051	0.055	0.215	0.072
PIV3-(D0 to D14)		12	88	*INRA5-INRA209*	4.94	4.851/6.297	-34.708	4.047	2.687	1.363	5.022	1.990
PIV3-(D0 to D28)		12	84	INRA5	6.76	4.381/5.520	-49.641	6.290	3.158	2.042	9.797	2.910
PIV3-(D0 to D42)		12	81	INRA5	5.49	4.522/6.314	-59.794	9.367	4.936	3.062	12.750	4.413
BRSV-(Day 0)		13	33	*ABS10-DIK93*	5.72	4.071/5.584	3.136	0.572	-0.600	0.191	-0.284	0.262
BRSV-(Day 14)-G2		14	12	*CSSM66-RM11*	5.45	4.590/7.289	-0.356	0.238	0.187	0.075	0.197	0.110
BRSV-(Day 49)-G2		14	14	*CSSM66-RM11*	5.38	4.693/6.887	0.264	0.294	0.283	0.093	0.136	0.137
BRSV-(Day 35)	M	14	0	CSSM66	6.65	5.160/7.553	3.223	0.210	-0.194	0.068	0.138	0.082
BRSV-(Day 0)	M	16	59	*BM719-HUJ625*	6.06	4.152/6.009	2.855	0.511	0.010	0.182	0.693	0.256
BRSV-(Day 14)	M	16	60	*BM719-HUJ625*	6.37	4.615/6.930	2.848	0.527	-0.057	0.189	0.943	0.265
BRSV-(Day 28)	M	16	44	*ETH11-BM719*	5.69	4.547/5.702	2.022	0.559	0.007	0.209	1.009	0.300
BRSV-(Day 14)		17	81	INRA25	7.79	4.790/6.154	2.940	0.216	-0.201	0.058	0.143	0.077
BHV1-(Day 0)		17	63	IDVGA40	5.09	4.847/7.333	4.697	0.466	-0.321	0.126	0.327	0.168
BRSV-(Day 14)-G2	F	18	18	INRA121	5.77	4.716/6.471	-0.270	0.346	-0.300	0.089	-0.013	0.129
PIV3-(D28 to D63)	M	18	60	*HAUT14-DIK67*	4.59	4.120/5.566	-47.619	11.526	-6.048	4.241	16.612	6.024
BRSV-(Day 35)-G2		19	65	ETH3	4.43	4.279/5.768	0.935	0.258	-0.203	0.068	0.006	0.090
BRSV-(Day 49)-G2	F	20	54	*DIK15-BM5004*	4.41	4.246/6.153	0.354	0.466	0.257	0.125	0.390	0.191

Table 5.3(c) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL. If analysis subset is used: Male (M) and Female (F).

Trait	Analysis Subset	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
BRSV-(D0 to D14)	F	21	10	*HEL5-TGLA337*	6.22	4.094/6.685	-37.568	5.442	-6.660	2.212	4.966	3.328
PIV3-(Day 63)		21	36	*HEL5-TGLA337*	4.70	4.053/6.041	3.251	0.253	-0.061	0.066	0.315	0.107
PIV3-(Day 77)		21	33	*HEL5-TGLA337*	4.94	4.305/5.756	3.507	0.281	-0.009	0.077	0.401	0.128
BHV1-(Day 0)		21	62	IDVGA39	5.26	4.298/5.725	4.331	0.472	0.117	0.150	0.739	0.233
PIV3-(Day 63)		22	44	UWCA49	4.45	4.149/5.815	3.258	0.254	0.164	0.056	-0.024	0.077
BRSV-(Day 14)-G2		23	25	*IOBT528-BM1905*	5.53	4.303/5.967	-0.331	0.243	0.313	0.095	0.062	0.157
PIV3-(Day 63)		24	44	INRA90	4.99	4.048/6.299	3.195	0.252	0.075	0.055	0.227	0.077
BHV1-(Day 0)		24	22	ILSTS101	5.25	4.006/6.426	4.632	0.468	0.473	0.153	-0.187	0.227
BHV1-(Day 28)		24	0	TGLA351	5.09	4.101/6.346	2.839	0.311	0.217	0.081	0.230	0.113
BHV1-(Day 63)		24	44	INRA90	5.10	4.105/6.226	2.848	0.142	0.375	0.109	0.114	0.150
BHV1-(Day 77)		24	44	INRA90	4.57	4.224/5.899	3.022	0.436	0.294	0.114	0.260	0.156
PIV3-(Day 0)	F	25	20	INRA222	5.40	4.278/5.848	5.275	0.400	0.261	0.101	-0.252	0.134
BRSV-(Day 0)-G2		26	13	*ABS12-HEL11*	4.51	3.870/5.981	1.810	0.230	0.210	0.074	0.099	0.111
PIV3-(Day 14)	F	27	47	*RM209-BM203*	5.46	3.834/6.682	0.746	0.249	0.206	0.071	0.189	0.113
PIV3-(D0 to D14)	F	27	42	*RM209-BM203*	6.03	4.431/6.879	-53.533	6.389	6.658	2.445	8.817	3.923
BHV1-(Day 14)		27	1	BM3507	5.22	4.062/6.030	0.532	0.213	0.075	0.056	0.225	0.074

Table 5.3(d) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL. If analysis subset is used: Male (**M**) and Female (**F**).

Trait	Analysis Subset	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
BRSV-(Day -28)		28	0	BP23	5.42	4.225/6.529	4.678	0.607	0.506	0.181	-0.401	0.231
BRSV-(Day -14)		28	0	BP23	5.25	3.820/6.105	4.570	0.641	0.540	0.192	-0.387	0.244
BRSV-(Day 0)		28	0	BP23	4.49	4.015/5.617	3.814	0.632	0.467	0.189	-0.407	0.241
PIV3-(Day 42)	F	28	23	IDVGA43	5.04	3.862/5.311	3.185	0.350	-0.192	0.069	-0.142	0.091
PIV3-(Day 63)	F	28	23	IDVGA43	5.69	4.125/6.305	3.406	0.366	-0.226	0.072	-0.122	0.095
PIV3-(Day 77)	F	28	23	IDVGA43	5.60	4.074/5.860	3.742	0.393	-0.255	0.078	-0.074	0.102
PIV3-(Day 42)	M	28	2	BP23	8.14	3.911/5.296	3.899	0.292	0.170	0.066	0.286	0.095
BRSV-(Day -28)	F	29	32	DIK94	5.05	3.652/4.723	3.289	0.400	-0.032	0.113	0.521	0.165
BRSV-(Day -14)	F	29	32	DIK94	4.33	4.183/6.232	3.255	0.419	0.000	0.119	0.508	0.173

Table 5.3(e) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL. If analysis subset is used: Male (**M**) and Female (**F**).

Trait	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
ANTIGEN-(Day 5)	7	1	BP41	5.46	4.853/6.216	10.579	0.583	0.651	0.201	0.234	0.290
CONTROL-(Day 4)	7	0	BP41	5.49	5.073/8.073	5.641	0.414	0.388	0.142	0.414	0.204
CONTROL-(Day 5)	7	1	BP41	5.11	4.205/6.567	5.055	0.364	0.373	0.125	0.237	0.181
ANTIGEN-(Day 9)	7	0	BP41	5.77	4.463/7.283	6.872	0.650	0.621	0.223	0.673	0.321
ANTIGEN-(Day 10)	7	0	BP41	5.25	4.613/7.117	5.799	0.639	0.562	0.219	0.670	0.315
CONTROL-(Day 9)	8	95	*DIK74-CSSM47*	5.75	4.688/5.754	4.198	0.243	0.318	0.094	-0.049	0.143
CONTROL-(Day 10)	8	100	CSSM47	6.55	5.082/6.939	3.786	0.246	0.326	0.091	0.061	0.141
CONTROL-(Day 4)	9	17	UWCA9	4.55	4.846/6.568	6.398	0.409	-0.365	0.129	-0.195	0.173
MITOGEN- (Day 3)	10	68	TGLA272	4.58	5.314/6.476	10.565	0.269	-0.109	0.082	-0.300	0.109
MITOGEN-(Day 4)	10	70	TGLA272	5.49	5.007/6.494	9.964	0.312	-0.076	0.090	-0.392	0.121
MITOGEN-(Day 5)	10	70	TGLA272	6.68	5.425/7.064	9.099	0.347	0.042	0.100	-0.485	0.134
MITOGEN-(Day 9)	10	68	TGLA272	7.52	4.600/5.962	5.861	0.340	-0.004	0.096	-0.499	0.129
CONTROL-(Day 4)	11	46	ILSTS100	4.60	4.543/7.209	5.716	0.410	0.311	0.142	0.382	0.211
CONTROL-(Day 10)	12	84	INRA5	4.79	4.118/6.174	3.792	0.240	0.085	0.079	0.333	0.113

Table 5.4(a) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL for lymphocyte proliferation following stimulation with staphylococcal antigen (ANTIGEN), phytohaemagglutinin (MITOGEN) and no stimulation (CONTROL).

Trait	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
MITOGEN-(Day 9)	16	9	*BMS121-TGLA53*	4.54	4.547/6.339	5.856	0.353	-0.332	0.112	-0.059	0.149
MITOGEN-(Day 10)	16	6	*BMS121-TGLA53*	5.71	4.576/6.755	6.390	0.353	-0.365	0.109	-0.053	0.148
ANTIGEN-(Day 3) / CONTROL	17	94	BM1233	5.40	4.779/6.989	7.236	0.475	-0.235	0.098	-0.305	0.128
ANTIGEN-(Day 5) / CONTROL	17	94	BM1233	4.80	4.745/7.014	8.526	0.716	-0.432	0.156	-0.316	0.204
ANTIGEN-(Day 3)	18	70	DIK67	4.98	4.296/5.465	9.617	0.396	0.149	1.285	0.524	0.191
ANTIGEN-(Day 4)	18	70	DIK67	5.65	4.576/5.984	10.299	0.469	0.066	0.159	0.764	0.235
ANTIGEN-(Day 5)	18	70	DIK67	5.42	4.553/6.963	10.426	0.588	0.014	0.191	0.919	0.283
CONTROL-(Day 2)	18	63	*HAUT14-DIK67*	7.08	4.620/7.463	6.420	0.264	0.236	0.088	0.321	0.133
CONTROL-(Day 3)	18	63	*HAUT14-DIK67*	9.51	4.283/6.373	5.981	0.365	0.428	0.129	0.491	0.194
CONTROL-(Day 4)	18	67	*HAUT14-DIK67*	6.18	4.668/6.199	5.632	0.417	0.326	0.138	0.477	0.206
CONTROL-(Day 5)	18	54	*HAUT14-DIK67*	5.07	4.398/6.608	5.062	0.364	0.292	0.118	0.331	0.172
CONTROL-(Day 10)	19	58	*CSSM65-ETH3*	4.92	4.475/6.418	3.877	0.236	0.235	0.075	0.046	0.105
CONTROL-(Day 10)	20	9	*BM3517-TGLA126*	7.67	4.534/6.389	3.745	0.242	0.283	0.081	0.205	0.115

Table 5.4(b) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL for lymphocyte proliferation following stimulation with staphylococcal antigen (ANTIGEN), phytohaemagglutinin (MITOGEN) and no stimulation (CONTROL).

Trait	BTA	Position cM	Marker *(bracket)*	F-statistic	Significance 5% / 1%	Mean Effect	(± s.e.)	Additive Effect	(± s.e.)	Dominance Effect	(± s.e.)
ANTIGEN-(Day 2)	21	53	*TGLA337-IDVGA39*	4.17	4.413/6.045	4.087	0.396	0.148	0.087	0.501	0.147
ANTIGEN-(Day 3)	21	58	*TGLA337-IDVGA39*	6.50	4.308/6.264	9.513	0.399	0.374	0.130	0.458	0.198
ANTIGEN-(Day 4)	21	56	*TGLA337-IDVGA39*	6.33	4.062/5.549	10.228	0.493	0.468	0.161	0.542	0.247
ANTIGEN-(Day 5)	21	58	*TGLA337-IDVGA39*	6.02	4.425/6.271	10.334	0.592	0.547	0.193	0.531	0.295
ANTIGEN-(Day 9)	21	26	*HEL5-TGLA337*	4.86	4.460/6.165	6.810	0.674	0.713	0.258	0.655	0.438
ANTIGEN-(Day 10)	21	25	*HEL5-TGLA337*	4.02	4.107/5.593	5.799	0.665	0.364	0.256	0.525	0.436
CONTROL-(Day 3)	24	26	ILSTS101	4.69	4.173/6.133	6.052	0.391	0.182	0.123	0.508	0.180
ANTIGEN-(Day 2)	26	0	ABS12	4.67	4.266/6.706	8.947	0.290	-0.279	0.099	0.190	0.147
ANTIGEN-(Day 3)	26	0	ABS12	4.74	4.454/6.098	10.000	0.400	-0.409	0.136	0.164	0.203
CONTROL-(Day 2)	27	44	*RM209-BM203*	4.82	4.112/5.946	7.016	0.266	-0.205	0.110	-0.432	0.176

Table 5.4(c) *Bos taurus* autosome (BTA), autosomal position (centiMorgans), nearest marker or *flanking marker bracket*, single QTL model F-statistic, autosome-wide 5% and 1% significance thresholds and estimated mean, additive and dominance effects of putative QTL for lymphocyte proliferation following stimulation with staphylococcal antigen (ANTIGEN), phytohaemagglutinin (MITOGEN) and no stimulation (CONTROL).

5.4.2 Significant QTL findings

Figures 5.3 to 5.7 show F-statistic curves for a number of traits, selected due to high peak levels of significance. They act as examples, illustrating how the patterns of F-statistics typically vary according to chromosomal position.

5.4.2.1 BRSV antibody

A significant QTL ($p < 0.01$) was detected on BTA8, located between markers DIK106 and HUJ174, with a peak F-statistic of 8.96 and a positive additive (±se) effect of 0.336 (±0.086) for post-vaccination levels of BRSV-specific IgG antibody in the male calf group (Figure 5.3). The position of this QTL was estimated as 42.9 cM with a 95% confidence interval (CI-95) between 25 cM and 92 cM.

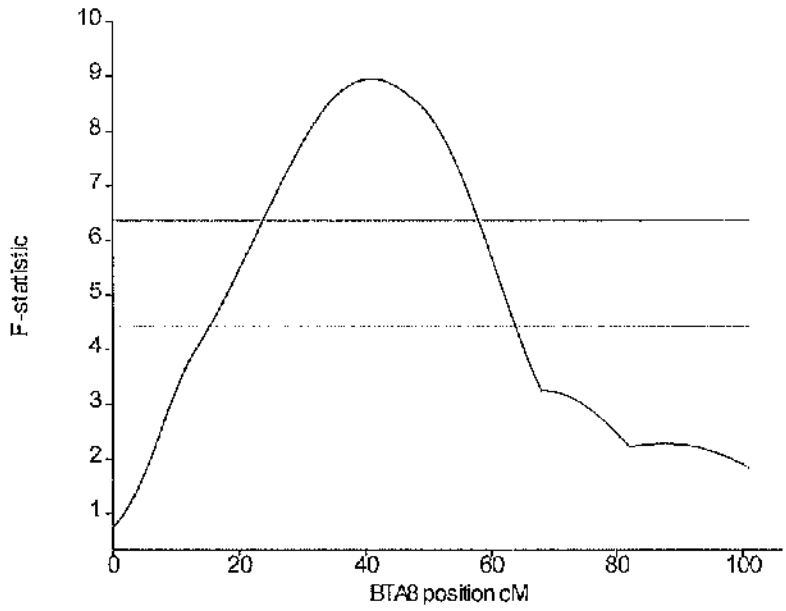


Figure 5.3 F-statistic curve of single QTL (BTA8) for level of BRSV-IgG antibody on Day 49, relative to first vaccination (male calves only). The x-axis indicates the chromosomal position in centimorgans (cM). The y-axis represents the F-statistic at each chromosomal position. Horizontal lines indicate chromosome-wide significance thresholds of $p < 0.05$ (green) and $p < 0.01$ (red).

5.4.2.2 PIV3 antibody

Another significant QTL ($p < 0.01$) was detected on BTA10 between markers CSRM60 and TGLA272, with a peak F-statistic of 6.75 and a dominant negative effect (±se) of -0.410 (±0.114) for post-vaccination levels of PIV3-specific IgG antibody in the male calf group (Figure 5.4). The position of this QTL was estimated as 58.9 cM with a CI-95 between 12 cM and 67 cM.

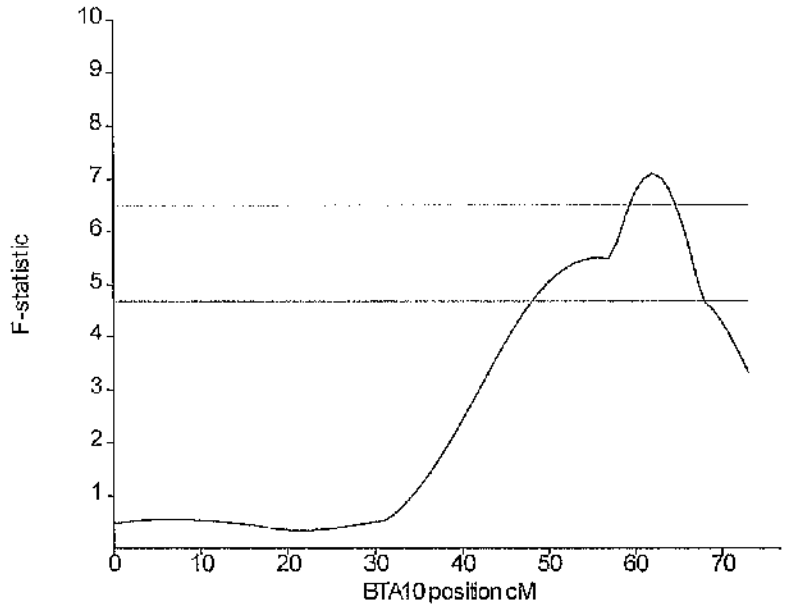


Figure 5.4 F-statistic curve of single QTL (BTA10) for level of PIV3 IgG antibody on Day 77, relative to vaccination (male calves only). The x-axis indicates the chromosomal position in centimorgans (cM). The y-axis represents the F-statistic at each chromosomal position. Horizontal lines indicate chromosome-wide significance thresholds of $p < 0.05$ (green) and $p < 0.01$ (red).

5.4.2.3 BHV1 antibody

A significant QTL ($p < 0.01$) was detected on BTA1 between markers INRA128, with a peak F-statistic of 7.03 and an additive negative effect (±se)of -0.331 (±0.117) for post-vaccination levels of BHV1-specific IgG antibody (Figure 5.5). The position was estimated as 48.6 cM with a CI-95 between 9.5 cM and 106.5 cM.

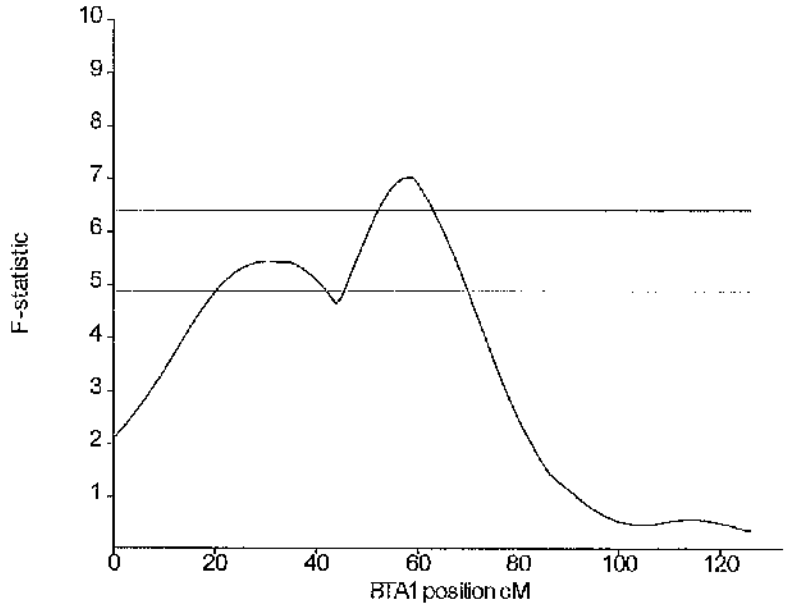


Figure 5.5 F-statistic curve of single QTL (BTA1) for level of BHV1 IgG antibody on Day 63 relative to first vaccination. The x-axis indicates the chromosomal position in centimorgans (cM). The y-axis represents the F-statistic at each chromosomal position. Horizontal dotted lines indicate chromosome-wide significance thresholds of $p < 0.05$ (green) and $p < 0.01$ (red).

5.4.2.4 Lymphocyte proliferation (CONTROL)

A significant QTL ($p < 0.01$) was detected on BTA18 between markers HAUT4 and DIK67, with a peak F-statistic of 9.51 and a positive additive (±se) effect of 0.336 (±0.086) for levels of lymphocyte proliferation in the CONTROL group (Figure 5.6). The position was estimated as 59.8 cM with a CI-95 between 32 cM and 70 cM.

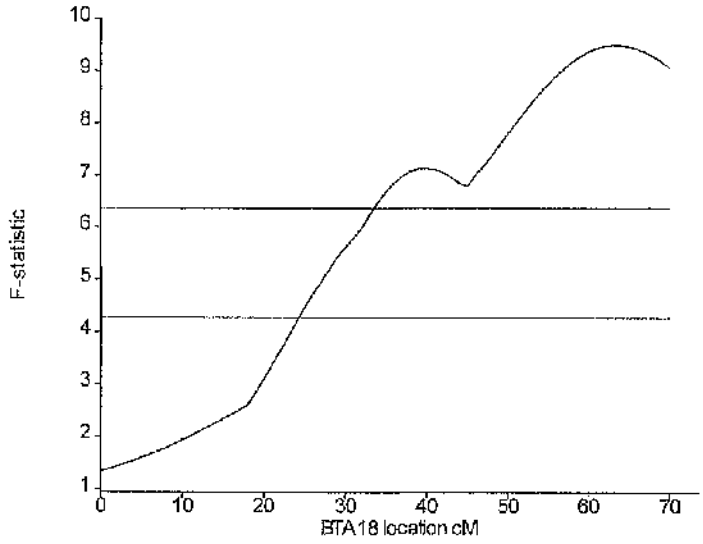


Figure 5.6 F-statistic curve of single QTL (BTA18) for level of lymphocyte proliferation (CONTROL) on Day 3, relative to stimulation. The x-axis indicates the chromosomal position in centimorgans (cM). The y-axis represents the F-statistic at each chromosomal position. Horizontal dotted lines indicate chromosome-wide significance thresholds of $p < 0.05$ (green) and $p < 0.01$ (red).

5.4.2.5 BCV antibody

A putative QTL ($p < 0.05$) for levels of BCV antibody due to presumed exposure to wild-type virus had a peak F-statistic of 5.47 and a pattern suggestive that the detected QTL may lie just outside the available marker map (Figure 5.7). The 95% confidence intervals for this QTL tended to be quite large, with minimum and maximum values of 38cM and 97cM, respectively.

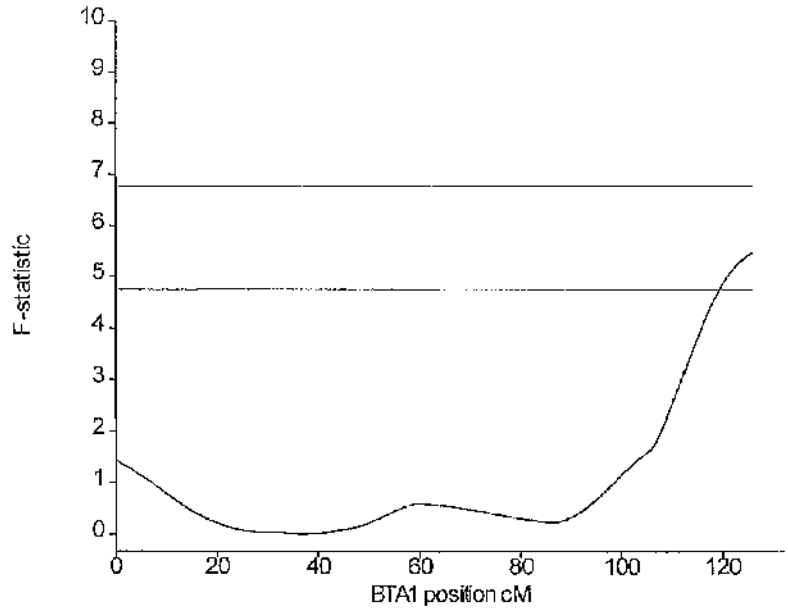


Figure 5.7 F-statistic curve of single QTL (BTA1) for level of BCV IgG antibody. The x-axis indicates the chromosomal position in centimorgans (cM). The y-axis represents the F-statistic at each chromosomal position. Horizontal dotted lines indicate chromosome-wide significance thresholds of $p < 0.05$ (green) and $p < 0.01$ (red).

5.5. Discussion

A comprehensive genome scan was performed to detect QTL associated with antibody responses to vaccination and antigen/mitogen stimulated lymphocyte proliferation in a young, crossbred cattle population. Quantitative phenotypic data generated using standard laboratory techniques (ELISA and [^{3}H]thymidine-incorporation lymphocyte proliferation assay) were used to explore the novel area of immune system genetic control. With ~ 80% of the genome covered, the results strongly support a multilocus model for both genetic control of antibody response to respiratory vaccination and genetic control of lymphocyte proliferation in cattle. In particular, QTL on BTA1, -2, -6, -7 for antibody response and QTL on BTA10, -18 and -21 for lymphocyte proliferation, show high significance. This is consistent with the paradigm discussed by Glass (2004), with the contribution of many genes with small and interacting effects determining overall vaccine responsiveness. Most of the other immune QTL reported satisfy lower significance thresholds only and so must be regarded as prospective rather than definitive.

This study was novel in many respects. Much previous research on the role of genetic variation on the functioning of the immune system has concentrated on qualitative issues of high or low responses, essentially phenotypic extremes (Mallard *et al.*, 1998). By introducing a continuum model for antibody responsiveness, it is hoped to provide a more inclusive representation of *in vivo* immune processes. Unlike most cattle QTL experiments which are based on the grand-daughter design (Weller *et al.*, 1990), this project used a F2/backcross (two commercial cattle breeds) test population explicitly designed for QTL detection. This approach has advantages: 1) as three genotypes can be found at loci, both additive and dominance effects can be estimated; 2) increased power for QTL analysis than backcross (alone) design; 3) any identified QTL may be more precisely investigated in subsequent crossing experiments (Liu and Zeng, 2000). However as the experimental population size was limited, both in size and structure, some relevant alleles and genes may be inadvertently excluded from the study.

Unlike most previous antibody response studies, which were based on selected lines; mice (Puel *et al.*, 1996), pigs (Magnusson *et al.*, 1997), chickens (Yunis *et al.*, 2002b) and turkeys (Sacco *et al.*, 1994a) divergent for antibody production, the current study was based on breed lines developed for production traits alone. As such, all results are relevant and pertinent to commercial cattle production. Eighty-two QTL F-statistics for antibody response met or exceeded the 5% chromosomewide significance threshold in this study, 34 more than the 48 QTL expected due to stochascity alone, given the number of trait and chromosome combinations. Similarly, although 26 QTL were expected due to stochascity, based on the lymphocyte proliferation data, 41 (an excess of 15) were detected by this experiment. Although in terms of both antibody and cellular traits, many more QTL were detected than could be expected by chance alone, it is probable that some of the putative QTL represent Type I errors (false positives adopted). This limitation is accepted but all available QTL were reported both to allow future research access to the fullest set of "raw" results possible and to lower the probability that true QTL for antibody response to vaccination were ignored, a Type II error (true positives rejected).

The immune system is under complex balance and control. Each immune response to vaccination is dependent on host immune status, immunological memory, administration route, antigen type, inoculate dose, pathogen predilection sites and many other factors. Although the three viruses used are together described as respiratory pathogens, each virus has a unique niche: epidemiological, immunological and pathological and this is reflected in their corresponding vaccines (Chapters 3 and 4). Sources of variation additional to those discussed in those chapters include parenteral versus mucosal administration; DNA-type versus RNA-type viruses; primary, secondary and subsequent immune responses and prevailing levels of pathogen-specific herd immunity and infections. For these reasons, as well as the complexity and multiplicity of cellular interactions in an immune reaction, it is unsurprising that a wide variety of genes could be involved in the immune responses to vaccination. The three antibody responses studied here seem to represent distinct immunostimulatory events, perhaps under separate genetic influence and control.

As discussed in Young *et al.* (2005), MITOGEN lymphocyte responses tend to be independent of both ANTIGEN and CONTROL lymphocyte responses. A similar disparate pattern is reflected in the QTL results for lymphocyte proliferative responses reported here, emphasizing how the innate and acquired types of immunity interact in cattle. The divergent and unique characteristics of each type of immune response tends to reflect their genetic origin. It would seem that many non-MHC QTL (not located on BTA23) are involved in the immune responses tested, which is consistent with the hypothesis that MHC genes have a supervisory role: modifying and regulating the influence of non-MHC genes (Behnke *et al.*, 2003).

Prior to this experiment, QTL mapping for immune function traits, especially humoral immunity, has been limited in cattle, with other species being much more thoroughly investigated. Mouse chromosome (Mmu) 6 (located at 20-36 cM), Mmu12 (at 23-66 cM) and Mmu17 (at 18-27 cM) were found to influence antibody responsiveness following immunisation with sheep erythrocytes (Puel *et al.*, 1995). The same author found that Mmu4 (at 49-54 cM), Mmu10 (at 65cM) and Mmu8 (at 30-52 cM) were also involved in control of antibody response though to a lesser extent (Puel *et al.*, 1996). On Mmu3 (at 49 cM), Mmu8 (at 38cM) and Mmu9 (at 34 cM), QTL for antibody responses against Salmonella flagellar antigens were reported by De Souza (2004). The ITAM (immunoreceptor tyrosine-based activation motif), interleukin (IL)12a and IL6Rα loci were proposed as likely candidate genes controlling antibody phenotype in mice (Takai, 2002). Due to the lack of resolution in the current bovine map, it was difficult to accurately align the prospective cattle QTL found here, relative to the published mouse map.

Fourteen QTL were described for antibody responses to *Escherichia coli* and *Salmonella enteritidis* in chickens (Yunis *et al.*, 2002b), six of which were common to both types of antigen. That study reported quite large genetic contributions of up to 1.84 phenotypic standard deviations and implicated a homologue of the human BMP2 gene. In baby chicks, QTL were detected by Siwek *et al.* (2003a) for antibody responses against keyhole limpet hemocyanin (KLH) on *Gallus gallus* autosome (GGA)-14 and GGA18 and against *Mycobacterium butyricum* on GGA14, GGA16 and GGA18. The same author was able to classify QTL

controlling antibody responses into private (antigen-specific) and public (non-specific). Such classification of QTL was not possible in the current study, probably due to an overdiversity among the phenotypes studied, leading to a low number of common QTL.

Another study in chickens (Yunis *et al.*, 2002a), found strong correlations between genetic control of antibody responses to both bacterial and viral antigens, suggesting a non antigen-specific common mechanism of antibody response in that species. No correlation was found between antibody response and body weight in that population tested, a finding also confirmed by Siwek (2004). In chickens, transforming growth factor (TGF-β) genes were found to exert genetic control over antibody responsiveness (Zhou and Lamont, 2003), a finding further refined to the TGF-β2 gene by Malek and Lamont (2003). The latter group also found associations between antibody levels induced by *Salmonella spp* vaccination and polymorphisms of inducible nitric oxide synthase (INOS) and immunoglobulin G light chain (IgL) genes in young chickens. In pigs, polymorphisms on MHC class II (*DRB, DQB*) and complement factor B genes were found to influence levels of antibody to AD virus (Wimmers *et al.*, 2004). Furthermore, QTL determining antibody responses to *Escherichia coli* antigens K88 and O149 were detected on pig chromosomes SSC5 (at 64 cM) and SSC6 (at 69 cM), respectively (Edfors-Lilja *et al.*, 1998).

The vast majority of the QTL related to antibody response in cattle reported here are original, simply because there has been virtually no previous QTL research for these types of immune trait. In cattle, Elo *et al.* (1999) described a QTL on BTA23 (linked to marker RM185) associated with general veterinary treatments although metabolic-type as well as infectious disease was included. There was evidence of a QTL for prevaccination levels of antibody (Day 0-PIV3) on BTA25 (INRA222) in the current study and this corresponds to a QTL proposed on the same chromosome related to diseases other than mastitis and fertility problems by Holmberg and Andersson-Eklund (2004). Significant QTL were characterised by the same author for diseases other than mastitis and fertility problems, associated with BTA11 (linked to INRA177) and BTA25 (linked to ILSTS102). Although INRA177 was

included in the current study, there was no direct evidence of linkage and only putative evidence that BTA11 contained any immune function QTL, those for late antibody response (Day 63-PIV3) antibody and unstimulated lymphocyte proliferation (Day 3-CONTROL). Schulman *et al.* (2004) found QTL (position) for veterinary treatments (other than mastitis or infertility) on BTA1 (22 cM), BTA2 (28 cM), BTA5 (76 cM), BTA8 (121 cM), BTA15 (101 cM) and BTA23 (1 cM). None of these were confirmed by the QTL results from the current study.

Along with many other immunomediators, viral infections induce type I interferons (IFN-α and -β) which in turn stimulate the myxovirus (Mx) protein family designed to provoke apoptosis of virus-infected cells. Genes for bovine Mx1 are on BTA1 (BM1824; 128 cM) and have been implicated in resistance to the viral pathogens BVDV, BHV1 and bovine rotavirus (Muller-Doblies *et al.*, 2002; Muller-Doblies *et al.*, 2004). The current study found a concordant QTL on BTA1 (BMS4044; 126 cM) for levels of antibody induced by field infections with bovine coronavirus. There appeared to be no evidence of similar QTL associated with levels of antibody induced by BRSV, PIV3 or BHV1 vaccination.

In the current study, BTA8 contains both QTL for antibody response and lymphocyte proliferation. BTA8 holds four IFN loci (Eggen and Fries, 1995) which have been demonstrated to influence the severity of disease induced by BHV1 (Ryan and Womack, 1997). Quantitative trait loci for both antibody and lymphocyte proliferation traits, early ANTIGEN/CONTROL rates of lymphocyte proliferation and late post-vaccination levels of PIV3 antibody, coincided with QTL on BTA18 (~60 cM). The gene for the immunomodulant cytokine, transforming growth factor (TGF-β1), is located at this point on BTA18 (Sonstegard *et al.*, 2000).

Many cattle-based QTL studies include somatic cell count (SCC), which is linked to the cellular immune response within the mammary gland. Somatic cell count is an indirect measure of udder health, reflecting both subclincial and clinical mastitis (Koivula *et al.*, 2005). Previously published results related to SCC and incidence of mastitis (*), are shown in Table 5.5 along with any corresponding lymphocyte proliferation QTL as determined by the current study. The following list is not exhaustive but does illustrate that QTL for SCC in cattle are accumulating through consensus and continuously evolving research. The number of genetic markers used

by each study is also listed. (**A**) (Reinsch *et al.*, 1998), 45 markers; (**B**) (Schrooten *et al.*, 2000), 277 markers; (**C**) (Zhang *et al.*, 1998), 206 markers; (**D**) (Ashwell *et al.*, 1998b),16 markers; (**E**) (Ashwell *et al.*, 1996),17 markers; (**F**) (Ashwell *et al.*, 1998a), 16 markers; (**G**) (Schulman *et al.*, 2004), 150 markers; (**H**) (Van Tassell *et al.*, 2000), 105 markers; (**I**) (Heyen *et al.*, 1999), 174 markers; (**J**) (Rodriguez-Zas *et al.*, 2002), 174 markers; (**K**) (Kuhn *et al.*, 2003), 263 markers; (**L**) (Holmberg and Andersson-Eklund, 2004), 116 markers; (**M**) (Van Tassell *et al.*, 2004), 22 markers; (**N**) (Ron *et al.*, 2004), 73 markers; (**P**) (Bennewitz *et al.*, 2003), 133 markers; (**Q**) (Ashwell *et al.*, 2004), 367 markers, (**R**) (Schulman *et al.*, 2002), 101 markers, (**S**) (Klungland *et al.*, 2001), 288 markers.

Quantitative trait loci identified for lymphocyte proliferation on BTA12, BTA17, BTA19, BTA20 and BTA24 are novel to the current study and unreported elsewhere. The cellular phenotype tested in the current study is a much more precise and limited trait than the rather rather broad indicator trait, SCC used by the aforementioned studies. SCC is a complex summary trait, potentially influenced by the environmental, physiological, immunological, pathological and genetic backgrounds. It is therefore unsurprising that a more restricted number of QTL were identified in the current study, than those which dealt with SCC. This phenomenon, where some studies confirm a QTL, while others provide conflicting results, is relatively common and is due to a lack of consensus about how phenotypic data should be collected, organised, analysed and the appropriate levels of significance to apply when reporting. Furthermore, biological variation between populations means that real differences may exist in the relative importance of specific immunological QTL.

Other studies				Current study			
BTA	Study[1]	Marker[1] (marker bracket)	Position[1]	Marker[2] (marker bracket)	Position[2]	BTA	Type
1	A	MAF46	125			1	
1	G	TGLA57	59			1	
1	J		80,125			1	
2	P	(BMS829-BMS2267)	(91-100)			2	
3	B	BMC5227	171			3	
3	G	HUJ1177	105			3	
4	C	(RM188-TGLA116)	43			4	
5	I	BM315	*108*			5	
5	J		36			5	
7	H	BM6117	*66*			7	
7	I	BMS1979	*131*			7	
7	J		60			7	
7	K	(BMS2258-AE129)	107			7	
7	N	(BM6105-TGLA303)	*(36-39)*	BP41	*16.3*	7	A/C
7	Q	(BM6117-BMS2258)	*67*			7	
8	A	BM3419	*19*	(DIK74 - CSSM47)	*(98-121)*	8	C
8	S	(TGLA13-INRA122)	*31*			8	
10	K	(TGLA378-TGLA102)	49 *(44-62)*	TGLA272	*98*	10	M
11	L	BM7169	*52*	ILSTS100	*60*	11	C
13	C	(TGLA381-AGLA232)	91			13	
13	J		0			13	
14	C	(ILSTS11-BM302)	21			14	
14	G*	(BMS1747-BMS740)	25			14	
16	J		51	(BM121 - TGLA53)	*(26-39)*	16	M
18	D	BM2078	*80*	(HAUT14 - DIK67)	*(42-68)*	18	C
18	B	(BM7109-ILSTS002)	70			18	
18	G*	TGLA227	111			18	
18	K	TGLA227	117			18	
18	Q	TEXAN10	*21*			18	
20	Q	(RM310-TGLA126)	*29*			20	
21	I	ETH131	*33*	(TGLA337 - IDVGA39)	*(54-71)*	21	A
21	R		*51*			21	
22	I	BM3628,CSSM26	*47,0*			22	
23	E	513				23	
23	D	513				23	
23	F	BM1258	*28*			23	
23	A	RM033	*21*			23	
23	I	MGTG7	*49*			23	
26	Q	BM1314	*27*			26	
26	C	(TGLA429-BM804)	14	ABS12	*3*	26	A
27	K	(BM3507-TGLA179)	8	(RM209 - BM203)	*(15-27)*	27	C
27	M	BMS2116	54			27	
29	G	ILSTS057	16			29	

Table 5.5 Published QTL for SCC traits and mastitis (*) traits over *Bos taurus* autosomes (BTA) 1 to 29; [1]Lymphocyte proliferation QTL taken from other studies in literature (shaded), coded as in preceding text (p206); [2]Lymphocyte proliferation QTL taken from current study (unshaded), typed ANTIGEN (A), MITOGEN (M) and CONTROL (C). Where necessary marker positional data taken from Band *et al.* (2000).

Cellular immune traits have been investigated for likely QTL in other species too. Using flow cytometry, significant QTL (positioned at) for resting lymphocyte counts were found on human chromosomes HSA1 (200-231 cM), HSA2 (28-54 cM), HSA8 (15-18 cM) and HSA9 (38-39 cM) (Hall *et al.*, 2002). Chen and Harrison (2002) found QTL on Mmu1 (63 cM) and Mmu19 (12 cM) for peripheral blood percentages of B-cells and cytotoxic T-cells, respectively.

BTA23 carries the bovine MHC (BoLA), which is intricately linked to infection, resistance and immunity (Ashwell *et al.*, 1998b; Elo *et al.*, 1999; Heyen *et al.*, 1999). However, the extremely low density of markers on this autosome precluded evaluation of its immunological role in the current study. Due to technical difficulties, BTA23 was mapped with only three markers, giving a very low map resolution and coverage. Only one QTL on BTA23 was identified (related to early postvaccination levels of BRSV IgG_2), a probable reflection of the low QTL detection rate possible. Where marker spacing exceeds 15 cM, particularly with BTA23, additional markers need to be selected and genotyped to prevent missing QTL due to inadequate genotypic information.

The power and mapping resolution of all QTL searches is constrained by the size and genetic content of the individual mapping population (Snelling *et al.*, 2005). As there were limited numbers of progeny in the project, a certain degree of imprecision must be accepted for QTL located on chromosomes; thus their exact positions must be regarded as provisional only. Similarly the non-detection of QTL on any specific chromosome may be due to the restricted number of alleles and markers available. The interval mapping technique for QTL discovery uses information from consecutive informative markers simultaneously to estimate QTL position and effect (Knott *et al.*, 1992). A significant problem is that QTL outside the interval tested may remain undetected or give false positive results.

Although dictated by experimental practicalities, only two cattle breeds were used in this experiment and some QTL, either described or excluded, may be breed-specific. If, as the heritabilities (Chapters 3 and 4) suggest, antibody responses to vaccination are sire-specific rather than breed-specific, using twelve sires may have restricted the number of alleles available. In the strictest sense, the sires used were not representative of the entire *Bos taurus* genome as their very availability is based on many cycles of production-based progeny testing. As this was preliminary work

in a new area there was no way of devising a breeding strategy to knowingly ensure parental lines were homozygous for loci related to relevant traits: future studies may concentrate on the loci identified here to make maximum use of their experimental populations.

Despite differences in the resource population tested, the experimental approach and the method of analysis from previous related experiments, several previously identified QTL were confirmed by the current study. More than twenty of the QTL identified were common to both sexes, operating across both dairy and beef suckler farming systems. Such QTL are therefore robust and flexible enough to be of practical value across quite disparate production schemes. Many of the analysed traits included in the study, such as antibody levels on successive sampling days, are well correlated. A possible improvement would be to use multi-trait phenotypic analysis which would increase power and permit the detection of pleiotropic effects. Intrachromosome searches for multiple QTL had limited usefulness in this exploratory study. Unless two QTL are separated by two informative markers, they prove difficult to discriminate. More focussed marker placement would help resolve this weakness. Confidence intervals for QTL positions tended to be rather large. Due to the limited number of informative meiotic events inherent in the project's design, such imprecise results are expected. Expanding the number of informative families as well as increasing the number of markers would improve the precision of the experiment.

The approach of integrated gene-mapping for heritable molecular phenotypes in large organised pedigrees is a very powerful tool for unravelling the complex effects of polymorphic genes in health and disease. Based on the results presented here, there is strong evidence for the segregation of QTL for immune function, even with the rather coarse marker map used.

To prevent declines in health and immunity, QTL for antibody and cellular immune function should be incorporated into cattle breeding goals. The detection here of immune function QTL ensures that this will be, in principle, possible although further fine mapping is likely to be necessary for implementation. Mapping of QTL is an ideal method of identifying regulatory proteins or rate-limiting enzymes in very complex physiological and biochemical pathways, such as serum antibody

response, with the eventual aim of identifying causative genes at a molecular level (Korstanje and Paigen, 2002).

Even before that stage, the QTL identified here, which are closely linked to genetic markers, could be used to introduce immune function into traditional progeny testing leading to more healthy and productive cattle with improved welfare.

Chapter 6

General discussion

6.1 General Discussion

Reducing losses due to infectious disease is an excellent method of improving production efficiency and animal welfare. Selection for disease resistance in farm animals is a sustainable approach which avoids many of the difficulties of traditional disease control methods. Resistance to infection is under polygenic control. Functional polymorphisms in host resistance genes manifest themselves as a spectrum of immunological responses. The kinetic profiles of IgG antibody discussed in Chapters 2, 3 and 4 typify the extent of this variation occurring even after a standardised respiratory virus vaccination protocol. The opportunity to normalise environmental conditions is a critical advantage of experimental genetic studies such as this, so diminishing somewhat the complexity of multifactorial phenotypes such as antibody response (Kramnik and Boyartchuk, 2002).

The results presented here show that when calves were reared using dairy-type as opposed to beef-type systems, there were no long term effects on their ability to develop an antibody response to vaccination. Even though this contrast in calf-rearing management was also confounded by calf-sex, the eventual convergence of post-vaccination antibody levels in both groups suggests a common mechanism controlling humoral responses in male and female calves, aged between 60 and 160 days.

Maternally-derived passive antibody progressively decays as calves grow older. Calf-age and serum levels of passive antibody are therefore inherently linked and as pre-existing serum antibody can suppress the subsequent response to vaccination, this is important. The current study found that in calves over two months of age, the effect of calf-age was primarily to determine the level of pre-existing antibody before vaccination. The direct effects of age on the induced antibody response following vaccination were much weaker, suggesting that the bovine immune system is competent beyond 60 days. The use of logisitic regression analysis to establish the thresholds at which the inhibitory effects of pre-existing ELISA antibody act is novel to the current study. If it is accepted that serum antibody is an indicator of vaccine responsiveness, maximising post-

vaccination levels of serum antibody is a legitimate aim in all calf production systems. An accurate cut-off threshold for interference of pre-existing antibody with vaccine-induced antibody, as derived here, would therefore prove extremely useful when optimising existing vaccination regimes.

Due to the design of the project, it was possible to observe the transitions within the phenotypic variance induced by vaccination in particular detail for BRSV IgG. Total transmissible variance accounted for about a third of the overall phenotype variance before and after vaccination. Before vaccination the majority of this was due to the maternal heritable component but after vaccination the additive heritable component dominated. This period coincides with the window of vulnerability for BRSV clinical disease in calves. However, in a more general sense, a fuller understanding of this juncture is critical as disease resistance becomes a breeding goal in farm animals. If "super" resistant parents transfer short-term passive immunity to progeny, sufficient to interfere with endogenous immune responses, break-downs in immunity could occur. As all farm species rely heavily on transfer of passive immunity for neonatal survival, it is vital to investigate this area in tandem with progress on other heritable resistance traits.

The heritability of IgG responses to vaccination was established as ~0.3 for BRSV and ~0.5 for PIV3. If host genetic variation can account for a third or more of antibody responses, a priority for all future vaccine studies must be to ensure as genetically diverse a test population as possible to prevent biased results. In contrast, no heritable effects were detected for antibody levels to presumed field infections of bovine coronavirus (although seroprevalence was high) or to the poorly sero-active BHV1 vaccine. Antibody responses were more sire-specific than breed-specific, a useful finding allowing the overall productive character of particular breeds to be retained while incorporating beneficial immune function traits into progeny selection.

All disease resistance can be regarded as a complex genetic trait in that there is no direct correlation between particular genetic polymorphisms and traits (Lander and Schork, 1994). Instead gene-gene and gene-environment interactions contribute to and determine the emerging immune response, with perhaps the primary environmental component being the infecting pathogen itself. Each

pathogen has a unique set of epidemiological and virulence characteristics. To counter this, the immune system has devised qualitatively and quantitatively adjusted responses, all of which emanate from quantitative trait loci on the host genome. Although constituents of the same multivalent vaccine, the PIV3 and BIIV1 components studied in Chapter 4 were independent entities immunologically, with strikingly different vaccine response, the final contribution of immune system genes varies according to the pathogen, the environment and the host physiology.

Antibody response is a complex trait involving networks of different molecules and pathways, all of which have genetic origin. Eighty-two QTL for antibody response were reported by the current study which, even after excluding Type 1 errors, is a reflection of the multitude of cell-types, receptors, cytokines, chemokines, growth factors and others involved in each antibody response. A further forty-one QTL related to lymphocyte proliferation were identified, again demonstrating the complex nature of the cellular immune response. Indicator traits such as serum antibody are likely to remain the chief *in vitro* method for immune phenotype detection in farm animal species. Deliberately exposing large scale populations to pathogens, necessary for genetic studies of clinical disease, is hugely expensive and is fraught with standardisation and ethical issues.

Genetic influences on immune responses can also be more oblique. For respiratory virus responses, less direct considerations include the morphology of the respiratory tract such as chest volume, tracheal cross-section, etc; non-specific immunity such as the chemical nature of the bronchial mucus, the efficiency of the mucociliary escalator, the number of active patrolling leukocytes in the lungs and respiratory mucous membrane and the destructive efficiency of these cells. Genetics can control animal behaviour with factors such as maternal recognition, stress-handling, aggression, avoidance of excretory products while grazing, and levels of social interaction allowing pathogen transmission (Le Neindre *et al.*, 1998; Mackenzie *et al.*, 1997). Finally as every immune response is largely mediated via protein molecules, there is an energy and resource cost. The partitioning and processing of nutrients is under genetic

control in cattle (Funk, 1993; Galyean *et al.*, 1999) and as such the immune system is dependent on those genes.

Marker-assisted selection is the obvious objective following confirmation of relevant QTL. Cattle-breeding is particularly suited to marker-assisted selection (MAS), as in this species conventional phenotypic selection is hampered by a long generation interval, the predominance of sex-limited production traits, expensive progeny testing programmes for artificial insemination (AI) bulls and the generally low heritability of disease resistance traits. The widespread use of AI in cattle production means a small nucleus of key breeding animals exists which facilitates implementation of MAS. Furthermore MAS could act as a useful genetic filter to reduce the number of genetically undesirable bulls before they enter production-based progeny testing schemes (pre-selection). Dekkers and Hospital (2002) concluded that it would too risky to abandon phenotypic evaluation completely once MAS is introduced, as high levels of environmental variation, genotype/environment interaction or epistasis could each subvert the intended breeding goals. The same authors suggested that applying MAS in conjunction with *in vitro* fertilisation and embryo transfer technologies, effectively "pre-pregnancy pre-selection", could accelerate the production of prospectively high merit animals ten-fold.

Although QTL with major effects are easily identified, genome scans may not detect those QTL with smaller effects if they fail to reach the stringency level applied. A secondary phase of positional candidate cloning following initial QTL mapping will continue. It would be extremely costly to generate pedigrees large enough to provide a map resolution capable of gene-level precision. The many chromosomes and QTL identified here, related to antibody response in cattle, imply that selection based on MAS from a F2 generation may be the more effective method of introducing these genetic elements into a population, rather than attempting marker assisted introgression.

The ultimate aim when mapping trait loci has to be confirmation of causative genes and causative mutations. The results presented here have the potential to help delineate physiological pathways involved in humoral immunity to

infectious disease and steer intelligent searches for relevant polymorphic molecules and candidate genes. The emergence of detailed comparative genome maps means that these results are useful outside the test species - cattle. Comparative mapping between species using expressed sequence tags (ESTs) is continuing and gene expression correlations between human and bovine genes is approximately 0.52(±0.07) (Cosseddu *et al.*, 2004). In conjunction with expanding micro-array technologies (Donaldson *et al.*, 2005; McGuire and Glass, 2005), this synteny between cattle and other species means that homologues of immune function genes and models of mammalian immune processes can be transferred between species. Such cross pollination will greatly enhance progress towards a fuller understanding of the immune system in all mammalian species.

6.2 Future Work

In this study the earliest post-vaccination sampling point was fourteen days. To test for specific IgM and IgA, both of which have peaked and subsided by Day 10 post-infection, an earlier sample or preferably set of samples would be required. Even if sera was only collected in the first ten days after vaccination, additional phenotypic datasets could be generated if interferon-γ and/or the acute phase proteins such as serum amyloid A, haptoglobin and alpha 1-acid glycoprotein were measured. Another informative extension would be to test for IgG_2 antibody specific to BHV1 and PIV3 vaccination. Mucosal immune responses are notoriously difficult to assess as they are often unrelated to serum antibody levels (Mestecky, 1987). Assays based on circulating antibody–secreting cells have been used as a correlative indicator of mucosal immunity in sheep (Premier *et al.*, 2004). A logical progression of the current study would be to evaluate mucosal immune responses e.g. IgA. To do this on a scale sufficient for genetic experiments, the methodology used by Premier *et al.* (2004) could be adapted to cattle.

The RoboGen herd was virtually clean of BVDV but because of the profound immunosuppressive effects of this virus, some research is necessary on how it affects antibody responses to vaccination, particularly with inclusion of genetics.

Due to technical problems, BTA23 was only weakly mapped in the current study. As it is the primary location of the *BoLA*, this is a considerable defiency. It is important to reanalyse the immune system phenotypic datasets for QTL using an adequate, preferably dense, map of BTA23. Generally, more markers over the whole genome would improve our results, in terms of precision and also informativity as not all sires were heterozygous at the selected markers used. Expanding the number of cattle-breeds (only two were used in the current study) would inevitably increase the number of possible alleles per gene and could change the emphasis onto different QTL.

It is crucial to determine if any negative effects exist on other economically important traits such as milk yield and feed conversion rates before embarking on MAS based on any QTL for immunological function. Unfavorable genetic correlations have been reported between milk productivity and some functional traits in dairy cows (Castillo-Juarez *et al.*, 2000). With the wealth of production data available for the RoboGen herd, it is an ideal opportunity to study this controversial relationship in cattle. It is unknown how long term selection for disease resistance may influence the equilibrium between subclinical and clinical disease.

6.3 Conclusion

Eradication of bovine respiratory disease pathogens is unlikely to be an economic or feasible option on a commercial scale unless production practices change radically. Selecting for resistant or vaccine hyper-responder animals does not mean all animals need to be perfectly resistant within a population to prevent an epidemic – just a sufficient number to lower virus transmission rates below epidemic self-sustenance (Bishop and MacKenzie, 2003). Vaccination will continue to be used as an effective disease management tool in many types of cattle production systems. Host genetics must now be integrated into vaccine development programmes to optimise and maximise their efficacy.

Abbreviations

1°	primary
2°	secondary
AD	Aujeszky's Disease
AI	artifical insemination
ANOVA	one-way analysis of variance
APC	antigen processing cell
B-cell	B lymphocyte
BCH	backcross Charolais
BCR	B-cell receptor
BCV	bovine coronavirus
BH	backcross Holstein
BHV1	bovine herpesvirus 1
bIgG	bovine respiratory syncytial virus IgG
BLAD	bovine leukocyte adhesion deficiency
BMP2	bone morphogenetic protein 2
BoLA	bovine lymphocyte antigen system
BRD	bovine respiratory disease
BRSV	bovine respiratory syncytial virus
BTA	*Bos taurus* autosome
BVDV	bovine viral diarrhoea virus
CD	cluster of differentiation
CI-95	95% confidence interval
cM	centiMorgan
CMI	cell-mediated immunity
COD	corrected optical density
CpG	non-methylated cytosine guanine DNA motifs
cpm	counts per minute
CR3	complement receptor ; integrin
cR_{5000}	centiRay
d.f.	degrees of freedom
DC	dendritic cell
dl	decilitre
DNA	deoxyribonucleic acid
dQ	interquartile range
ELISA	enzyme linked immunosorbent assay
Eos	eosinophil
EQ	equation
EST	expressed sequence tag
F0, F1, F2	filial (generation) -1, -2 and -3
Fab	antigen binding fragment (antibody)

Fc	crystalizable fragment of antibody
FcRn	neonatal Fc receptor
FcγRIIB	Fc receptor II-B
g	gram
GGA	*Gallus gallus* autosome
h2	heritability
HLA	human leukocyte antigen complex
hRSV	human respiratory syncytial virus
HSA	*Homo sapiens* autosome
IFN	interferon
IgA	immunoglobulin A
IgD	immunoglobulin D
IgG	immunoglobulin G
IgM	immunoglobulin M
iIgG	bovine herpesvirus 1 IgG
IL	interleukin
INOS	inducible nitric oxide synthase
ITAM	immunoreceptor tyrosine-based activation motif
KIR	killer-inhibitory receptor
KLH	keyhole limpet hemocyanin
LAK	lymphocyte-activated killer
log	logarithm
Ly49	lectin inhibitory receptor
M	molarity
m2	maternal heritability
MAC	membrane-attack-complex
MAS	marker assisted selection
max	maximum
MDPA	maternally derived passive antibody
Mem	memory B-cell
mg	microgramme
MHC	Major Histocompatibility Complex
min	minimum
MIP	macrophage inflammatory protein
Mmu	*Mus musculus* autosome
MΦ	macrophage
n.s.	not significant
NK	natural killer cell
NRAMP	natural resistance associated macrophage protein
OD	optical density
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PHA	phytohemagglutinin
pIgG	parainfluenza virus 3 IgG
pIgR	polymeric immunoglobulin receptor
PIV3	parainfluenza virus 3
PRR	pattern recognition receptors
postvac	post-vaccination
prevac	pre-vaccination
QTL	quantitative trait loci
r	correlation
r_g	genetic correlation
r_r	residual correlation
RANTES	regulated on activation, normal T-cell expressed and secreted
REML	residual maximum likelihood linear analysis
RLB	reverse line blot typing
RNA	ribonucleic acid
ROD	relative optical density
RSV	respiratory syncytial virus
SCC	somatic cell count
sd	standard deviation
SE	sheep erythrocytes
se	standard error
SNT	serum neutralisation test
SSC	*Suis scrofus* chromosome
T-cell	T lymphocyte
TFPGA	Tools for Population Genetic Analyses
Th1	helper T-cell type 1
Th2	helper T-cell type 2
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
β_2M	β_2-microglobulin
σ^2	variance

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Appendices

Skewness is a measure of the asymmetry of data around the sample mean. If skewness has a negative value, the long tail of data distribution is more on the left of the mean than on the right. If skewness has a positive value, the long tail of the data distribution is more on the right of the mean than on the left.

Skewness of a distribution is defined as

$$\frac{\sum_{i=1}^{n}(Y_i - \overline{Y})^3}{(n-1)s^3}$$

Where $\overline{Y}$ is the mean of Y, s is the standard deviation of Y and n is the sample size.

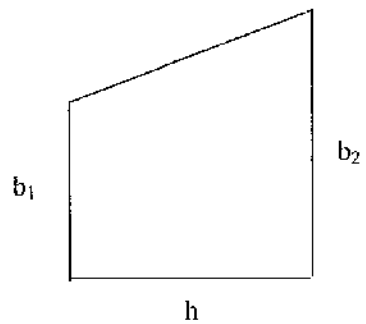
Appendix A.1 Definition of sample skewness

The trapezoid rule permits accurate estimation of the area under a line. It is based on the formula used in geometry to find the area of a trapezoid of height (h) and base length (b_1) and base length (b_2). By subdividing any area under a line into trapezoids and then summing each trapezoid area, the total area under a line can be calculated.

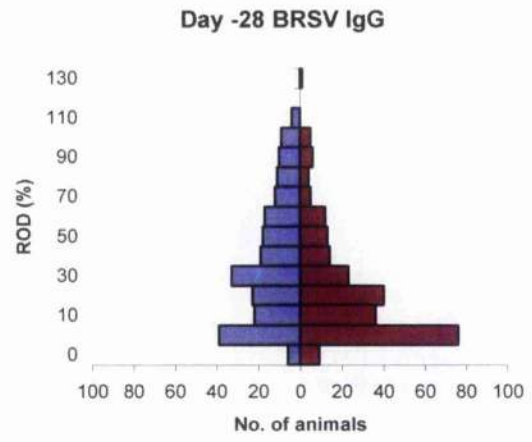
Area of trapezoid is

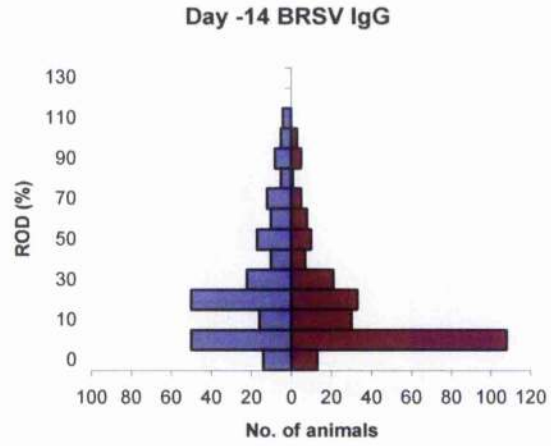
$$\frac{1}{2}h \cdot (b_1 + b_2)$$

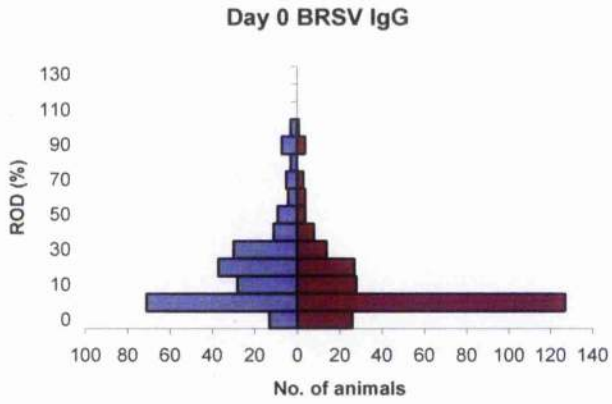
Where



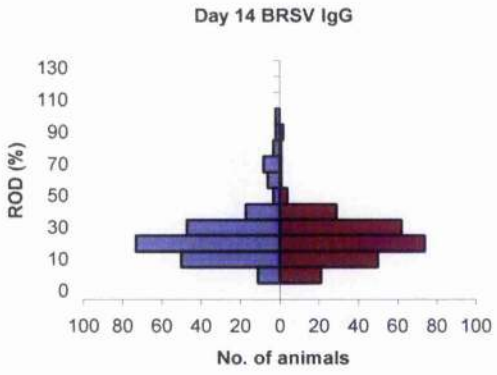
Appendix A.2 The trapezoid rule for calculation of area of a trapezoid.

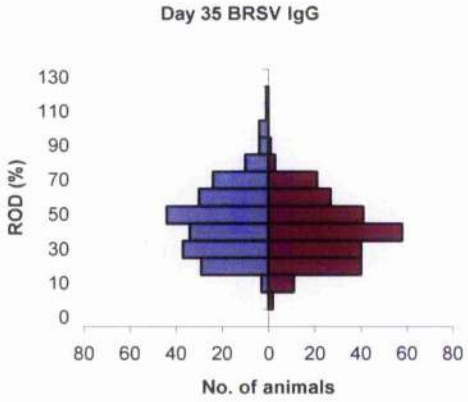


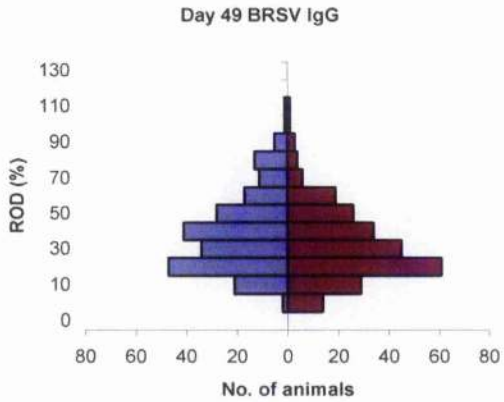




Appendix A.3 Raw data for Chapter 3. Trellis plots for levels of total BRSV-IgG (non-transformed) on pre-vaccination Days -28, -14, 0, grouped on **sex**.

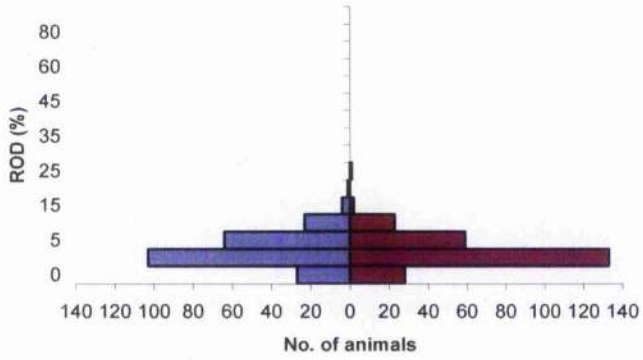




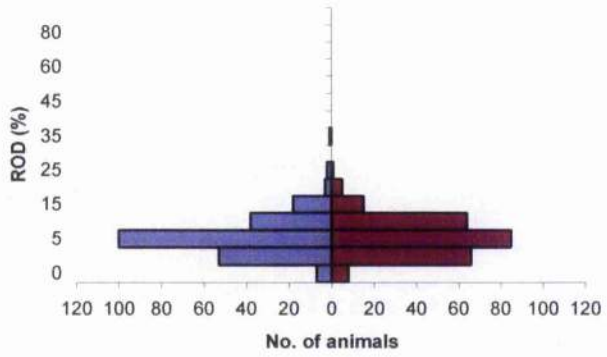


Appendix A.3 (contd) Raw data for Chapter 3. Trellis plots for levels of total BRSV-IgG (non-transformed) on post-vaccination Days 14, 35 and 49, grouped on **sex**.

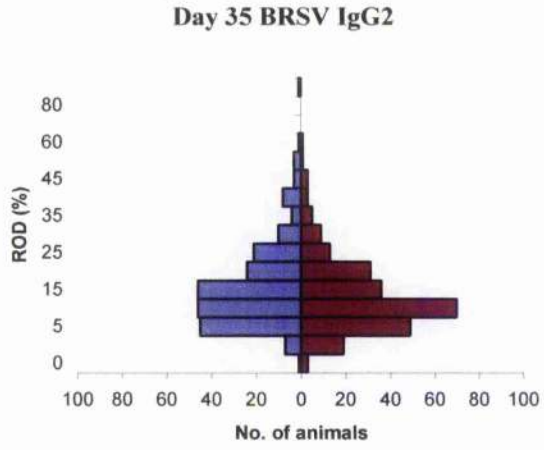
Day 0 BRSV IgG2

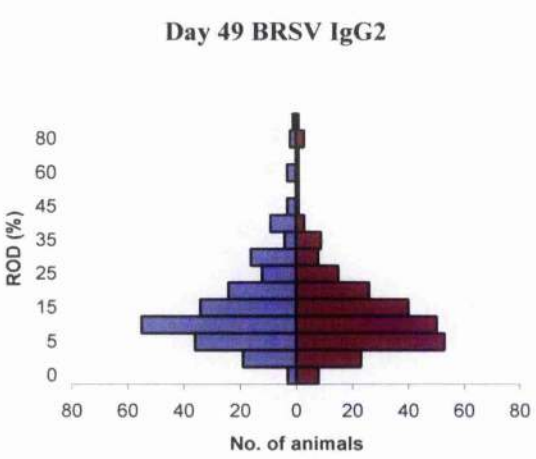


Day 14 BRSV IgG2

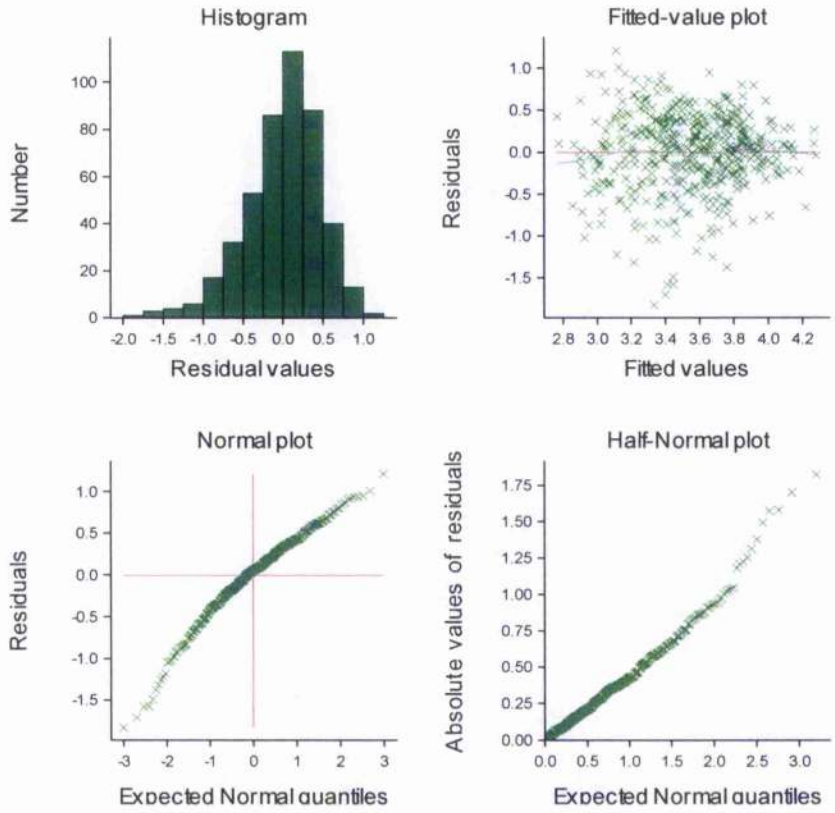


Appendix A.4 Raw data for Chapter 3. Trellis plots for levels of BRSV-IgG_2 (non-transformed) on Days 0 and 14, grouped on **sex**.

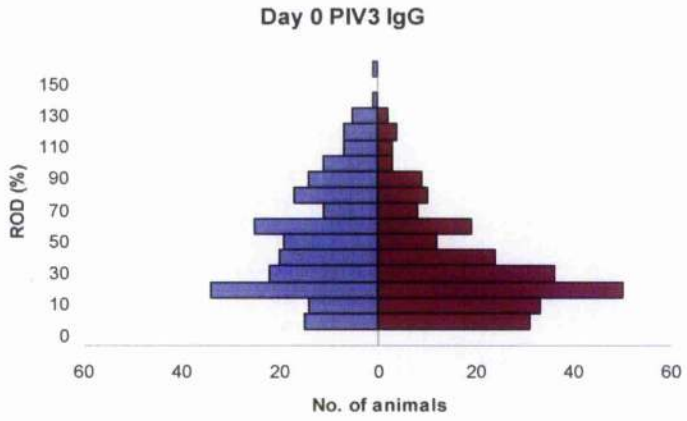


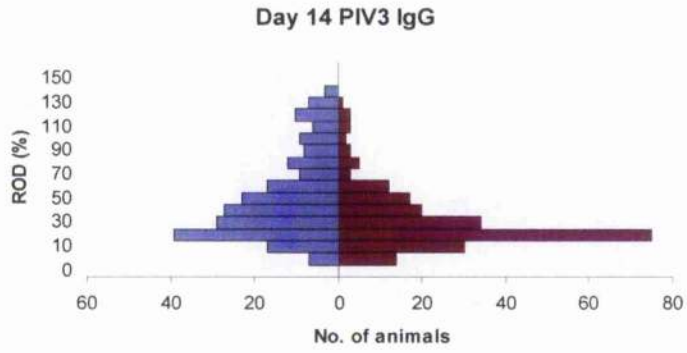


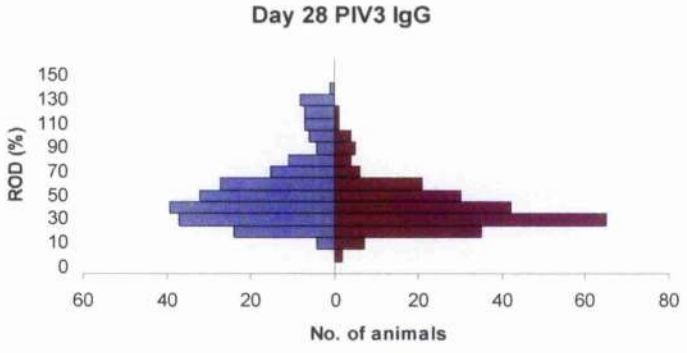
Appendix A.4 (contd) Raw data for Chapter 3. Trellis plots for levels of BRSV-IgG_2 (non-transformed) on post-vaccination Days 35 and 49, grouped on **sex**.



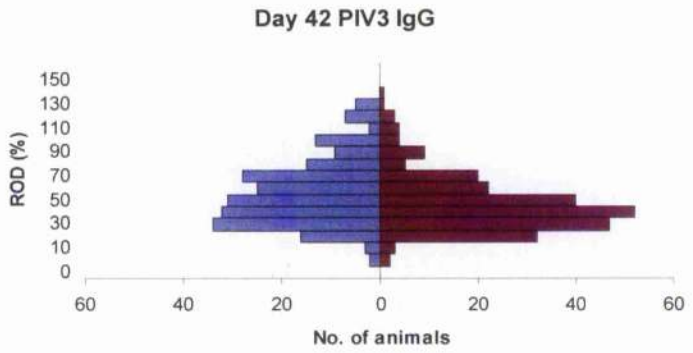
Appendix A.5 Normal order statistics for Day 35 BRSV-IgG REML model as an example. Normality, random nature and balance of residual data was verified for each REML model by examining their linearity against normal order statistics

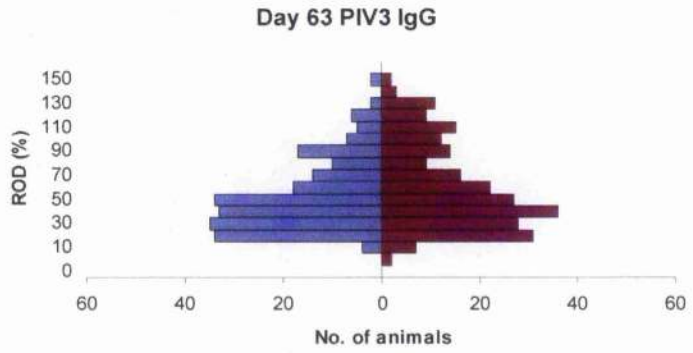


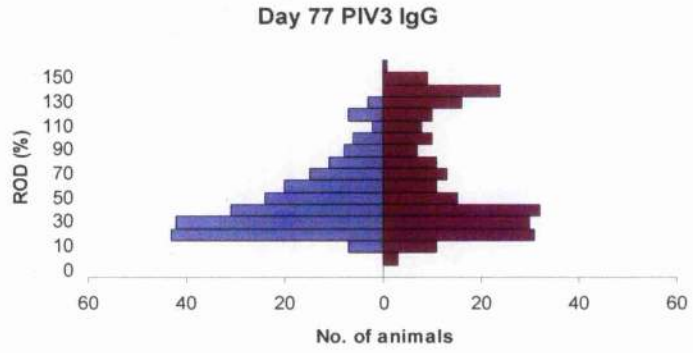




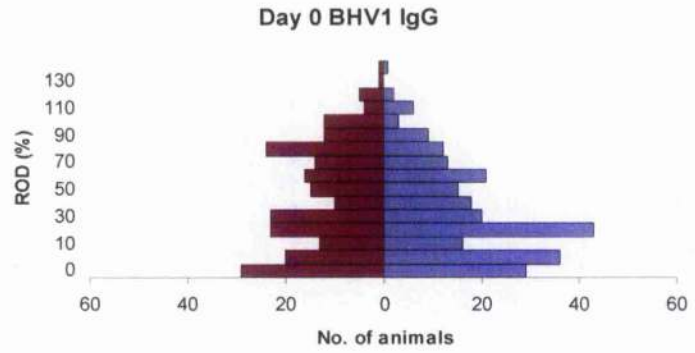
Appendix B.1 Raw data for Chapter 4. Trellis plots for levels of PIV3-IgG (non-transformed) on Days 0, 14 and 28, grouped on **sex**.

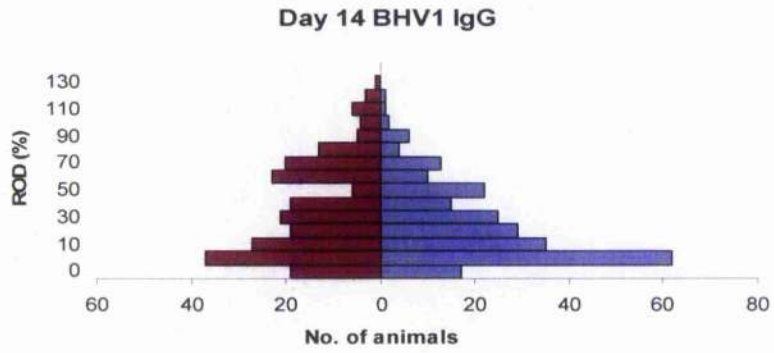


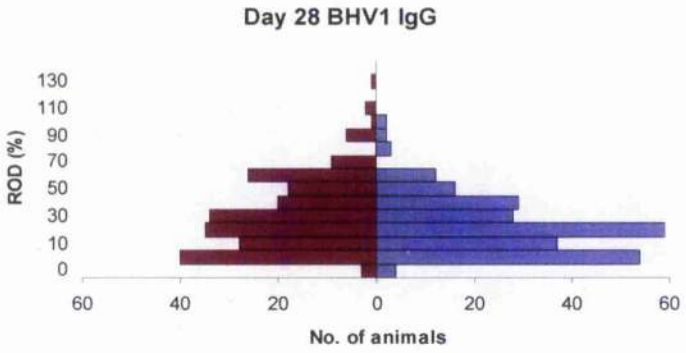




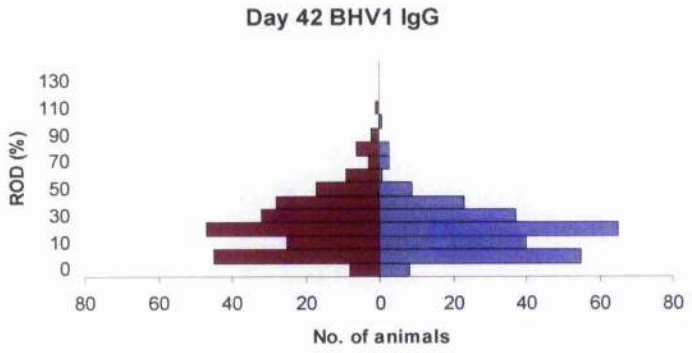
Appendix B.1 (contd) Raw data for Chapter 4. Trellis plots for levels of PIV3-IgG (non-transformed) on post-vaccination Days 42, 63 and 77, grouped on **sex**.

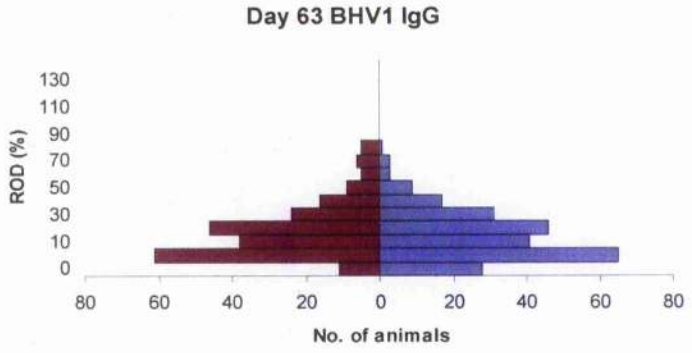


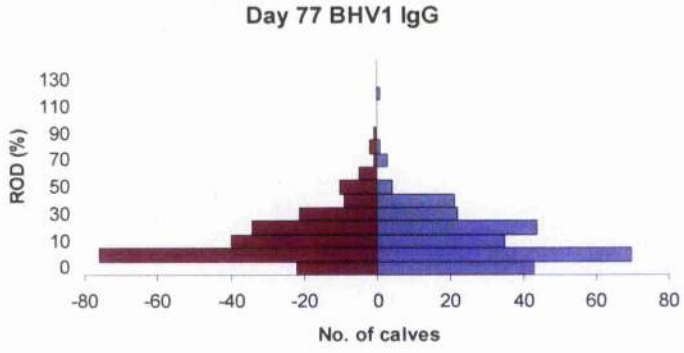




Appendix B.2 Raw data for Chapter 4. Trellis plots for levels of BHV1-IgG (non-transformed) on Days 0, 14 and 28, grouped on **sex**.







Appendix B.2 (contd) Raw data for Chapter 4. Trellis plots for levels of BHV1-IgG (non-transformed) on Days 42, 63 and 77, grouped on **sex**.

Chromosome	Genetic markers with intermarker distances (cM)														
BTA1	TGLA49	32.9	BMS4017	11.4	TGLA57	14.8	INRA128	27.2	BM864	20.1	CSSM19	20.4	BMS4044		
BTA2	TGLA431	25.3	CSSM42	20.6	BM4440	25	TGLA226	26.2	BM2113	11.6	IDVGA2				
BTA3	INRA130	29.7	ILSTS96	26.4	TGLA263	10.1	INRA123	9.7	IOBT250	11.5	HUJ1177	15.5	IDVGA35	20.1	IDVGA27
BTA4	BMS1788	39	MAF50	36.2	DIK26	0.5	IDVGA51	10.7	RM88	7.2	MGTG4B				
BTA5	BM6026	21.9	RM103	35.7	BR2936	5.7	ETH10	48.3	ETH152						
BTA6	BM1329	15.9	DIK82	31.2	CSN3	8.6	BP7	22.2	BMS739	7.3	BM2320				
BTA7	BP41	8.2	RM6	63.2	BM1853	30.7	ILSTS6	7.5	INRA53						
BTA8	IDVGA11	13.8	DIK106	35.3	HUJ174	18.8	HEL9	14	DIK74	19.8	CSSM47				
BTA9	ETH225	17.1	BM2504	19.7	UWCA9	32.5	MM12E6	6.9	INRA84						

Appendice C.1 Marker maps used for QTL analysis listed in order with inter-marker distances between adjacent markers in centiMorgans (cM) per chromosme (BTA1-BTA29).

Chromosome	Genetic markers with intermarker distances (cM)										
BTA10	BMS528	16.1	TGLA378	15.3	BM888	26	CSRM60	10.4	TGLA272	6.1	CSSM46
BTA11	BM716	16.2	INRA177	30.2	ILSTS100	21	IDVGA3	8.5	HUJV174	19.9	BMS607
BTA12	RM162	56	BM6404	27.1	INRA5	22.9	INRA209				
BTA13	HUJ616	0.2	DIK54	26.3	ABS10	17.7	DIK93				
BTA14	CSSM66	24.7	RM11	33.1	PZ271	1.7	BM4513	3.9	BM2934		
BTA15	BR3510	19.8	JAB1	14.1	BMS2684	16.7	IDVGA10	41.8	BMS429		
BTA16	BM121	16.2	TGLA53	15.9	ETH11	21.5	BM719	11.5	HUJ625		
BTA17	URB48	27.8	BMS1373	23.6	TGLA231	11.6	IDVGA40	18.7	INRA25	12.9	BM1233
BTA18	IDVGA31	18.1	ABS13	13.7	INRA121	13	HAUT14	25.7	DIK67		
BTA19	HEL10	28.6	BMS2142	21	CSSM65	15.8	ETH3				

Appendice C.1(contd) Marker maps used for QTL analysis listed in order with inter-marker distances between adjacent markers in centiMorgans (cM) per chromosme (BTA1-BTA29).

Chromosome	Genetic markers with intermarker distances (cM)						
BTA20	BM3517	31.2	TGLA126	14.7	DIK15	18.4	BM5004
BTA21	HEL5	43.3	TGLA337	18.8	IDVGA39		
BTA22	BM3406	15.8	BM3628	14.1	HAUT24	14.3	UWCA49
BTA23	IOBT528	60.8	BMS2269	3.5	BM1905		
BTA24	TGLA351	9.8	CSSM23	16	ILSTS101	18.8	INRA90
BTA25	BM4005	15.5	BM737	5	INRA222		
BTA26	ABS12	20.7	HEL11	16.6	RM26	22.7	IOBT730
BTA27	BM3507	15	RM209	49.1	BM203		
BTA28	BP23	23.2	IDVGA43	7.9	BMS2658	6.3	IDVGA8
BTA29	TGLA86	16.3	RM44	16.6	DIK94		

Appendice C.1(contd) Marker maps used for QTL analysis listed in order with inter-marker distances between adjacent markers in centiMorgans (cM) per chromosme (BTA1-BTA29).