Characterisation of physiological and immune-related biomarkers of weaning stress in beef cattle

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Declaration of authorship

I hereby declare that this thesis, submitted in candidature for the degree of Doctor of Philosophy of Biology with the National University of Ireland, has not been previously submitted for a degree to this, or any other University. I further declare that work embodied in this thesis is my own and any assistance is acknowledged.

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Lynch, E.M., McGee, M., Doyle, S. and Earley, B. (2009). Abrupt weaning alters leukocyte subsets and functional activities of granulocytes in beef calves. *J Anim Sci* 87, E-Suppl. 2, 14.

List of Abbreviations

ACD	Acid citrate dextrose
ACTH	Adrenocorticotropin hormone
ADG	Average daily gain
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Antigen presenting cell
ATP	Adenosine triphosphate
AVP	Arginine-vasopressin
βHB	β-hydroxybutyrate
Bo	Blank only
BRD	Bovine respiratory disease
BW	Body weight
ºC	Degrees Celsius
CD	Cluster of differentiation (i.e., CD4)
CD62L	L-selectin
C:D ratio	Cortisol: dehydroepiandrosterone ratio
CRF	Corticotrophin-releasing factor
CRH	Corticotrophin-releasing hormone
CNS	Central nervous system
Con A	Concanavalin
d	Day
DHEA	Dehydroepiandrosterone
dL	Decilitre

DNA	Deoxyribonucleic acid
DM	Dry matter
EDTA	Ethylenediamine tetra-acetate
ELISA	Enzyme linked immunosorbent assay
EU	European Union
FSC	Forward scatter (90° angle)
fg	Femtogram(s)
g	Gram(s)
GLDH	Glutamate dehydrogenase
G6PDH	Glucose-6-phospahte dehydrogenase
GR	Glucocorticoid receptor
GRE	Glucocorticoid regulatory element
h	Hour(s)
HCT	Haematocrit
HGB	Haemoglobin
HPA	Hypothalamic-pituitary-adrenal axis
IFN-γ	Interferon-γ
IgG	Immunoglobulin isotype G
IL	Interleukin
i.m.	Intramuscularly (injection/vaccination site)
kg	Kilogram
KLH	Keyhole limpet haemocyanin
K_3EDTA	Tripotassium ethylenediamine tetra-acetate
L	Litre(s)
LPS	Lipopolysaccharide

Lsmeans	Least squares means
Lym	Lymphocyte
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MFI	Mean fluorescence intensity
mg	Milligram(s)
MHC II	Major histocompatibility complex type II
Min	Minute(s)
mL	Millilitre(s)
mo	Months
MR	Mineralocorticoid receptor
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium test
NEFA	Non-esterifed fatty acids
Ng	Nanogram(s)
NK	Natural killer cell
N:L ratio	Neutrophil: lymphocyte
nm	Nanometer
nmol	Nanomole(s)
NSB	Non-specific binding
OD	Optical density
PBS	Phosphate buffered saline
pg	Picogram(s)

PHA	Phytohaemagglutinin
PIPES	Piperazine-N,N'-bis(ethanesulfonic acid)
pNpp	P-nitrophenyl phosphate
POMC	Pro-opiomelanocortin
ppm	Parts per million
PVN	Paraventricular nucleus
RBC	Red blood cell
rpm	Revolutions per minute
ROS	Reactive oxygen species
SSC	Side scatter (45° angle)
SAM	Sympathetic-adrenomedullary
SAS	Statistical Analysis Software
s.c.	Subcutaneously (injection/vaccination site)
s.e.	Standard error
s.e.m.	Standard error of the mean
Tc	Cytotoxic T cell
Th 1	T helper cell type 1
Th 2	T helper cell type 2
TNF	Tumour necrosis factor
TOOS	N-ethyl-N-(2hydroxy-3-sulphorpropl)m-toluidine
UK	United Kingdom
USA	United States of America
vs	Versus
v/v	Volume per volume
w/v	Weight per volume

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Abstract

Weaning is a necessary husbandry practice in which nutritional, social, physical and psychological stressors are imposed on the beef calf causing alterations in behavioural and physiological responses. To date, few studies have examined the effect of weaning on the extended physiological and immunological responses of beef calves and cows. Furthermore, no research has examined the effect of weaning stress on leukocyte immunophenotypes expressing selected differentiation antigens and the functional activity of neutrophils in calves. A series of studies were conducted to investigate i) the effect of weaning strategy (at pasture or housing) on the extended physiological and immunological responses of calves, ii) the effect of weaning and subsequent housing on physiological and immunological responses of cows and iii) the effect of weaning on leukocyte immunophenotypes and neutrophil activity of calves. Results indicate that weaning in combination with immediate housing decreased total leukocyte numbers, reduced *in vitro* production of interferon-gamma and increased concentrations of acute phase proteins compared with deferring housing for 35 days post weaning. In cows, transitory neutrophilia (increase in neutrophil number) and lymphopenia (decrease in lymphocyte number), reduced interferon-γ production, and increased concentrations of acute phase proteins were evident post weaning, whereas post-housing, changes were less marked. Weaning caused neutrophilia and impaired the functionality of these cells to traffic and efficiently phagocytise bacteria up to 7 days post weaning. Altered lymphocyte subsets and MHC Class II^+ cell percentage were evident in weaned and non-weaned calves on day 2 post-weaning, however the magnitude of change was greater in weaned calves. Thus, there is a greater transitory reduction in immune

function biomarkers in calves immediately post weaning. These immune biomarkers may be used in the future to help identify animals susceptible to weaning stress and that are more likely to succumb to respiratory infection.

Chapter 1

Introduction

Background and review of the literature

1.1. Animal Welfare

1.1.1. Introduction

The welfare of an animal refers to the state of an animal in relation to its environment, whereby, a state of health, prosperity, and well-being is achieved by its ability to cope with external stimuli (Broom, 1986). This state includes the feelings of the animal, various physiological and behavioural responses, as well as its overall health. The role of feelings in welfare was stated in the Brambell Report (1965) "*We accept that although pain, sufferning and stress are certainly not identical in animals and men, there are sound reasons for believing they are substantial in domestic animals and there is no justification for disregarding them…We accept that animals experience emotions such as rage, fear, apprehension, frustration and pleasure...*". Thus, feelings, particularly the experience of pain or suffering have always been part of the definition of welfare. This definition is probably closest to the public perception of animal welfare, and the reason that farm animal welfare first received public attention.

The evaluation of welfare may be scaled from very poor to excellent, and hence scientific assessment of welfare is possible (Broom, 1988a; 1991a; 1999; Broom and Johnson, 1993, Canali *et al.*, 2005; Laister *et al.*, 2007; Winckler *et al.*, 2007a; 2007b). In a broad philosophical, socio-ethical, economic and political context, and separate from a purely biological perspective, animal welfare is deemed an important topic (Bennett, 1996; Stafleu *et al.*, 1996; Fraser, 1999; 2008; Fraser *et al.*, 1997; McGlone, 2001; Appleby, 2005; Chilton *et al.*, 2006), one that warrants substantial attention with regard to legislation (Winter, *et al.*, 1998; Horgan and Gavinelli, 2006; Mench, 2008;

Veissier *et al.*, 2008) and the establishment of animal welfare policies (King, 2004; Botreau and Perny, 2009; Canali and Keeling, 2009; Phillips *et al.*, 2009).

In recent years, the intensification of cattle production (Swanson, 1995; Wilson *et al.*, 2002) has led to increased concern with regard to the welfare and management of farm animals by humans. It is well established that both the physical and mental well-being of domestic animals should be considered when evaluating animal welfare (Rushen, 2003; Boissy *et al.*, 2007; Mendl *et al.*, 2009). The UK Farm Animal Welfare Council (1993; 2009) in their guidelines outlined the five 'freedoms' required to cater for the basic needs of an animal. These five 'freedoms' are:

1) freedom from thirst, hunger and malnutrition
2) freedom from discomfort
3) freedom from pain, injury and disease
4) freedom to express normal behaviour
5) freedom from fear and distress

(Farm Animal Welfare Council (UK), 1993; 2009)

Defining animal welfare in terms of the five 'freedoms' has provided an all-encompassing framework for the evaluation of the welfare of an animal or group of animals in different environments. However, in intensive production systems these five 'freedoms' may not be optimally achieved as cattle contend with integral elements of these systems such as i) high stocking densities or mixing of animals which leads to heightened social pressures, ii) the inability to carry out a range of normative behaviours due to physical and psychological stress, and, iii) frequent interaction with human care-givers (Webster, 2001). Refinement to Broom's definition of welfare to incorporate the long-term consequences of an animal in its current state led Webster

(1994) to describe animal welfare as an individual animal's 'capacity to avoid suffering and sustain fitness'. In addition, Duncan and Petherick (1991) argued the importance of emotive and cognitive needs of the animal following a threat and concluded that 'animal welfare is dependent solely on the cognitive needs of the animal concerned'. The nature of the emotional response results from a series of evaluations of the triggering situation made by the individual based on criteria including novelty, predictability and controllability (Scherer, 2001). Recent work has emphasised that good welfare is not simply an absence of negative experiences rather increased occurrences of pleasurable or positive experiences (Boissy *et al.*, 2007; Christiansen and Forkman, 2007). Increased knowledge of the repertoire of emotions experienced by animals has been compiled via the examination of the relationship between the threat evaluation process and the behavioural and physiological responses in the animal (Désiré *et al.*, 2002; Veissier and Boissy, 2007; Fraser, 2009).

Despite the five 'freedoms' concept proving very useful when considering practical animal welfare issues, no definition of animal welfare is universally accepted. Definitions that do exist are based on what are considered the most important attributes of domestic animals and, thus the most important determinants of their welfare. The three main classifications are based on 1), the production capacity of an animal, 2), the physiological function of an animal and 3), the feelings of an animal. Production based definitions reflect the efficiency of an animal in terms of economically important traits, whereby, when all other things are equal, a high producing animal will have better welfare than a lower producing animal (Hewson, 2003). Function based definitions are determined by an animal's physiological state and an additional definition by Broom (1986) stated that an animal is only in a state of poor welfare when physiological systems are disturbed to the point that survival or reproduction are

impaired (McGlone, 1993). The final classification of feelings-based definitions takes into account the cognitive power of the animal as previously stated. Additional and on-going research in this area may lead to better management systems for domestic animals that favour animal welfare.

The European Union (EU) officially recognises animals as sentient beings in the sense that they are able to feel and suffer. In this context, the Community Action Plan on the Protection and Welfare of Animals, adopted by the European Commission in January 2006, embodies the Commission's commitment to EU citizens, stakeholders, the European Parliament and the Council for a clear map of the Commission's planned animal welfare initiatives for the coming years. It also responds to the principles set out by the Protocol on Protection and Welfare of animals annexed to the EC Treaty by the Amsterdam Treaty (1997).

1.1.2. General assessment of animal welfare

A variety of general animal welfare indictors are listed in Table 1.1. Some of these measures describe short-term (acute) effects, whilst others may be more relevant for long-term or chronic welfare problems. More recently, the EU funded Welfare Quality® project aimed to accommodate societal concerns and market demands, to develop reliable on-farm monitoring systems, product information systems, and practical species-specific strategies to improve animal welfare. The project efforts were focused on three main species and their products: cattle (beef and dairy), pigs, and poultry (broiler chickens and laying hens) (http://www.welfarequality.net/). After discussions with consumers and scientists, stakeholders, and policy makers, Welfare Quality® defined 4 animal welfare principles: good housing, good feeding, good health, and appropriate behaviour. Within these 4 principles, 12 animal welfare

criteria were identified. The 12 animal welfare criteria are listed in Appendix 1. The practical assessment systems measure each of these 12 criteria in dairy cattle, beef cattle, veal calves, sows, fattening pigs, laying hens and broilers.

Table 1.1. General indicators of animal welfare

Physiological indicators of pleasure
Behavioural indicators of pleasure
Extent to which strongly preferred behaviours can be shown
Variety of normal behaviours shown or suppressed
Extent to which normal physiological processes and anatomical development are possible
Extent of behavioural aversion shown
Physiological attempts to cope
Immunosuppression
Disease prevalence
Behavioural attempts to cope
Behavioural pathology
Body damage prevalence
Reduced ability to grow or breed
Reduced life expectancy

(Adapted from Broom, 2000)

1.1.3. Factors affecting animal welfare

Many factors contribute to the overall welfare of domestic farm animals. These factors may be endogenous or exogenous, directly and indirectly influencing subsequent responses in the animal and are summarised in Table 1.2. The genetic component and physical conditions of an animal are included in the endogenous factors and the exogenous factors refer to the external environmental and social factors.

Table 1.2. Endogenous and exogenous factors affecting farm animal welfare

Endogenous		Exogenous	
Genetic or physical conditions	Social conditions	Physical environment	Social environment
Breed	Competition	Temperature	Housing
Sex	Aggression	Humidity	Feeding
Age	Leadership	Wind	Age
Weight	Dominance	Photoperiod	Stocking rate/
Temperament	behaviour		space allowance

(Grandin, 2000; Moberg and Mench, 2000)

1.1.4. Areas of concern for animal welfare

Over the last century, our understanding of cattle physiology and behaviour has improved. Through our increased knowledge of the complex regulatory processes, elaborate social structure and sophisticated learning ability of cattle, it has been necessary to re-evaluate the effects of conditions and procedures at farm level, both in terms of efficiency of production and animal welfare. General areas of concern that affect animal welfare and consequently production include:

1) ill-treatment of animals referring principally to physical abuse,
2) neglect which may be calculated, accidental or due to lack of knowledge,
3) inadequacies in design of accommodation/housing – insufficient space, unsuitable floor type, poor feed and/or water access,
4) inadequate management systems or poor husbandry practices,
5) unnecessary or poorly executed mutilations (e.g. tail-docking, disbudding, dehorning, castration) of the animals,
6) poor conditions or procedures during transport, at market or at slaughter.

(Broom, 1991a; 2000)

Animal husbandry practices that are potentially stressful and increase the incidence of reduced productivity, injury or disease, include; handling (Grandin, 1997), castration (Earley and Crowe, 2002; Ting *et al.,* 2003; Pang *et al.,* 2008), weaning (Hickey *et al.,* 2003a); inadequate housing (Fisher *et al.,* 1997; Gupta *et al.,* 2005), mixing of animals (Bøe and Færevik, 2003; Gupta *et al.,* 2007) and transportation (Knowles, 1999; Buckham Sporer *et al.,* 2007a). Although, a reduction in productivity and/or increased mortality are suggestive of poor animal welfare, a greater evaluation of animal welfare in terms of scientific evidence may be achieved using an depth investigation of the behavioural, physiological, immunological and metabolic functions in the animal to external stimuli.

Ultimately, the welfare of an animal hinges on its capacity to cope or adapt to its environment and the biological cost associated with this adaptation and maintenance of this response (Broom, 1998b; Moberg and Mench, 2000). A very closely associated concept to animal welfare and one that is often seen as an opposing concept is, that of stress. This juxtaposition originates from the fact that good welfare cannot be achieved under stress and that the absence of stress is necessary to ensure satisfactory animal welfare. Both concepts share many common features. Firstly, each has been described in terms of physiological and behavioural responses that largely overlap and, secondly, the concepts are dependent on how an animal perceives its environment. There is an assumption that animals possess some degree of cognitive ability and therefore, the nature of the response of an animal, whether it is used to assess the stress response or state of welfare, depends on the animal's cognitive evaluation of its environment.

1.2. Stress

1.2.1. Concept of Stress

Stress is a broad and complex concept that is difficult to concisely define. Prior to, and throughout, the twentieth century, this definition received much attention from the scientific community with numerous attempts made to develop more, increasingly appropriate definitions, and thus, many new concepts relating to stress emerged. The Greek philosopher, Hippocrates, may have been the first to describe the term stress with respect to balance and disharmony. He conceived this balance as an essential state of health and that perturbation to this internal balance would manifest as disease (Chrousos *et al.*, 1988). In 1878, Claude Bernard recognised that the internal environment ('le milieu intérieur') was much more stable than the ever-changing external environment surrounding an organism and that the maintenance of this stability was essential for free and independent life. Improvements to this description were pioneered by the work of Walter B. Cannon (1914), with the expansion of this concept to provide a mechanism of achieving this steady state via physiological regulation and modulation, a process which he termed 'homeostasis' (Cannon, 1914, 1929). This concept stated that all sorts of physiological variables are kept at an optimal level or within a narrow range by the actions of the homeostatic control mechanisms. Cannon's classical studies paved the way for future work by Seyle, who provided the first comprehensive biological theory of stress called 'The General Adaptation Syndrome' (Seyle, 1946). This theory consists of three universal stages of coping with a external stimulus or threat, i) an initial 'alarm reaction', analogous to Cannon's 'fight or flight' response, ii) a stage of adaptation which was associated with resistance to an external stimulus or threat, and finally, iii) an exhaustion stage

culminating in death of the organism if the adverse stimuli was not removed. Furthermore, the General Adaptation Syndrome postulates that the physiological response to every stimulus is relatively uniform and is defined as a 'non-specific response of the body to any demand made upon it' (Seyle, 1973).

The concept of allostasis was put forward by Sterling and Eyer (1988) to refer to the active process by which the animal responds to daily events and maintains homeostasis. Allostasis means achieving stability through changes, whereby these changes prepare the animal to better cope with further challenges or external stimuli and this concept is not intended to replace that of homeostasis (McEwen, 2008) but to help explain the long-term negative effects of chronic stress. If an animal experiences an external stimulus that they have encountered and controlled with relative ease in the past, the response will be measured accordingly; habituation will ameliorate the physiological perturbation and the biological cost will be minimal. The response will be mediated via the sympathetic-adrenomedullary (SAM) axis and the hypothalamo-pituitary-adrenocortical (HPA) axis, and if the stimulus is mild or short-lived, homeostatic mechanisms will modulate the perturbations within optimal ranges until the parameter(s) have returned to stable set points (Moberg and Mench, 2000). Both the HPA and SAM axes are discussed in greater detail later (Section 1.3.3.). Some circumstances, however, require that several interacting systems (e.g. neuroendocrine, metabolic, immune, and cardiovascular systems) vary their homeostatic set points to ensure their complex interactions and activities are not dysregulated (McEwen and Wingfield, 2003). In short, the new state is established through a panel of readjusted set points in counter-balancing systems. Numerous mediators, through a network of non-linear regulation, modulate the activities of the interacting systems required for allostasis (Figure 1.1). Overstimulation or dysregulated allostatic systems result in the

phenomenon known as allostatic load or overload (McEwen, 1998), which can lead to disease in instances such as when the response is not terminated after it is required, when an inadequate response was initiated at the onset of the stimulus or when progressive habituation to the stimulus is not achieved (Korte *et al.*, 2005; McEwen, 2008).

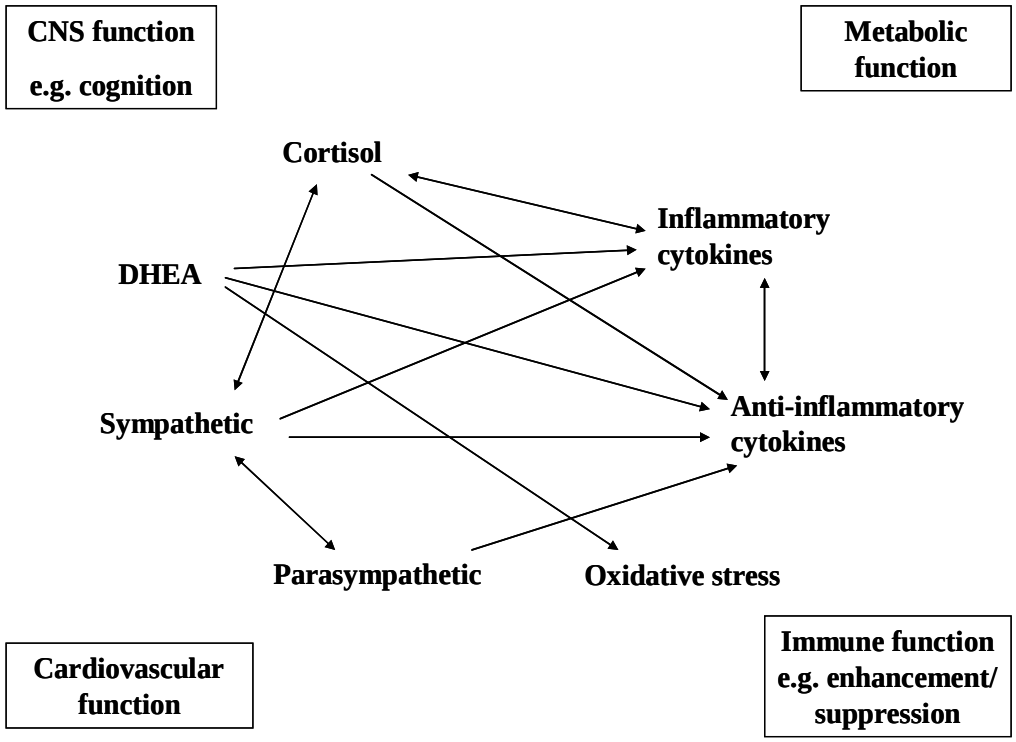

Figure 1.1. Non-linear networks of mediators of allostasis involved in the stress response.

The complexity of regulation of this response is illustrated by the multiple pathways e.g. inflammatory cytokine production is negatively regulated by anti-inflammatory cytokine production and by the parasympathetic and glucocorticoid pathways also. Arrows indicate that each system regulates the other. Bidirectional regulation indicated by double headed arrows. Dehydroepiandrosterone (DHEA) (Adapted from McEwen, 2008).

1.2.2. Definition of stress and stressors in animals

In a veterinary context, the term stress in farm animals is characterised by abnormal or extreme adjustment in the physiology of the animal to cope with adverse changes in its environment and management (Fraser *et al.*, 1975). Taken together with the other definitions of stress outlined above, the author summarises that stress in farm animals involves a disruption to homeostasis by an external stimuli or stressor that may consequently have negative effects on an animal's ability to grow and reproduce. A stressor can be any factor, endogenous or exogenous in nature, that is capable of provoking an altered state of homeostasis and may be classified as psychological, physical, or environmental, but usually contain a combination of all three classifications (Chrousos, 2000; 2009; Moberg and Mench, 2000).

1.3. Biological response of animals to stress

1.3.1. Introduction

In domestic animal research, with particular emphasis on livestock (cattle) research, it is not appropriate to categorise stressors as entirely physical or psychological. Many stressors commonly encountered by cattle combine both these elements, thus they both need to be considered as an integrated response when evaluating animal welfare. Extremes in temperature, feed and/or water deprivation, electric shock, surgery, pain, and disease are examples of physical stressors that may be encountered by cattle (Dantzer and Morméde, 1983; Grandin, 1998). Examples of psychological stressors include fear, novelty to routine and/ or environment, mixing with unfamiliar peers, restraint, confinement, isolation, presence of a perceived predator, and novel human interaction (Grandin, 1997, 1998).

Within the central nervous system (CNS), two distinct systems, the SAM and HPA axes, link the initial perception of the stressor to the subsequent stress response. These axes orchestrate the release of neurotransmitters and pituitary and adrenal hormones required to regulate the physiological, immunological, metabolic, and behavioural responses to the stressor (Chrousos, 2000).

1.3.2. Model of animal stress

A comprehensive biological model (Figure 1.2) outlining the general stages involved in the stress response in animals has been developed and redefined by Moberg (Moberg, 1985; 1996; Moberg and Mench, 2000), and is divided into three general stages:

1) recognition (perception) of a stressor,
2) biological defence against the stressor, and,
3) consequence of the stress response.

Recognition of a stressor occurs within the CNS, which organises an adaptive defence encompassing behavioural, physiological (autonomic, metabolic, neuroendocrine) and immunological responses used by the animal in its attempts to cope with the stressor. The final stage of this response or the consequence, determines whether an individual is suffering from distress/poor welfare or merely experiencing a brief episode that will have no significant impact on its welfare. Failure to regain homeostasis results in altered biological function that can lead to a pre-pathological state such as immunosuppression or metabolic disorder or, ultimately, pathology and/or death (Moberg and Mench, 2000).

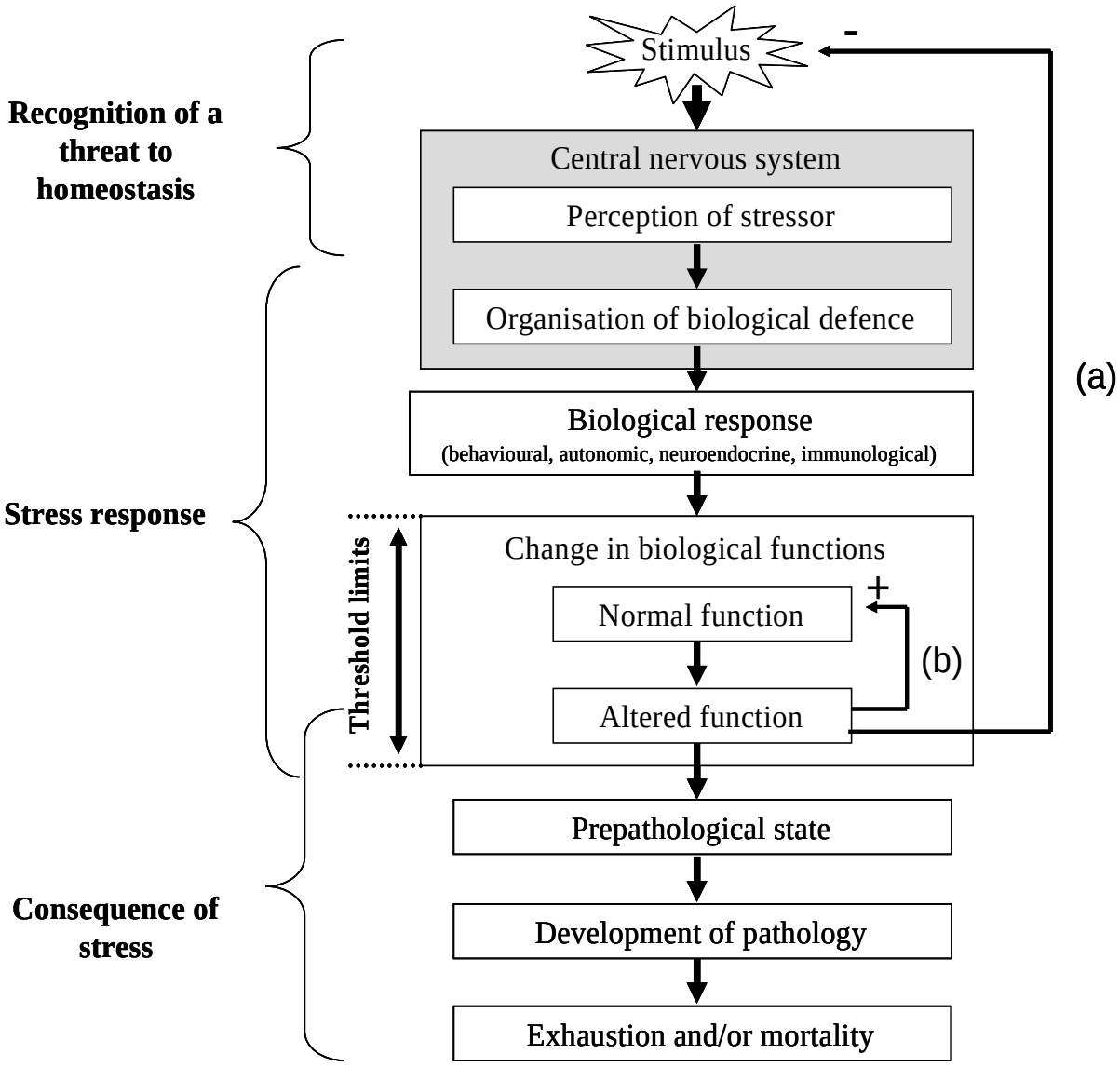

Figure 1.2. A model of the biological response of animals to stress.

After perception of a stimulus as a threat, (a) changes in biological function are required to cope with the threat and so to reduce it negative effects. Normal biological function may be restored (recovery) if the animal has successfully regained homeostasis following a stress (b). Otherwise, a pre-pathological state may ensue, which may progress to pathology. When a severe imbalance between homeostatic mechanisms and the coping capacity of the animal occurs, exhaustion and mortality are inevitable. (Adapted from Moberg and Mench, 2000; Ting *et al.*, 2004).

1.3.3. General organisation of the stress system

For didactic purposes, neuroendocrine responses evoked by stressors are often collectively referred to as the products of the 'stress system', and include many interacting neural and hormonal mediators that integrate these systems. At the core of the system are the SAM and HPA axes, linking neural and neuroendocrine activities (Charmandari *et al.,* 2005).

1.3.3.1. Sympathetic-adrenomedullary (SAM) axis

The 'fight or flight' response described by Cannon (1929) is controlled by the SAM axis (Cannon, 1935; Griffin, 1989). Cognitive stimuli are processed by the limbic system within the cerebrum in the brain, producing neurological impulses that cause hypothalamic stimulation of the autonomic nervous system. This triggers the associated production of sympathetic neurotransmitters, adrenaline, and noradrenaline, known also as epinephrine and norepinephrine, respectively. Release of these catecholamines is extremely rapid, within seconds (Sapolsky *et al.,* 2000), and occurs by discrete pathways, i) directly, by the release of noradrenaline from the sympathetic nerve endings, resulting in greater mental acuity, and ii) indirectly, by the release of adrenaline from the medullae of the adrenal glands, resulting in the changes in metabolism and activities of cardiovascular and respiratory systems to increase cardiac output, facilitate oxygenation of blood and to raise plasma glucose concentration and deliver enriched blood selectively to tissues and/or organs upon which the stressors are making the greatest demands. Accordingly, there is active mobilisation of energy during the stress response, as evidenced by the elevated glucose concentrations in circulation. These activities are mediated through ligand binding to two forms of adrenergic receptors, α and β (both of which are classic seven transmembrane domain

G protein-coupled receptors) on target cells such as dendritic cells, lymphocytes, and macrophages. Although adrenaline and noradrenaline are associated with increased alertness, adrenaline secretion is also more generally associated with fear and anxiety (Axelrod and Reisine, 1984; Fell *et al.*, 1985; Charney, 2003; Goldstein, 2003), whereas noradrenaline release is associated with, and may be an indicator of, physical stress (Leblanc *et al.*, 1980) and active avoidance or attack (Goldstein, 2003, Neumeister *et al.*, 2005) in humans. Similar effects have been evidenced in cattle following freeze- and hot-iron branding (Lay *et al.*, 1992). In addition to the aforementioned functions, catecholamines substantially influence the HPA axis and the overall response to a stressor by regulating hormone release, namely corticotrophin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, adrenocorticotropin hormone (ACTH) from the anterior pituitary and cortisol from the adrenal cortex (Axelrod and Reisine, 1984).

1.3.3.2. Hypothalamic-pituitary-adrenocortical (HPA) axis

Inability by the SAM axis to resolve a stressful event stimulates the activation of the HPA axis, to restore homeostasis. Via neuronal signals and inputs, the cerebral cortex in the brain perceives an event as a stressor stimulating the release of CRH and to a lesser extent, arginine-vasopressin (AVP) from PVN of the hypothalamus (Johnson *et al.*, 1992). Both CRH and AVP are transported via the hypophyseal portal circulation from the axon terminal in the capillary bed of the median eminence to the anterior pituitary where they synergistically stimulate the synthesis and secretion of ACTH from the precursor molecule, pro-opiomelanocortin (POMC), into the systemic circulation (Ulrich-Lai and Herman, 2009). The ACTH regulator, CRH, is historically referred to as corticotrophin-releasing factor (CRF) in accordance to the conventional

nomenclature at the time of discovery (Saffran and Schally, 1955), though the use of CRF is still prevalent in today's scientific vocabulary. Increased concentrations of ACTH in peripheral circulation stimulate the outer adrenal cortex of the adrenal gland to synthesis and secrete steroid hormones, namely glucocorticoids (Chrousos, 2000; Tsigos and Chrousos, 2002). Peak glucocorticoid levels occur within ten minutes and one hour after the initiation of the stress response (Sapolosky *et al.*, 2000; Droste *et al.*, 2008).

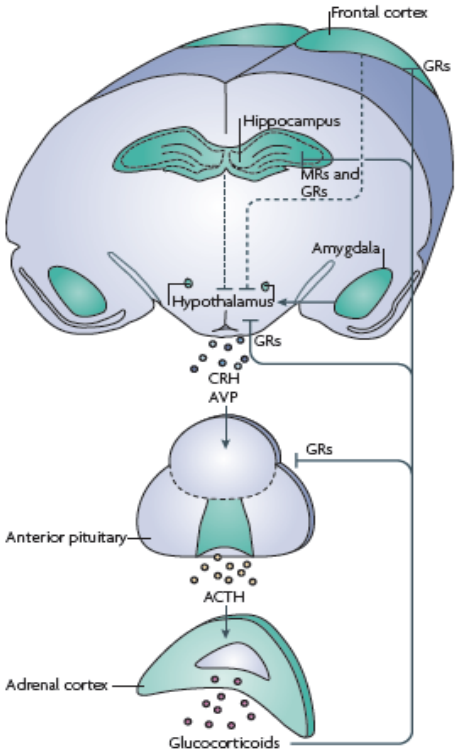

Figure 1.3. A schematic representation of HPA architecture and activation. Components of the autonomic, neuroendocrine, metabolic and immune systems orchestrate a coordinated physiological response upon perception of a threat by the brain. Hypophysiotrophic neurons in the PVN of the hypothalamus secrete CRH and AVP. These hormones act on the anterior pituitary promoting the secretion of ACTH, which in turn stimulate the release of glucocorticoids from the adrenal cortex. (Adapted from Lupin *et al.*, 2009).

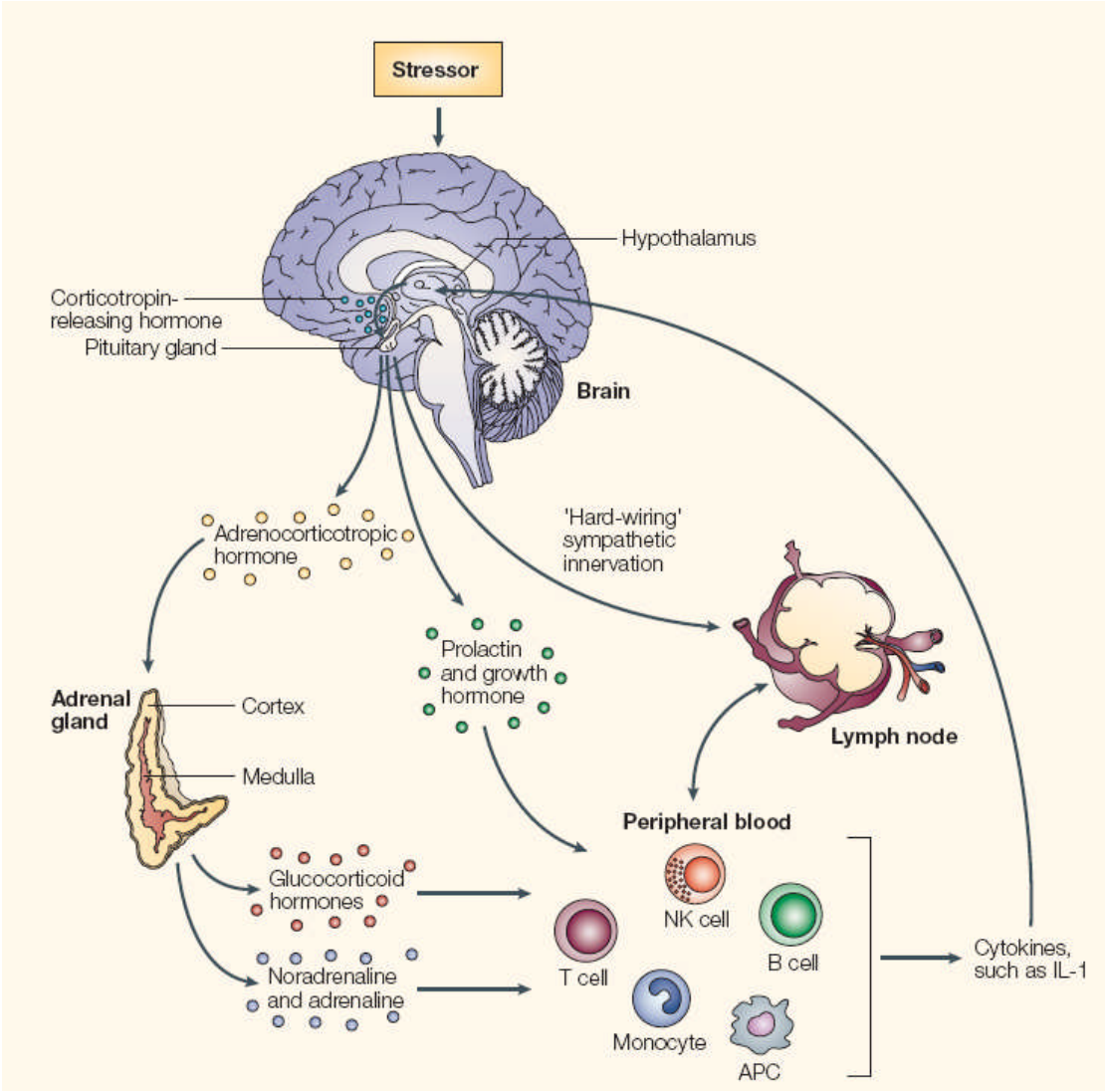

Figure 1.4. Stress associated modulation of the hormone response by CNS.

Stress is perceived in the brain, stimulating activation of HPA and SAM axes. Production of ACTH by the pituitary gland results in the production of glucocorticoids. The SAM axis is activated by stimulation of adrenal medulla producing catecholamines, adrenaline and noradrenaline, and by sympathethic-nervous system innervation of lymphoid organs. Binding of glucocorticoids and catecholamines to glucocorticoid and adrenergic receptors on leukocytes modulate the distribution and function of these cells in the periphery. The interactions are bidirectional in that cytokines produced by immune cells modulate the activity of the hypothalamus (Adapted from Glaser and Kiecolt-Glaser, 2005).

1.3.4. Glucocorticoids

1.3.4.1. Action of glucocorticoids

In cattle, the main active glucocorticoid is cortisol, a cholesterol-derived steroid (Morméde *et al.,* 2007), superseding corticosterone in concentration at ratios of 1.5, 2.8 and 4.0 in Guernsey, Holstein and Jersey cattle, respectively (Venkataseshu and Estergreen, 1970). Glucocorticoids elicit a plethora of biological functions within the body including metabolism of carbohydrates (gluconeogenesis) (McMahon *et al.,* 1988) and proteins (Raynaert *et al.,* 1976; Umpleby and Russell-Jones, 1996; Vegiopoulos and Herzig, 2007), alterations in growth (Elsasser *et al.,* 1997; Sartin *et al.,* 1998), reproductive axes (Davenport *et al.,* 1989; Tempel and Leibowitz, 1994), cardiovascular output (Yang and Zhang, 2004; Brotman *et al.,* 2007), regulation of the stress response (Munck *et al.,* 1984; Minton, 1994), overall immune function (Blecha, 2000; Carroll and Forsberg, 2007; Webster Marketon and Glaser, 2008), and maintenance of homeostasis (von Borell, 2001).

1.3.4.2. Molecular modes of action

As final effectors of the HPA axis, glucocorticoids participate in the overall control of homeostasis, playing key roles in the basal activity of the HPA axis, and in the magnitude and termination of the stress response (Keller-Wood and Dallman, 1984; Tsigos and Chrousos, 2002; Tasker *et al.,* 2006). Stress and non-stress activities of glucocorticoids are regulated through two types of glucocorticoid receptors; a high affinity mineralocorticoid receptor (MR) and a low affinity glucocorticoid receptor (GR). Glucocorticoid affinity for MR is ten times greater than GR (De Kloet *et al.,* 1998; Kino and Chrousos, 2001) and these receptors are present on numerous target

cells widely distributed throughout tissues and in circulation (Munck *et al.*, 1984; Bamberger *et al.*, 1996; Sapolsky *et al.*, 2000). At basal glucocorticoid levels, such as those at the circadian nadir of cortisol secretion, MR predominantly binds to glucocorticoids, thus non-stress fluctuations (permissive effects) are mediated by MR. However, at high levels of endogenous glucocorticoids, MR occupancy is saturated and GR becomes extensively bound, as in the case of a major stress response (Reul and de Kloet, 1985; Sapolsky *et al.*, 2000). Upon ligand binding, GR, acting directly as a transcription factor or indirectly by associating with other transcription factors (Newton, 2000), translocates to the nucleus of the cell, and interacts with the glucocorticoid regulatory elements (GRE) within the promoter regions of glucocorticoid regulated stress-responsive genes, thereby activating or repressing their transcription (Pratt, 1990; Bamberger *et al.*, 1996; Falkenstein *et al.*, 2000). The action of GR is also mediated by membrane-bound receptors coupled to downstream G protein-dependent signalling cascades (Tasker, 2006; Tasker *et al.*, 2006). This mode of GR action is postulated as the rapid mechanism of negative feedback-inhibition of the HPA axis as it occurs within minutes of the rise of circulating glucocorticoids, much quicker than that possible via genomic regulation (De Kloet *et al.*, 1998; Tasker, 2006; Ulrich-Lai and Herman, 2009).

1.3.4.3. Termination of the stress response

The HPA axis is an excellent example of a negative feedback system in which the end product (i.e. cortisol) inhibits the initiating substance (i.e. CRH) (Manteuffel, 2002). Termination of the stress response operates through three independent mechanisms, i) a rate sensitive fast feedback, ii) an intermediate feedback and iii) a delayed feedback (Vázquez, 1998). A rate sensitive rise in glucocorticoids inhibits

CRH and ACTH secretion from the hypothalamus and anterior pituitary, respectively. This feedback mechanism is rapid, occurring within minutes of the elevated steroid response. Immediate feedback is progressively slower than the rate sensitive feedback mechanism while delayed feedback occurs at a transcriptional level and thus over the course of hours. The glucocorticoid-receptor (GR) complexes translocate to the nucleus and down-regulate the expression of key molecule and hormone regulatory genes involved in the HPA axis via transrepression (Vázquez, 1998; Sapolsky *et al.*, 2000). Inhibition of CRH/AVP and ACTH secretion limits the duration of the total tissue and target cell exposure to glucocorticoids, thus minimising the catabolic, lipogenic, anti-reproductive, and immunosuppressive effects of these hormones. Dysregulation of negative feedback mechanisms, which result in elevated levels of glucocorticoids, are implicated in immunosuppression and consequently numerous disorders (Chrousos, 2000). The feedback mechanism within the HPA axis is illustrated in Figure 1.4.

1.3.4.4. Bovine specific aspects

Under normal resting conditions, approximately 80 % of cortisol is bound by corticosterone binding protein, 10 % by albumin with the remaining 10 % unbound (Gayrard *et al.*, 1996). Typically, basal plasma cortisol concentration is lower than 10 ng/ml (Venkataseshu and Estergreen, 1970; Thun *et al.*, 1981) and secretion is pulsatile, with a periodicity of approximately ninety minutes (Thun *et al.*, 1981) that varies as the animal matures sexually (Verkerk and MacMillan, 1997; Earley and Crowe, 2002, Ting *et al.*, 2003; Pang *et al.*, 2006). However, Lefcourt *et al.* (1993), using an intensive sampling schedule of every fifteen minutes for forty-eight hours, showed that cortisol secretion in dairy cattle exhibited weak circadian rhythms but

strong ultradian rhythms. The apparent presence of circadian rhythms reported in some studies may be attributable to the management of the experimental animals. In high-yielding dairy cows, peak plasma cortisol concentrations were found after milking, particularly in the morning (Blum *et al.*, 1985).

1.4. Stress and immune function

1.4.1. Introduction

A prompt, efficient, and regulated immune response is required for an animal to cope with stressful environments and/or stimuli and to alleviate the increased susceptibility to disease associated with stress in domestic animals (Blecha *et al.*, 1984; Griffin, 1989). Stress has complex effects on the immune system, influencing both innate and adaptive immunity. These components are often divided into distinct categories, however, the roles and functions are intrinsically linked and there is a complex interplay of communication between the innate and adaptive immune response. Innate immunity refers to the non-specific mechanism that serves as the first line of defence against pathogens, occurring rapidly upon their appearance in the body. Immune cells such as phagocytes (neutrophils, monocytes and macrophages), cells that secrete inflammatory peptides (eosinophils, basophils, and mast cells), NK cells and elements of the complement system, acute phase proteins, and cytokines are the major mediators of the innate response and many serve as a link between the innate and adaptive arms of the overall system (MacKay and Rosen, 2000a; 2000b; Janeway *et al.*, 2005). Adaptive or acquired immunity refers to the antigen-specific response that develops over time and is more complex than the innate system alone. Specialised antigen-presenting cells (APCs) such as dendritic cells and macrophages display

antigen to naïve lymphocytes thereby initiating a cell-mediated or humoral response. More specifically, antigens recognised by antigen-specific receptors on T and B cells drive target effector responses resulting in cell priming, activation, and differentiation and subsequently, the homing of the activated T cell to the site of infection and the release of antibody from activated B cells (plasma cells) (Parkin and Cohen, 2001). Activated T cells secrete autocrine, endocrine, and paracrine messengers (cytokines) that alter the behaviour of the activated T cell and/or other leukocytes thus modulating the immune response against specific antigens or stress stimuli (Elenkov and Chrousos, 1999, Lippolis, 2008).

1.4.2. Stress-associated immunomodulation

Stress has traditionally been associated with impaired functioning of an animal's immune system (Kelley, 1980; Dantzer and Morméde, 1983; Broom, 1991b) which is mediated to a large extent by glucocorticoids and catecholamines following activation of the HPA and SAM axes (Griffin, 1989; Elenkov *et al.*, 2000) and their subsequent anti-inflammatory activities (Schimmer and Parker, 1996). However, the plethora of immune system activities that are influenced by glucocorticoids and catecholamines suggest that stress induced elevation in these steroids may also be, in some circumstances such as short-term acute stress, immuno-enhancing (Ashwell *et al.*, 2000; Elenkov and Chrousos, 2002; Sorrells and Sapolsky, 2007; Sorrells *et al.*, 2009), confirming the assumptions put forward by Golub and Gershwin (1985) that this may also be the case in domestic animals. Such immuno-enhancing activities include the promotion of cytokine gene expression and secretion, stimulation of immune cell proliferation and differentiation, and regulation of effector cell function (Ashwell *et al.*, 2000; Elenkov and Chrousos, 2002; Sternberg, 2006). An extensive

body of research has examined the detrimental effects of stress on both the innate and adaptive arms of the immune system and includes changes in leukocyte subset populations and kinetics, decreased mitogen proliferation, decreased cytokine production, decreased antibody production and reactivation of latent viral infections (Fleshner *et al.,* 1989; Bohus *et al.,* 1993; De Groot *et al.,* 1999; Kelley, 2004; Webster Marketon and Glaser, 2008). Synthetic analogs of adrenal glucocorticoids, such as dexamethasone, have been used for many years as potent anti-inflammatory compounds in both humans and domestic animals and many studies have employed these compounds to examine the immunosuppressive properties of glucocorticoids, *in vivo* and *in vitro* (Cupps and Fauci, 1982). Thus, dysregulation by either excessive or inadequate activation of the immune system following stress plays a crucial role in an animal's susceptibility to disease and overall health and welfare.

Immune cells are very sensitive to elevations in circulating glucocorticoids as they all possess cytosolic GRs (Griffin, 1989) although the density of the receptors and their affinity of glucocorticoids do not always correlate to the sensitivity of the cell (Parrillo and Fauci, 1979). Elevations in endogenous or exogenous glucocorticoids cause a dramatic shift in the haematological profile of peripheral circulation in cattle resulting in leukocytosis which is marked by neutrophilia and lymphopenia (Roth, 1985). There may also be a reduction in the number of monocytes, eosinophils, and basophils in circulation (Burton *et al.,* 2005). These changes reflect the alterations in cell trafficking induced by glucocorticoids (Roth, 1985). The direct and indirect effects of glucocorticoids on some immune cell populations are briefly illustrated in Figure 1.5 and Table 1.3.

There is good evidence to indicate that the process of differentiation from uncommitted Th cell to a mature Th1 or Th2 cell is highly plastic. Many factors

influence the decision to become a Th1 or Th2 cell. The cytokines IL-12 and IL-4, acting through signal transducer and activator of transcription 4 (STAT4) and STAT6, respectively, are key determinants of the outcome (Liberman, 2007). Antigen dose, co-stimulators, genetic modifiers and other non-cytokine factors also have crucial roles in determining the dominance of a Th-cell response. How each signal influences the differentiation process is an area of active investigation and, often, lively debate. In situations of stress, glucocorticoid acting through GR in antigen-presenting cells (APCs), directly suppress transcription of the main inducer of Th1 responses, IL-12 (Figure 1.5). Since IL-12 is extremely potent in enhancing IFN-γ and inhibiting IL-4 synthesis by T cells, the inhibition of IL-12 production may represent a major mechanism by which glucocorticoids affect the Th1/Th2 balance that is, by enhancing humoral (Th2) and suppressing cell mediated (Th1) immunity (Elenkov *et al.*, 2004).

Cytokines play a major role in the bi-directional communication between the immune system and the neuroendocrine system. Upon activation of the immune system, released cytokines activate the HPA axis and increase peripheral glucocorticoids, and in a feedback mechanism glucocorticoids inhibit the synthesis and secretion of cytokines. This feedback system prevents the over-activation of the immune system which would otherwise be destructive. In general, glucocorticoids inhibit pro-inflammatory cytokine synthesis and promote the synthesis of those that have immunosuppressive potential (Wiegers *et al.*, 2005). It is this bias against pro-inflammatory cytokines that may be responsible for the shift towards a Th2 response following stress. The Th1/Th2 imbalance may be due to the inhibition of IL-12 production and responsiveness by glucocorticoids and catecholamines and by the increased IL-10 production by catecholamines (Elenkov, 2002). Thus, through their effects of Th1 and Th2 cytokine secretion, glucocorticoids and catecholamines

working in concert may cause a suppression of cell-mediated immunity and cause a shift towards Th2-mediated humoral immunity (Elenkov and Chrousos, 1999) which has been associated with immunosuppression (Padgett and Glaser, 2003; Viveros-Paredes *et al.*, 2006). The anti-inflammatory and immunosuppressive effects are mediated by toll-like receptor signalling pathways (Moynagh, 2003).

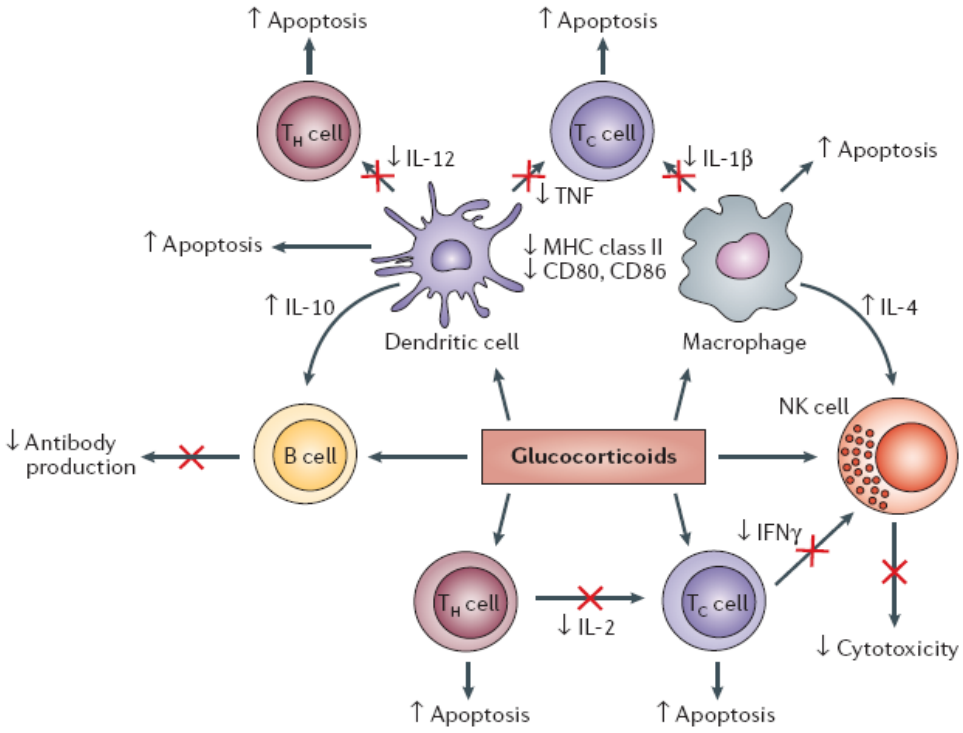

Figure 1.5. Effects of glucocorticoids on immune cell populations.

Glucocorticoids inhibit the production of plethora of cytokines, including but not limited to IL-4, IL-5, IL-6, IL-12, INF-γ, and TNF-α. In general, glucocorticoids inhibit pro-inflammatory cytokine synthesis (IL-1β, IL-6, and TNF- α) and induce those that have anti-inflammatory potential (IL-10) by macrophages and dendritic cells. Inhibition of immune response is also mediated through the pro-apoptotic properties of glucocorticoids on macrophages, dendritic cells and some T cells. (Adapted from Sternberg, 2006; Liberman *et al.*, 2007).

Table 1.3. Glucocorticoid effects on blood cells

Cell type	Effect
Neutrophils	↑ Number of circulating cells ↓ Chemotaxis (decreased IL-1, IL-8, and leukotriene B4) ↓ Endothelial adherence (CD62L surface expression)
Lymphocytes	↓ Number of circulating cells (redistribution effects) ↓ Proliferation / activation (by initiating IL-2 and other cytokines) ↓ NK cell activity Lympholysis induced through apoptosis
Monocytes	↓ Circulating numbers altered ↓ Secretion of IL-1, TNF-α, and monocyte chemotactic factors ↓ Synthesis of collagenase, elastase, and tissue plasminogen activator ↓ Expression of MHC class II molecules and Fc receptors ↓ Synthesis of pro-inflammatory cytokines (IL-2, IL-6, TNF-α)
Eosinophils	↓ Number of circulating cells ↓ Survival (↓ release of endothelial colony stimulating factors) ↓ Endothelial adherence (by IL-1 inhibition)
Basophils	↓ Number of circulating cells ↓ Histamine and leukotriene release ↓ Mast cell expansion inhibited ↓ Endothelial adherence

↑ = Increase / induces, ↓ = Decrease / inhibits. (Adapted from O'Connor *et al.*, 2000; Buttgereit *et al.*, 2005; Stahn *et al.*, 2007)

1.4.3. Acute phase protein response

1.4.3.1. Introduction

Glucocorticoids are also necessary perquisites for the induction of the acute phase protein (APP) response (Wilckens, 1995). The production of a variety of proteins and proteases during the inflammatory response is considered part of the complex defence mechanisms to re-establish homeostasis. The APPs are a group of blood proteins that change in concentration in animals subjected to internal and external challenges, such as infection, inflammation, trauma, and stress (Baumann and Gauldie, 1994; Murata *et al.*, 2004). This response is considered as a component of

the innate immune response involved in the re-establishment of homeostasis and restraint of microbial growth, before animals develop acquired immunity to the challenge. As such, the circulating concentrate of these proteins is directly related to the severity of challenge and the extent of the damage to tissue in the animal, and thus, their quantification can provide prognostic and diagnostic information (Gruys *et al.*, 1994; Godson *et al.*, 1996; Peterson *et al.*, 2004; Gruys *et al.*, 2005; Ganheim *et al.*, 2007; Nikunen *et al.*, 2007). Acute phase proteins are induced primarily in the liver but can also be produced in non-hepatic sites including the mammary glands, intestinal epithelium and the lung (Murata *et al.*, 2004). Often, they are described as positive (up-regulated) or negative (down-regulated) acute phase proteins in response to the challenge. The former have important roles in the inflammatory response, whereas the latter are important carrier proteins such as albumin, corticosteroid binding protein, and transferrin (metal-binding protein). Examples of positive APPs include haptoglobin, fibrinogen, serum amyloid A, C-reactive protein, and α-1-glycoprotein (Gruys *et al.*, 1994).

The APP response is typically characterised by systemic inflammatory signs such as fever, altered synthesis of liver products to APPs, increase peripheral total leukocyte numbers, reduction in iron and zinc in serum, increased gluconeogenesis, altered behaviours including; increased lethargy, decreased feed and water intake, and decreased social and sexual behaviours (Moshage, 1997; Hirvonen *et al.*, 1999). Induction of the response is stimulated by the release of pro-inflammatory cytokines, namely IL-1, IL-6 and TNF-α (Alsemgeest *et al.*, 1996; Gabay and Kushner, 1999), primarily from macrophages but also from other immune cells in response to external and internal stimuli (Yoshioka *et al.*, 2002). The acute phase response is regulated by a series of feedback mechanisms which control the further release of cytokines. These

processes are summarised in Figure 1.6. Non-inflammatory, psychophysical stress can also induce discernible APP responses in healthy animals (Murata and Miyamoto, 1993; Arthington *et al.*, 2003; Pineiro *et al.*, 2007). The modulatory effects of endogenous pro-inflammatory cytokines and nutrient metabolism and animal growth have been well established (Johnson, 1997; Spurlock, 1997; Gabler and Spurlock, 2008; Lippolis, 2008).

1.4.3.2. Haptoglobin

Haptoglobin, produced by Kupffer cells of the liver, is an α_2-globulin constituent that binds free haemoglobin preventing the loss of iron (Eckersall, 2000; Wagener *et al.*, 2001) and reducing the oxidative damage associated with haemolysis (Yang *et al.*, 2003). Numerous immunomodulatory effects can be attributed to haptoglobin, mediated by the binding to CD11/CD18 receptors on effector cells (El-Ghmati *et al.*, 1996), including inhibition of granulocyte chemotaxis, phagocytosis, and bactericidal activity (Rossbacher *et al.*, 1999). Additionally, haptoglobin may inhibit mast cell proliferation (El-Ghmati *et al.*, 1996), prevent spontaneous maturation of epidermal Langerhans cells (Xie *et al.*, 2000) or suppress T-cell proliferation (Murata and Miyamoto, 1993; Arredouani *et al.*, 2003). In healthy cattle, the plasma concentration of haptoglobin is negligible, with dramatic increases reported following infections and stress (Horadagoda *et al.*, 1999; Chan *et al.*, 2004).

1.4.3.3. Fibrinogen

Fibrinogen is necessary for homesotasis and tissue repair, as it provides a substrate for fibrin formation and a matrix for adherence of migrating inflammatory cells (Thomas, 2006). Fibrinogen specifically binds to CD11/CD18 integrins on cell

surface of migrated phagocytes, triggering a cascade of intracellular signals that lead to enhancement of degranulation, phagocytosis, antibody-dependent cellular cytotoxicity and delayed apoptosis (Sitrin *et al.*, 1998; Rubel *et al.*, 2001). The normal range for fibrinogen is 300-700 mg/dL in adult cattle (Jones and Allison, 2007).

1.4.3.4. Other acute phase proteins

Serum amyloid A (SAA) is an acute phase apolipoprotein associated with high density lipoprotein in plasma (Eckersall, 2000) and has been used to identify inflammation in cattle (Horadagoda *et al.*, 1999). Some of the roles of SAA include; inhibition of T cell adhesion to extracellular matrix proteins (Urieli-Shoval *et al.*, 2000), chemotactic recruitment of inflammatory cells to sites of infection (Xu *et al.*, 1995) and down-regulation of the inflammatory process by inhibiting myeloperoxidase release and directed migration of phagocytes (Gatt *et al.*, 1998).

Ceruloplasmin is a major copper carrying molecule in blood that oxidises toxic ferrous iron to non-toxic ferric form (Patel *et al.*, 2002), and is predominately anti-inflammatory by reducing the number of neutrophils attaching to the endothelium and by acting as a peroxide scavenger (Broadly and Hoover, 1989; Segelmark *et al.*, 1997). Alpha-1-glycoprotein is a sialo-glycoprotein secreted mainly by hepatocytes, however, extracellular gene expression has been confirmed (Fournier *et al.*, 2001). Anti-inflammatory actions include increases in IL-1 receptor antagonists by macrophages and inhibition of neutrophil activation and respiratory burst activity (Rinaldi *et al.*, 2008).

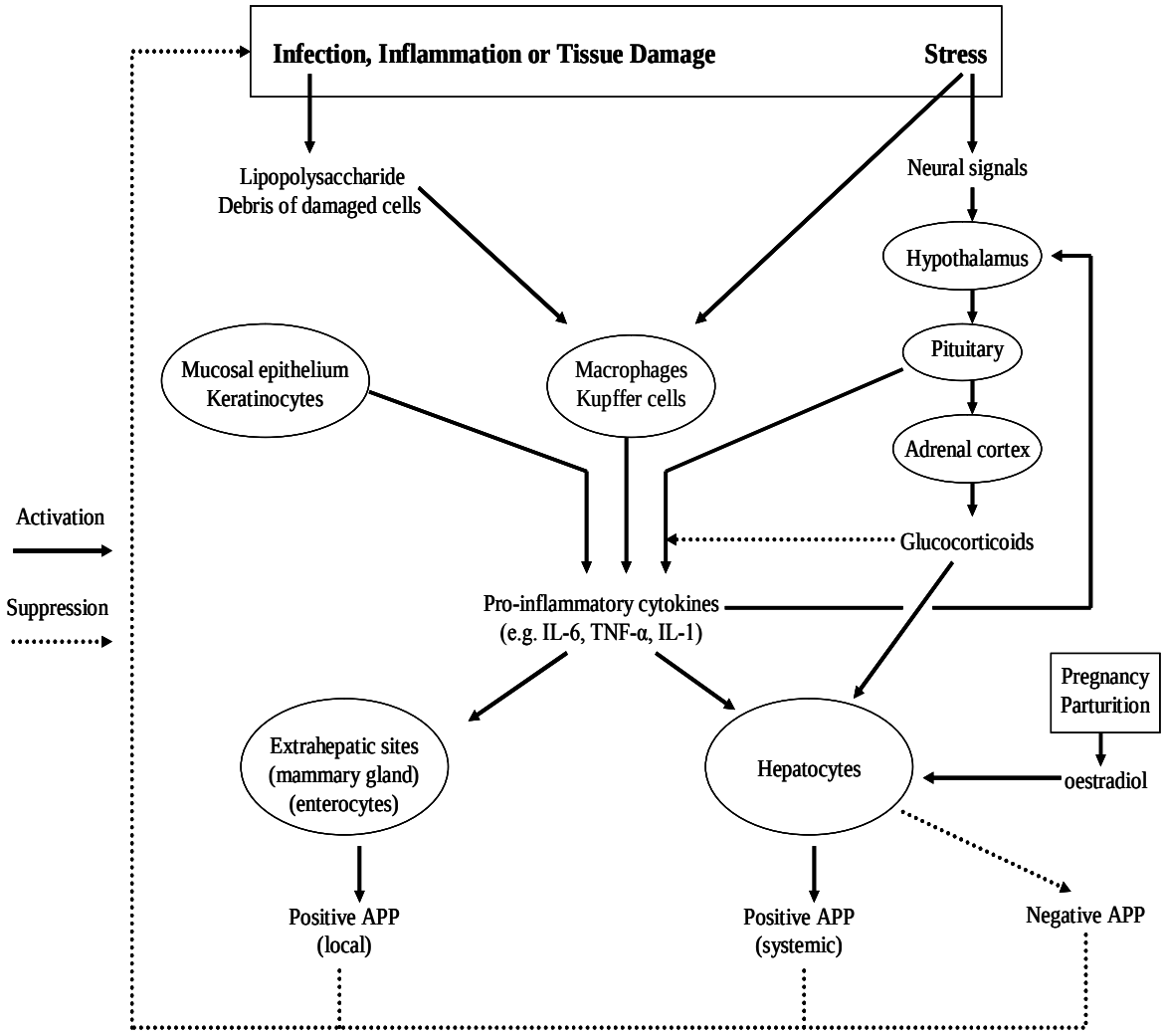

Figure 1.6. Induction and regulation network of acute phase protein (APP) synthesis in animals subjected to internal and external challenge.

Signals originating from stimuli (stress) are transmitted to the hypothalamus in the brain, activating neuroendocrine centres (HPA and SAM axes), which leads to release of catecholamines and glucocorticoids that, through the induction of pro-inflammatory cytokines by macrophages and lymphocytes, promote the production of APP in hepatocytes, thereby augmenting peripheral APP levels in stressed animals. Feedback mechanism of glucocorticoids within the HPA axis regulates the production of excessive pro-inflammatory cytokines and consequently the acute phase proteins (Murata *et al.*, 2004).

1.4.4. Other mediators of stress response affecting immunocompetence

Although glucocorticoid and catecholamines are very important mediators of the stress response, they are not the only players in this complex response, and moreover, they are not the only means by which stressors influence immunocompetence (Griffin, 1989; Biondi and Zannino, 1997, Yang and Glaser, 2000). Other hormones and neurotransmitters produced when animals are trying to cope with stimuli perceived as threats include β-endorphins, vasopressin, somatostatin, oxytocin, prolactin, growth hormones and releasing hormones, serotonin, dopamine and urocortins. These individual neurotransmitters, neuropeptides and steroid mediators have both specific spatial and temporal niches, but these niches overlap, affording opportunities for these mediators to interact and orchestrate the animal's ability to respond and adapt to stressors (Joëls and Baram, 2009). In terms of immunocompetence, β-endorphins can promote T-cell responses, whereas vasopressin and oxytocin stimulate T helper cells to produce IFN-γ (Moynihan *et al.* 2000), activating NK cells and macrophages (Griffin, 1989; Yang and Glaser, 2000; Salzet *et al.*, 2005; Tseng *et al.*, 2005).

1.4.5. Stress, immune function and nutrition

During periods of stress, a percentage of available nutrients from caloric intake are diverted away from processes such as growth and muscle deposition and have a role in stabilising other physiological processes (i.e. those required for survival, e.g. an immune response) (Elsasser *et al.*, 1997). Furthermore, intake and appetite are suppressed during stressful events (Haddad *et al.*, 2002) with intake of nutrients by stressed livestock often compromised and below that normally consumed by unstressed animals (Duff and Galyean, 2007). The metabolism of a given tissue in

terms of maintenance, accretion, or catabolism is diverted by signals from the immune and endocrine system as well as nutritional signals (Figure 1.7). Partitioning occurs according to priority, such that basal metabolic needs supersede tissue accretion, as per a polarised scheme of use whereby neural utilisation > visceral > bone > muscle > adipose (Touchberry, 1984; Husband, 1995). During stress, metabolism and caloric demands are often increased, however, nutrient intake may be low with deficiencies being made up by catabolic processes that metabolise muscle proteins and fat stores (Husband and Bryden, 1996). Severity of the compromised metabolism and subsequent diversion of nutrients are proportional to the severity of the stress in livestock (Elsasser *et al.,* 1997). Nutrition appears to affect immune function most dramatically through the regulation of cytokine production and expression (Cunningham-Rundles, 2002; Colditz, 2002; 2004; Elsasser *et al.*, 2008).

Various nutritional strategies that enhance the functioning of the immune system at different stages of production of livestock have been evaluated (Galyean *et al.*, 1999; Goff, 2006; Duff and Galyean, 2007; Carroll and Forsberg, 2007). Vitamins, minerals and other supplements (Nockels, 1996; Spears, 2000; Grimble, 2001; Farran *et al.*, 2008) have been shown to have immunomodulatory effects in livestock (Blecha, 1988; Galyean *et al.*, 1999) and humans (Calder and Kew, 2002; Romeo *et al.*, 2008; Calder and Yaqoob, 2009). A comprehensive review of the effects of supplementation on the nutrition- neuroendocrine- immune system interaction is beyond the scope of this literature review and therefore the main supplements with greatest potential of enhancing immune function in beef cattle are summarised in Appendix Table A.1. Important considerations for further work must address the current mineral or vitamin status and the physiological status of the animal at the time of supplementation, the concentration and bioavailability of the supplement,

and the type of biomarker used to investigate the immunological effects. It is most likely that many of the aforementioned issues have contributed to the conflicting data arisen to date.

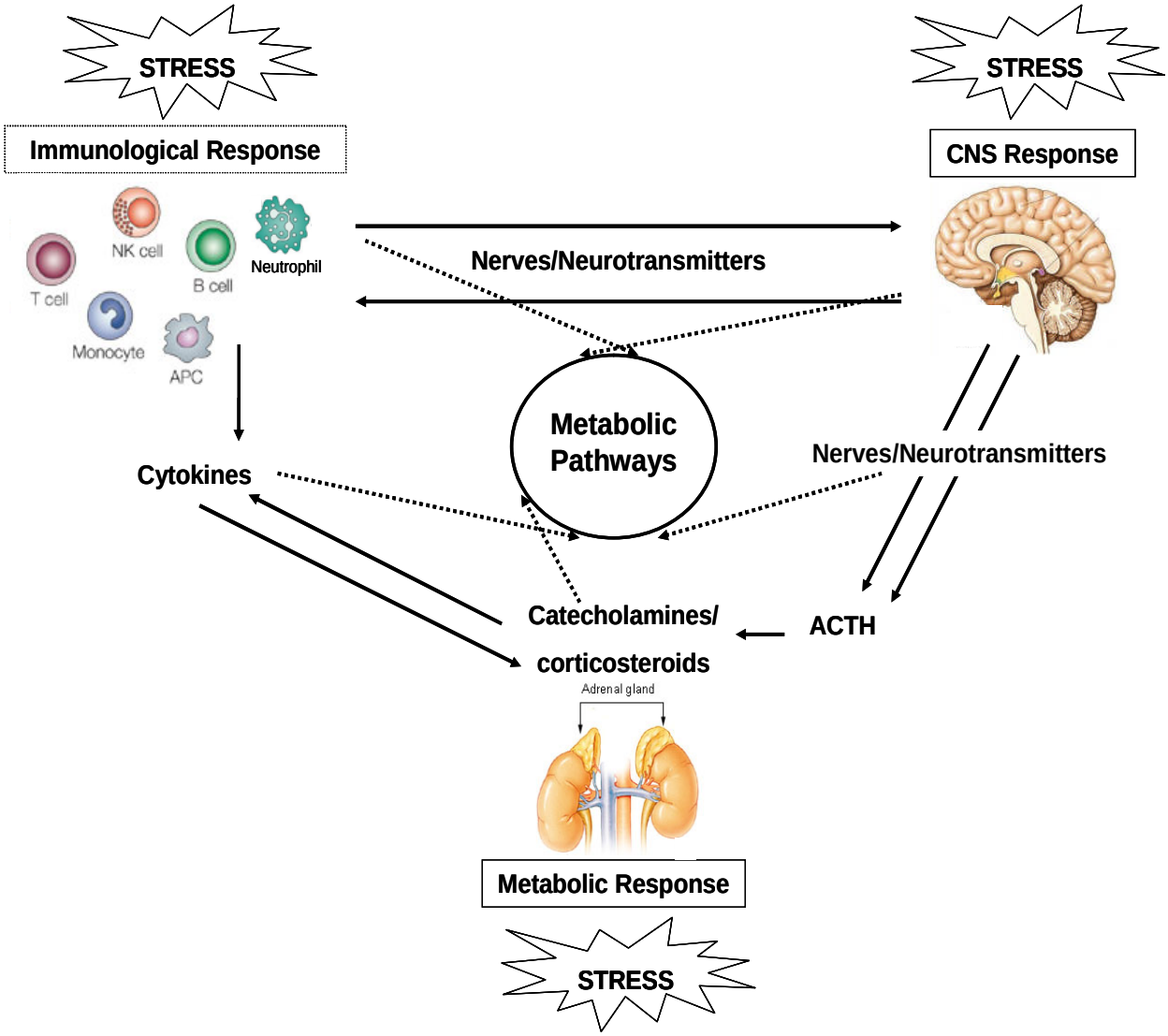

Figure 1.7. Communicative architecture between the endocrine, immune system and nutrition axes.

Perceived stress can impact on any single effector or multiple effectors culminating in changes in the interactions between these regulatory elements and consequently altered metabolism of cells (Modified from Bell *et al.*, 1987; Husband and Bryden, 1996).

1.4.6. Incidence of stress associated infection and disease

Stress-associated infection and disease is not a recent finding in domestic animals. From as early as 1878, Pasteur demonstrated that thermal (cold) stress increased the mortality of chickens infected with anthrax compared with chickens that were also infected with anthrax but not subjected to thermal stress (as cited by Kelley, 1985). As previously mentioned, the anti-inflammatory and immunosuppressive actions of glucocorticoids are primarily implicated as causative factors in exacerbation of infection and disease pathogenesis (Roth, 1985; Burton *et al.*, 2005). Examples of this include increased incidence and severity of bovine respiratory disease (BRD), coccidiosis, fatal bovine viral diarrhoea (BVD), and decreased resistance to parasitic infections (Filion *et al.*, 1984; Roth, 1985). The stress associated with weaning has been linked to immunosuppression (Griffin, 1989). Sharp rises in mortality rates have been documented in newly weaned cattle entering feedlots, resulting in greater losses through reduced performance and treatment costs (Hartland *et al.*, 1991). The higher rate of mortality is generally attributable to increased incidences of fibrinous pneumonia or 'shipping fever' (Hartland *et al.*, 1991) and the disease has been more commonly associated with transported cattle (Yates, 1982). The causative pathogens of BRD are numerous and are listed in Table 1.4 (Bowland and Shewen, 2000; Ellis, 2001). Ultimately, primary viral and/or mycoplasma infections compromise immune defences of the host, thus facilitating secondary infection by opportunistic bacteria (Lekeux, 1995).

Table 1.4. Etiological agents of bovine respiratory disease (BRD) complex

Viruses	Bacteria	Mycoplasma
Infectious bovine rhinotracheitis virus (IBRV) or bovine herpesvirus type 1 (BHV-1)	*Mannheimia (Pastuerella) haemolytica*[1]	*Mycoplasma bovis*
Bovine respiratory syncytial virus (BRSV)	*Pasteurella multocida*	*Mycoplasma dispar*
Bovine adenovirus	*Haemophilus somnus*	*Ureaplasma diversum*
Bovine parainfluenza virus type 3 (PI-3V)	*Actinomyces pyogenes*	*Mycoplasma hyorhinis*
Bovine viral diarrhea virus (BVDV)	*Streptococcus pneumoniae*	
Respiratory bovine cornonavirus (RBCV)	*Staphylococcus aureus*	

[1]*Mannheimia haemolytica* replaced *Pasteurella haemoltica* in 2000 (Angen *et al.*, 1999)

Many factors centred on the time of weaning may heighten the susceptibility of cattle to BRD or may exacerbate its outcome in cattle (Hodgson *et al.*, 2005; Aich *et al.*, 2009a). Conversely, numerous factors, such as preconditioning, may alleviate the negative effects on immunity and susceptibility to disease. The pre-weaning and post-weaning factors affecting BRD are illustrated in Figure 1.8.

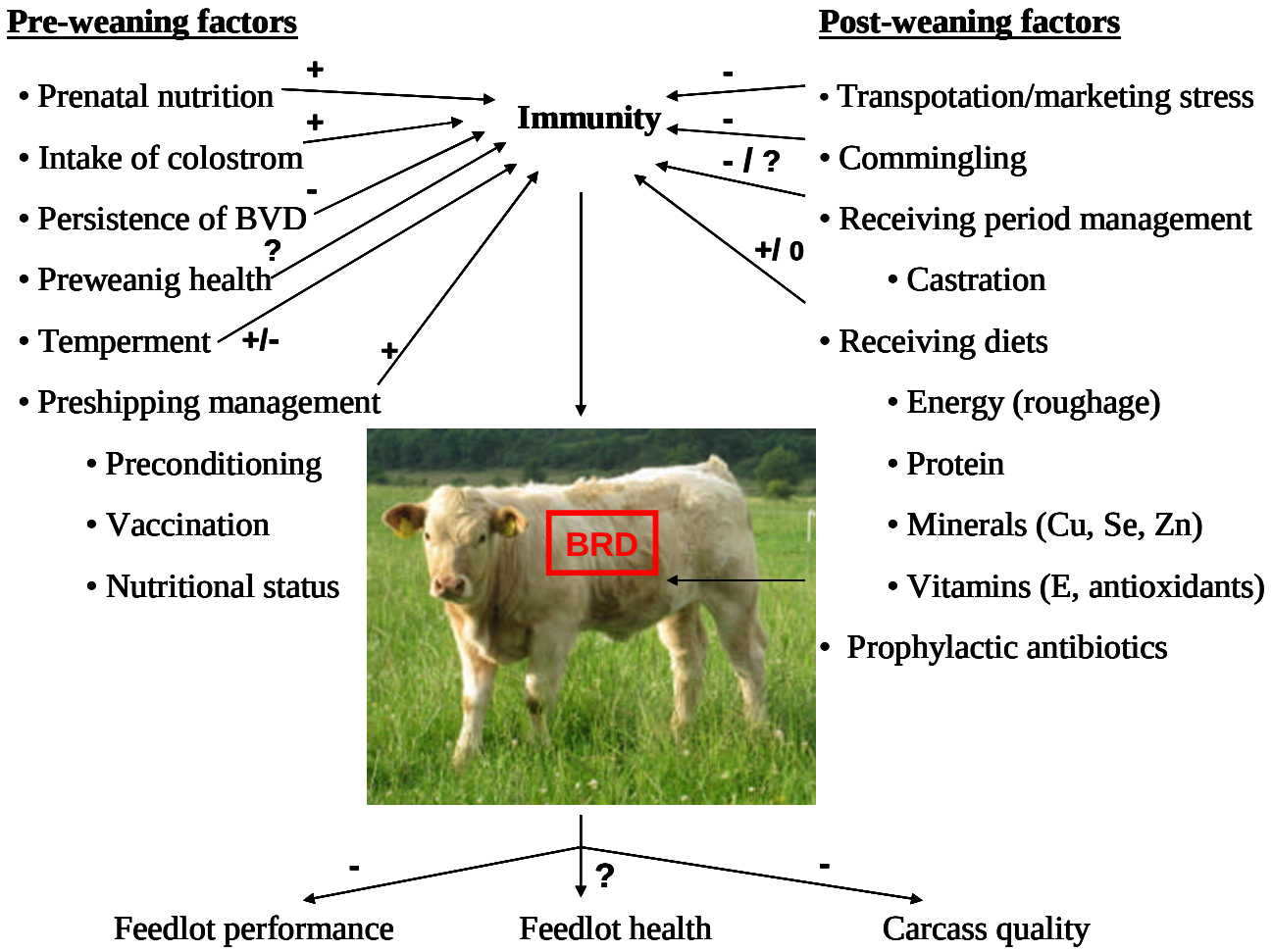

Figure 1.8. Pre- and post-weaning factors affecting bovine respiratory disease (BRD) in beef calves and resulting outcomes of the disease.

Stresses due to weaning, marketing, and transportation, previous planes of nutrition, genetics and health history interact with the exposure to viral, mycoplasmal, and bacterial agents and affect the overall immunity of cattle. + = positively affects immunity thus, ameliorating the incidences or consequences of BRD; - = negatively affects immunity thus, exacerbating the incidences or consequences of BRD; ? = effects are not fully understood based on available literature. BVD = bovine viral diarrhoea virus. (Source: Duff and Galyean, 2007).

1.5. Biomarkers/indicators of stress

1.5.1. Introduction

A biomarker is a measurable indicator of a specific biological state, particularly one relevant to the risk of contraction, and the presence or the stage of disease (Rifai *et al.*, 2006). Biomarkers can be used clinically to screen for, diagnose or monitor the activity of disease, and to guide molecularly targeted therapy or assess therapeutic responses (Biomarkers Definitions Working Group 2001; Etzioni *et al.*, 2003).

1.5.2. Characteristics and validation of biomarkers

The key characteristics of a biomarker depend upon its intended use (Aronson, 2005; La Baer, 2005). Firstly, it must be accurate, sensitive, and specific. It should be altered in or by the relevant process (in the case of this thesis: the stress response) and should be easy to discriminate between affected and non-affected (i.e. control) populations. Furthermore, it should also be possible to reliably quantify and reproducibly detectable. Another important factor for consideration is the accessibility of the biomarker; ideally it should be easily obtained from accessible body fluids such as blood plasma or serum, urine, saliva or sweat, or from other sources such as faeces (Morrow *et al.*, 2002), breath (Burciaga-Robles *et al.*, 2009), or hair (Queyras and Carosi, 2004).

Once identified, a biomarker must be rigorously evaluated to demonstrate that it will provide an acceptable measure of a biological process or pathological state in an animal. The species, sex, breed and age of the animal should be defined as such variations may contribute to marked differences in composition of body fluids and generalised responses. In addition, the reproductive status of the animals, diurnal

variations of hormones, and diet should be taken into account. Thus, implementation of standardised protocols for sample collection (including animal handling), processing, and storage are necessary in order to obtain reliable, reproducible data and to eliminate erroneous results due to confounding external factors (Moore *et al.*, 2007).

Biomarkers are particularly relevant in medical and veterinary biomedical research where they have an important role in the characterisation of human and animal disease (Hein and Greibel, 2003; Kues and Niemann, 2004; Rowan *et al.*, 2007; Aich *et al.*, 2009b). Within a veterinary setting, the optimisation of farm animal health and welfare is of major importance to agriculture. The discovery of novel biomarkers or biomarker profiles for animal health and welfare has the potential to further enhance clinical care and management of domestic animals. Conventional analyses target a selection of biochemical and molecular biomarkers that are related to, or, associated with a specific disease state, however some have poor prognostic and diagnostic specificity and are not characteristic across species, breed, sex and stage of animal maturity for the clinical state in question. Therefore, the identification and characterisation of novel, specific biomarkers and biomarker profiles of patho-physiological states will be an important step towards the management of infection in animals and the improvement of animal welfare.

1.5.3. Commonly used biomarkers of stress in cattle

1.5.3.1. Introduction

Traditionally, scientific assessment of stress in cattle has centred on the measurement of HPA axis activation and behavioural responses. The following sections of this chapter will deal with the most commonly used biomarkers of stress in

livestock animals and will outline the altered biological processes evident following stress in cattle. A more detailed review of the specific effects of weaning stress on the routinely used biomarkers of stress in beef calves is provided in Section 1.6.

1.5.3.2. Endocrine measures

Endocrine activity is routinely evaluated as a biomarker or indicator of the stress response in cattle (Möstl and Palme, 2002; Bristlow and Holmes, 2007; Morméde *et al.*, 2007). Activation of the HPA axis and resultant increase in circulating glucocorticoids, of which cortisol is the predominant steroid in cattle (Section 1.3.4.4.), has been measured in numerous studies including those examining the effects of lameness (Almeida *et al.*, 2007), transportation (Blecha *et al.*, 1984; Earley and O'Riordan, 2006; Gupta *et al.*, 2007; Yagi *et al.*, 2004), branding (Lay *et al.*, 1992); castration (Pang *et al.*, 2006), weaning (Hickey *et al.*, 2003a; Aich *et al.*, 2007; Blanco *et al.*, 2009), regrouping and relocation (Gupta *et al.*, 2007), isolation (Boissy and Le Neindre, 1997), restraint (reviewed by Grandin (1997)) and transfer from pasture to housing environment with tethering (Higashiyama *et al.*, 2007) in cattle.

Recently, the cortisol precursor, dehydroepiandrosterone (DHEA) has received some interest in the literature as a conjunct biomarker to cortisol (Hu *et al.*, 2000; Wolkowitz *et al.*, 2001; Carroll *et al.*, 2006; Newman *et al.*, 2008 Maringer *et al.*, 2010). In the steroid hormone pathway, 17α-hydroxy-pregnenalone can be converted to either DHEA, which is readily converted to DHEA-S, or to 17α-hydroxyprogesterone, which is converted to 11-deoxycortisol and subsequently, to cortisol (Fritsche and Steinhart, 1998; Schwartz, 2002). This androgen is implicated in the stress response (Zinder and Dar, 1999), and with its metabolites, plays a role in

immune system activation, counteracting the immunosuppressive actions of cortisol (Loria *et al.*, 1996; Svec and Porter, 1998; Saccò *et al.*, 2002; Bauer, 2005). The anti-inflammatory activities of DHEA are postulated to be mediated via their antioxidant properties, which prevent the activation of the pleiotropic transcription factor, NF-κB (Iwasaki *et al.*, 2004; Aragno *et al.*, 2006). Decreased concentrations of serum DHEA and DHEA-S coupled with stable and increased concentrations of cortisol have been reported in humans and animal studies (Straub *et al.*, 2002; Marx *et al.*, 2003; Butcher *et al.*, 2005). Buckham-Sporer *et al.* (2008) reported a profound increase in cortisol: DHEA ratio in young beef bulls that were subjected to 9 h of truck transportation. By 24 h post-transportation, cortisol:DHEA ratio had returned to pre-transport levels. As a marker of inflammation, this ratio has been used to describe the effects of inflammatory diseases such as lameness in dairy cows and surgical castration in piglets. Cows suffering with inflammatory foot lesions showed a 23 % decrease in serum DHEA concentrations compared with healthy cows (Almeida *et al.*, 2007). Decreased concentrations of DHEA-S coupled with subsequent rise in concentration of cortisol in surgically castrated piglets compared with increased concentration of cortisol and no change in DHEA-S in non-castrated piglets suggested that surgical castration was more stressful than handling alone as the precursor was sequestered from the steroid pathway at the expense of DHEA, in order to increase glucocorticoid production (Carroll *et al.*, 2006). A comprehensive study examining the pattern of DHEA and DHEA-S secretion and their response to ACTH administration in dairy cows concluded that DHEA secretion in cows is episodic and variable among animals, and not affected by ACTH stimulation (Marinelli *et al.*, 2007).

Additionally, activation of the sympathetic adrenomedullary axis in response to stress in cattle has been examined by measuring the concentration of circulating

adrenaline and noradrenaline (Minton, 1994; Lefcourt and Elsasser, 1995; Hickey *et al.*, 2003a). Increased endogenous concentrations of these hormones are associated with acute stress in livestock (Griffin, 1989). Plasma adrenaline was found to increase at the onset of transportation stress in calves (Odore *et al.*, 2004) and in goats (Nwe *et al.*, 1996). Other endocrine measures that have been used to monitor stress in livestock include β-endorphins which have been reported to increase in response to transportation stress in horses (Fazio *et al.*, 2008). Recently, β-endorphins have been used to describe stress and pain in horses with colic (Niinistö *et al.*, 2010).

1.5.3.3. Immune cell distribution and function

1.5.3.3.1. Introduction

The immediate innate immune response provides the first line of defence against foreign antigens, priming and directing the slower, more specific adaptive response. Among their many functions, innate immune responses lead to a rapid burst of inflammatory mediators which results in immune cells infiltrating to the site of stimulation (Medzhitov and Janeway, 1997). Alterations in proportions of leukocyte subsets and their ability to migrate to the appropriate locations can affect their ability to influence the risk of infection and disease, and contribute to the pathophysiology of disease (Narin *et al.*, 1995; Kehrli *et al.*, 1999). Sufficient leukocyte trafficking between body compartments and the periphery is important for optimal surveillance thus, enabling the immune system to initiate a rapid, appropriate, and effective response (Engler *et al.*, 2004a; 2004b). Figure 1.9 illustrates the origin and differentiation of immune cells and highlights the cell populations that are examined in this thesis. Alterations in circulating leukocyte subsets have been documented in beef cattle under various management practices such as restricted space allowance during

housing of steers (Gupta *et al.*, 2005), transportation of bulls (Yagi *et al.*, 2004; Buckham Sporer *et al.*, 2007a), and following castration (Pang *et al.*, 2006). Generally, elevations in endogenous glucocorticoids results in leukocytosis, defined by neutrophilia and concurrent lymphopenia (Griffin, 1989; Carroll and Forsberg, 2007). The main perturbations to peripheral immune cell distribution in response to many routine stressors encountered by livestock are summarised in Table 1.5.

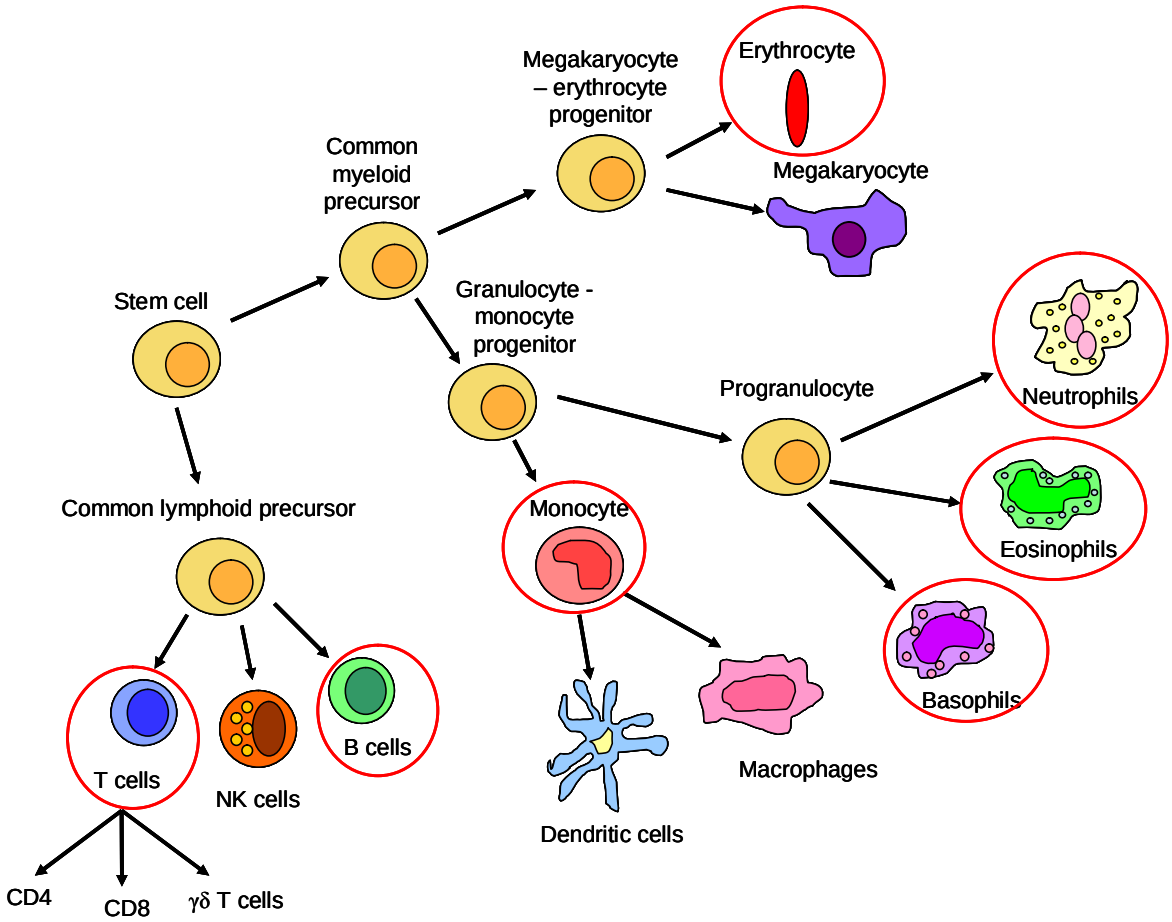

Figure 1.9. Origin and differentiation of immune cells.

Two main haematopoietic cell lineages, lymphoid cells and myeloid cells, are derived from pluripotent stem cells. Depending on their microenvironment, lymphoid progenitor cells can differentiate into T cells, B cells or NK cells. Cell populations circled in red indicate the cell populations examined in this thesis.

Table 1.5. Alterations to peripheral immune cells in livestock following routinely encountered stressors and the administration of synthetic glucocorticoids in order to mimic stress

Cell type	Stressor	Response	References
Total leukocytes			
	Transportation	↑	Murata *et al.*, 1987; Yagi *et al.*, 2004; Buckham Sporer *et al.*, 2007a; Riondato *et al.*, 2008
	Transportation (*Bos indicus*)	↓	Stanger *et al.*, 2005
	Transportation and isolation	↔	Fell *et al.*, 1999
	Feedlot induction	↑	Fell *et al.*, 1999
	Castration (surgical)	↑	Macauly and Friend, 1987; Chase *et al.*, 1995; Fisher *et al.*, 1997; Murata *et al.*, 1997; Earley and Crowe, 2002; Ting *et al.*, 2003; Pang *et al.*, 2006, 2009
	Castration (burdizzo)	↔	Ting *et al.*, 2003
	Pharmacological challenge[1]	↑↑	Menge and Dean Nystrom, 2008
Neutrophils			
	Transportation	↑	Buckham Sporer *et al.*, 2007a; Gupta *et al.*, 2007; Riondato *et al.*, 2008;
	Transportation and isolation	↑	Lomborg *et al.*, 2007
	Feedlot induction	↑	Fell *et al.*, 1999
	Restrictive space allowance at housing	↑	Gupta *et al.*, 2007
	Castration	↑	Fisher *et al.*, 1997; Murata *et al.*, 1997; Ting *et al.*, 2003; Pang *et al.*, 2009
	Pharmacological challenge[1]	↑↑	Burton *et al.*, 2005; Menge and Dean Nystrom, 2008

Table 1.5. Contd.

Cell type	Stressor	Response	References
Lymphocytes			
	Transportation	↓	Gupta *et al.*, 2007; Riondato *et al.*, 2008
	Feedlot induction	↓	Fell *et al.*, 1999
	Restrictive space allowance at housing	↓	Gupta *et al.*, 2007
	Castration	↓	Ting *et al.*, 2003
	Pharmacological challenge[1]	↓	Menge and Dean-Nytsrom, 2008
Monocytes			
	Transportation	↔	Riondato *et al.*, 2008
	Castration	↑	Ting *et al.*, 2003
Eosinophils			
	Transportation	↑	Gupta *et al.*, 2007
	Transportation	↓	Riondato *et al.*, 2008
	Restrictive space allowance at housing	↓	Gupta *et al.*, 2007
	Castration	↔	Ting *et al.*, 2003
Basophils			
	Transport	↔	Riondato *et al.*, 2008

[1]Pharmacological challenge utilised the synthetic glucocorticoid, dexamethasone.
↑ = increases, ↑↑ = profound increase, ↓ = decreases, ↓↓ = profound decrease, ↔ = no change.

1.5.3.3.2. Leukocyte trafficking

Leukocyte trafficking is regulated by various adhesion molecules and their ligands on endothelial cells, and involves the coordinated, sequential functions of several families of adhesion molecules, cytokines and chemoattractants. This multi-step process initially involves the transient attachment of leukocytes by surface selectins, namely L-selectin which is constituently expressed on leukocytes, and P-selectin and E-selectin, and their ligands. This interaction does not anchor cells rather allows them to roll along the endothelium, making and breaking contact (Smith *et al.*, 1994; Janeway *et al.*, 2005). Cells are 'arrested' along the endothelium via the tight adhesion and activation of surface β2-integrins, which bind to intracellular adhesion molecules (ICAMs). Cells then, extravastate through the intracellular junctions, following a chemotactic gradient generated at the infection locus. Chemoattractants include microbial peptides, prostaglandins, leukotrienes, complement components and cytokines including IL-1, -2, -6, and -8 and INF-γ (Smith *et al.*, 1994; Kehrli *et al.*, 1999; Paape *et al.*, 2003; Kobayashi, 2006). Lymphocyte trafficking is referred to as lymphocyte homing as these cells have a tendency to preferentially re-circulate through selected lymphoid tissues. In contract, neutrophils do not emigrate from tissues at the resolution of inflammation, rather they undergo apoptosis, being recognised by macrophages and removed via phagocytosis (Janeway *et al.*, 2005).

Studies examining the effects of stress on leukocyte function in cattle have focused on scenarios where endogenous glucocorticoids are greatly elevated, such as at parturition (Burton *et al.*, 2005), or following transportation (reviewed by Fike and Spire, 2006). Numerous studies have employed ACTH and synthetic glucocorticoid challenges in order to study the effects of stress on immune cell function. Findings from these studies have implicated the down-regulation of L-selectin as an important factor mediating neutrophilia (Burton and Kehrli, 1995a; 1995b). Moreover, recovery

of L-selectin on the surface of neutrophils and lymphocytes occurs over days after the challenge (Burton and Kehlri, 1995a; 1995b; 1996; Burton *et al.*, 1995). Weber *et al.* (2001) demonstrated that bovine neutrophils exposed to relatively subtle and severe glucocorticoid levels, during parturition and dexamethasone challenge, respectively, responded with pronounced inhibition of CD62L mRNA expression, suggesting pre-translation regulation by GR via its action as a transcription factor. Subsequent work by this group demonstrated that many effects of glucocorticoids on neutrophil expression and phenotype were modulated by GR, as evidenced by the reversal of these effects by the glucocorticoid antagonist, RU486 (Chang *et al.*, 2004; Madsen-Boutrese *et al.*, 2006; Weber *et al.*, 2006).

The demonstration that glucocorticoid and adrenergic receptors on lymphocytes are down-regulated in response to stress (Odore *et al.*, 2004) suggests that alterations of the lymphoid compartment may have a major role in the immune response to stress. Despite this, few studies have comprehensively examined changes in lymphocyte subsets in cattle following husbandry stress, with the majority of research focusing on parturition. Table 1.6 summarises the main perturbations to peripheral lymphocyte subsets in response to stressors encountered by livestock.

1.5.3.3.3. Leukocyte function

Neutrophils are essential for defence against opportunistic bacteria, with their main functions centering on phagocytosis which initiates two synergistic processes: respiratory burst and degranulation, which work to kill the pathogen ingested into the phagosome (Borregaard and Cowland, 1997). Highly reactive oxygen species namely superoxide anion, hydrogen peroxide and hydroxyl radical, are produced within the phagosome, and during degranulation, lysosomes fuse with the phagosome forming a phagolysosome. Within the phagolysome, myeloperoxidase mediates the generation

of hypochlorous acid and in conjunction with other proteases, efficiently degrade microbial components (Smith *et al.*, 1994; Paape *et al.*, 2003; Janeway *et al.*, 2005).

Various components of the neutrophil pathogen killing-function are impaired by administration of glucocorticoids. At pharmacologically high doses, dexamethasone impaired neutrophil phagocytosis, respiratory burst activity, myelopeoxidase activity, and antibody dependent cell-mediated cytotoxicity *in vivo* (Roth and Kaerberle, 1981). These findings were confirmed by another study which used ACTH challenge *in vivo* (Roth *et al.*, 1982). Hoeben *et al.* (1998) studied isolated bovine blood granulocytes and showed that pharmacologically high doses of infused hydrocortisone and therapeutic doses of glucocorticoids do not affect neutrophil oxidative burst activity. In terms of disease models, down-regulation of neutrophil surface adhesion molecules has been associated with impaired migration of neutrophils in mammary tissue during mastitis infection (Burton and Kehrli, 1995b; Burton and Erskine, 2003). In contrast, enhancing effects of glucocorticoids on neutrophil function have also been reported such as increased random migration and increased chemotaxis *in vivo* (Roth and Kaerberle, 1981). Furthermore, recent research on the effects of transportation on the neutrophil transcriptome in cattle has questioned the assumption that these effects are mainly immunosuppressive, by demonstrating that there is a reprogramming of gene expression for greater bactericidal capacity and hence, capacity for tissue damage (Buckham Sporer *et al.*, 2007a; 2007b). Further research is needed to examine if these effects on a transcriptome level are realised at a cellular phenotype and protein level in relation to this and other husbandry stressors in cattle. Using a castration-stress model, Pang *et al.* (2009) reported that surface expression of CD62L on neutrophils and neutrophil phagocytic activity and respiratory activity were not affected by either band and Burdizzo castration or hydrocortisone infusion. Reduced neutrophil phagocytosis has also been reported in

transported horses compared with non-transported controls (Raidal *et al.*, 1997), and in dairy cows that were subjected to once-a-day milking (Llamas Moya *et al.*, 2008).

As a general indicator of immunocompetence in an animal, lymphocyte reactivity tests have, traditionally, provided information on lymphoid contribution to animal health, and consequently, welfare. Many early, studies relied on *in vitro* lymphocyte proliferation assays to define the importance of immune function in the stress response (reviewed by Roth and Flaming, 1990; Minton, 1994; Blecha *et al.*, 1984). Antigenic and mitogenic stimulation was achieved by plant lectins such as concanavalin A (Con A), phytohaemagglutinin (PHA), and pokeweed mitogen (Kristensen *et al.*, 1982). A more rapid and robust indicator of general immunocompetence has been adopted, whereby lymphocytes are stimulated by plant lectins in a whole blood assay and cytokine production (IFN-γ; a major cytokine involved in the defence to numerous viral and microbial pathogens (Shtrichman and Sameul, 2001; Billiau and Matthys, 2009) is measured (Wood *et al.*, 1990; Rothel *et al.*, 1990; Antas *et al.*, 2004). A major advantage of this whole blood assay over lymphocyte assays is the preservation of the leukocytes within their natural environment which allows for local compartmentalised cytokine interactions (Ertel *et al.*, 1997) and also, as it does not require isolation of purified lymphocytes any possible random selection of populations is eliminated.

Mixing and relocation of ewes (Sevi *et al.*, 2001) and cattle (Gupta *et al.*, 2005) to different pens and social groups has been reported to result in a higher degree of activity, aggressive encounters, lower immune responses, unwillingness to eat novel feed (ewes) when compared with control animals maintained in a consistent physical and social environment. Hickey *et al.* (2003b) reported attenuated lymphocyte proliferation in *ex vivo* immune function test for cattle that were housed at space allowance of less than 2.0 m^2 per animal. This is in contrast with Fisher *et al.*, (1997)

who reported that no differences in cell-mediated immune function in heifers that were housed at 1.5, 2.0, 2.5, and 3.0 m^2 per animal. Reduced mitogen driven lymphocyte stimulation has been reported during transportation stress in cattle (Blecha and Minocha, 1983). Contrary to this finding, several cases of increased lymphocyte blastogenesis in response to mitogen stimulation have been reported following transportation in cattle (Murata *et al.*, 1987; Swanson and Morrow-Tesch, 2001).

Table 1.6. Alterations to peripheral lymphocyte subsets in livestock following husbandry management stressors and administration of synthetic glucocorticoids in order to mimic stress[1]

Cell type	Stressor	Response	Reference
CD2			
	Transportation	↓	Riondato *et al.*, 2008
	Parturition	↓	Shafer-Weaver *et al.*, 1996; van Kampen *et al.*, 1997
CD3			
	Transportation	↓	Riondato *et al.*, 2008
CD4			
	Transportation	↓	Riondato *et al.*, 2008
	Feedlot induction	↓	Fell *et al.*, 1999
	Repeated restraint and isolation	↓	Minton *et al.*, 1992
	Pharmacological challenge[2]	↔	Burton and Kehrli, 1996
	Parturition	↓	Shafer-Weaver *et al.*, 1996; van Kampen *et al.*, 1997
	Parturition	↑	Harp *et al.*, 2004
CD5			
	Transportation	↓	Riondato *et al.*, 2008
CD8			
	Transportation	↑	Riondato *et al.*, 2008
	Feedlot induction	↓	Fell *et al.*, 1999
	Pharmacological challenge[2]	↔	Burton and Kehrli, 1996
	Parturition	↓	Shafer-Weaver *et al.*, 1996; van Kampen *et al.*, 1997
	Parturition	↔	Harp *et al.*, 2004
WC1			
	Transportation	↓	Riondato *et al.*, 2008
	Feedlot induction	↔	Fell *et al.*, 1999
	Pharmacological challenge[2]	↓↓	Burton and Kehrli, 1996; Nonnecke *et al.*, 1997; Menge and Dean Nystrom, 2008
	Parturition	↔	van Kampen *et al.*, 1997

Table 1.6. Contd.

Cell type	Stressor	Response	References
MHC class II			
	Transportation	↑	Riondato *et al.*, 2008
	Pharmacological challenge[2]	↑↑	Burton and Kehlri, 1996
	Repeated restraint and isolation	↑	Minton *et al.*, 1992
	Parturition	↓	van Kampen and Mallard, 1997
CD21			
	Transportation	↓	Riondato *et al.*, 2008
CD4:CD8 ratio			
	Parturition	↑	Shafer-Weaver *et al.*, 1996; van Kampen *et al.*, 1997

[1]Due to the paucity of information on the effects of stressors on peripheral lymphocyte subsets in livestock, parturition is included as an event in which endogenous cortisol is elevated in livestock. Extrapolation of the effects of parturition on peripheral lymphocyte subset distribution in direct comparison with other husbandry practices must be viewed with caution due to numerous complex physiological, hormonal, and nutritional modifications active in the parturient cow.

[2]Pharmacological challenge utilised the synthetic glucocorticoid dexamethasone.

↑ = increases, ↑↑ = profound increase, ↓ = decreases, ↓↓ = profound decrease, ↔ = no change.

1.5.3.4. Acute phase proteins

Although stressors are considered to activate the acute phase response (Murata *et al.*, 2007; Lomborg *et al.*, 2008), there are conflicting reports, centred mainly on the nature of the stimuli and management and breed of the animals, regarding this response in livestock (Qui *et al.*, 2007). Following transportation, reduced concentrations of haptoglobin and fibrinogen were reported in bulls transported for 9 h

in a truck (Buckham Sporer *et al.*, 2008). In contrast, Phillips (1984) reported increased concentration of fibrinogen following assembly and transport of beef calves. Arthington *et al.* (2003) reported higher serum concentrations of SAA, fibrinogen, and ceruloplasmin and lower concentrations of haptoglobin in transported calves compared with non-transported calves. However, subsequent work by this group produced varied results leading these authors to acknowledge that further research is required before these proteins can be universally applied to all husbandry stressors, specifically co-mingling, weaning and transportation of calves. Elevated serum SAA levels are reported in cattle subjected to physical stress (Alsemgeest *et al.*, 1995), and in cattle transported for 4 – 6 h and then housed in tie stalls in isolation (Lomborg *et al.*, 2008). Ceruloplasmin is not as widely studied as other acute phase proteins however it has been used to identify infection in cattle (Chassagne *et al.*, 1998; Sheldon *et al.*, 2001).

1.5.3.5. Metabolites

Analysis of clinical biochemistry provides an objective means of assessing nutritional status in an animal and be used with other methods enabling specific and global nutritional problems to be identified. Activation of the HPA and SAM axes in response to stress prepare the animal to cope metabolically with the stimuli, generally in the form of altered protein and energy metabolism, thus, examination of these biomarkers provides valuable information on the nutritional status of the animal under stress. Initiation and maintaining the stress response is expensive in terms of energy, and in order to cover energy expenditure, a continuous supply of energy is required to maintain efficient growth, reproductive processes, and immune function. This supply is facilitated by anaerobic glycolysis and the aerobic breakdown of the main energy substrates; glucose and free fatty acids (Balm, 1999). As outlined in Section 1.4.5., activation of the HPA and SAM axes profoundly affect the levels of these substrates,

as the animal prepares to cope metabolically with stimuli. Social stress, achieved by the mixing of unfamiliar bulls in pens resulted in elevated concentrations of glucose and NEFA (McVeigh and Tarrant, 1983). In contrast, Gupta *et al.* (2005) reported that concentration of glucose increased after the initial mixing of unfamiliar steers, however, no changes in concentration of NEFA were observed. Similarly, unaltered concentrations of NEFA were reported following the mixing of beef cattle (Mench *et al.*, 1990) and sheep (Sevi *et al.*, 2001). Findings of increased energy metabolism is generally reported following transportation in cattle (Kent and Ewbank *et al.*, 1983; Knowles *et al.*, 1999; Tarrant *et al.*, 1992; Tadich *et al.*, 2005; Earley *et al.*, 2006). Reports on changes in concentration of blood beta-hydroxybutyrate, a ketone body, are variable, with decreases (Tadich *et al.*, 2000; Tadich *et al.*, 2005; Earley *et al.*, 2006) and increases (Warriss *et al.*, 1995; Knowles *et al.*, 1999) reported following transportation in cattle. Under extreme nutritional stress, such as early lactation in the cow, a state of negative energy balance exists whereby, there is a sudden increase is energy requirements imposed by onset of lactation and reduction in voluntary dry matter intake. Partly as a consequence, cows that are described as being in a state of negative energy balance around parturition are also immunosuppressed (Detilleux *et al.*, 1995). As the majority of available glucose in directed towards the mammary gland for milk synthesis and secretion, there is increased mobilisation of fat from body stores and release of NEFA into circulation as an alternative energy source (Sordillo *et al.*, 2009). Thus, the metabolic profile associated with negative energy balance (i.e. decreased concentrations of glucose and concurrent elevated concentrations of NEFA and beta-hydroxybutyrate in circulation) may provide information on the nutritional status of cattle under different physiological and nutritional stressors. In a study examining the effect of long-term cold climatic housing conditions in mature, pregnant suckler cows, blood metabolites were measured in order to detect sensitive indicators

of subclinical infections and imbalances in energy metabolism, that would be otherwise undetected by production performance characteristics (Mannien *et al.*, 2008). Additionally, Buckham Sporer *et al.* (2008) highlighted the use of metabolites as potential biomarkers of stress following transportation in beef bulls.

1.6. Weaning stress

1.6.1. Introduction

Under natural conditions, weaning for the offspring involves a complete transition from nutritional and social dependence on the mother to complete independence. This transition is understood to occur gradually as the availability of milk from the mother declines and solid food intake increases, consequently the offspring becomes more socially independent from the mother with greater social interaction with peers (Veissier and Le Neindre, 1989a; Veissier *et al.*, 1990a; 1990b; Lee, 1997). In farmed animals, the definitive point of natural weaning is somewhat unknown, varying within species and between species and sex (Gonyou and Stookey, 1987), and has been reported to be approximately between ten and twelve months of age in *Bos indicus* cattle (Reinhardt and Reinhardt, 1981) and *Bos taurus*, respectively.

In practice, livestock are generally weaned at a much younger age than that observed for natural weaning and, what is more, livestock may also encounter more associated stressors such as social disruption, new environments and additional processing or husbandry practices. In intensive livestock production systems, such as beef production, there is a need to artificially wean calves at approximately six to nine months of age in order to maximise the reproductive potential of the dam as cow-calf separation hastens the return of reproductive cycling and leads to the potential optimal yield of one calf each year. It also allows for the marketing or specialised feeding of

the calves (Myers *et al.,* 1999), or in the case of dairy cattle where calves are removed from their dams within hours or days of birth, it allows for an increased amount of milk available for human consumption.

Weaning under modern cattle production systems may be described as a multi-factorial stressor as it incorporates nutritional, physical and psychological elements (Weary *et al.*, 2008). Calves are often subjected to an array of husbandry practices which may compound their stress at the time of weaning. Additional husbandry practices can include; frequent handling and contact with humans, mixing with unfamiliar cattle, movement to new environments whether it be indoor housing or unfamiliar paddocks, switching to different, entirely solid diets, transportation and marketing. Some cattle may also undergo additional processing such as vaccinations, dehorning and castration. Studies suggest that modifying the management of beef calves pre- and post-weaning can reduce weaning stress. Such management practices include the use of anti-suckling devices (nose-clips) for a period prior to weaning (Haley *et al.*, 2005; Siegford *et al.*, 2007; Boland *et al.*, 2008), fence-line contact between calf and dam post-weaning (Stookey *et al.*, 1997; Price *et al.*, 2003; Siegford *et al.*, 2007; Burke *et al.*, 2009) and a combination of both practices before complete separation (Newberry and Swanson, 2008). Furthermore, research has examined the effect of yard-weaning with and without feed bunk training on the behavioural adaptation of beef calves to a feedlot environment, with particular emphasis on prevalence of BRD (Walker *et al.*, 2007). Calves that were yard-weaned and trained to source grain ration in a trough showed greater feeding behaviour during the first few days in the feedlot compared with those that were yard-weaned and not trained, however, this did not result in significantly greater weight gain. Moreover, there was less morbidity in yard-weaned calves compared with pasture-weaned calves following entry into a commercial feedlot (Walker *et al.*, 2007). Compared with beef calves that

were weaned in paddocks, yard-weaned calves showed increased social interactions with their peers post-weaning (Fell *et al.*, 1998).

1.6.2. Neurobiological mechanisms of attachment and separation (mother – young bonding)

Several neurochemicals are associated with attachment in animals including oxytocin, prolactin, dopamine, gonadal steroids, vasopressin and endogenous opioids. These neurotransmitters work in synergy to perform specialised functions required for attachment and maintenance of maternal care. Oxytocin is associated with suckling in many mammals and has been shown to be released in calves during nursing but was not released when fed milk from a bucket (Lupoli *et al.*, 2001) indicating that the presence of the dam is required for oxytocin release. Separation-induced calling has been reported to be reduced in oxytocin-deficient mice suggesting a role of this neurotransmitter in attachment-separation behaviour (Winslow and Insel, 2002) and is a major determinant for bonding of the ewe to her newborn lamb (Kendrick *et al.*, 1997). Working in concert with oxytocin, prolactin promotes maternal care through the activation of dopaminergic and opioid pathways responsible for the addictive and rewarding nature of social contact, lactation and other responses involved in maternal maintenance (Insel *et al.*, 2001; Insel, 2003). Cows' milk contains α-lactophorin, a tetra-peptide that is structurally similar to endogenous opioids and binds to opioid receptors (Yoshikawa *et al.*, 1986). Some theories suggest that the phases of attachment are similar to that of opiate addiction and withdrawal symptoms (Nelson and Panksepp, 1998). The fact that cows' milk contains opiate-like substances may provide some weight to these theories. Cholecystokinin (CCK) is involved in satiety (Crawley and Cronin, 1994), and is increased in calves by non-nutritive suckling following milk consumption (de Passillé *et al.*, 1993). Studies using rodents have

shown that the reduction in vocalisations achieved by an intra-oral diffusion of milk is blocked when rats are first given CCK antagonists (Blass and Shide, 1993). Thus, this peptide may be useful in identifying responses to nutritional and social components of stress.

1.6.3. Behavioural responses to weaning stress

Overt behavioural changes, such as increased vocalisations and locomotor activity, caused by weaning, have been documented for several days in the beef and dairy cow and calf, however, these changes are reported to be more intense and persist longer in the calf compared with the cow (Price *et al.*, 2003). A large volume of research has examined these behavioural responses and for the purpose of this review they are summarised in Table 1.7. The latency to initiate behavioural responses following weaning varies between species and with age. In general, responses are immediate in piglets (Weary *et al.*, 1999) and foals (McCall *et al.*, 1985), however, this contrasts with longer latency observed in cattle. Additionally, latency of behavioural responses can also be affected by age especially in young calves (Weary and Chua, 2000; Flowers and Weary, 2001). Another point to note on the perturbations to normative behaviours following weaning is that the intensity or magnitude of the responses decline with time. For instance, in abruptly weaned beef calves vocalisations over the first three days after the event were approximately 2000 - 4000 times greater than those of unweaned control calves (Price *et al.*, 2003). This point was further emphasised by the fact that weaned calves spent 28.1 % of their time walking and 23.7 % of their time eating compared with 8.6 % and 41.1 %, respectively, in unweaned control calves. Linked to the intensity or magnitude of the behavioural response is the persistence of such behaviour following weaning. Weaning is unlike many husbandry/ management practices in that distress behaviours

persist for several days after the event unlike, for example, the relatively short lasting (< 5 h) alterations in behaviour observed post-transportation. In the days following weaning, distress behaviours in beef calves lessen (Price *et al.*, 2003; Haley *et al.*, 2005; Boland *et al.*, 2008) and this may signal adaptation by the calves to their new situation. Alternatively, this could also signal an exhaustion of the system to maintain increased locomotor activity and elevated rates of calling, which may be due in part to irritation of the larynx. The greatest changes in behaviour were observed within the first forty-eight (Price *et al.*, 2003; Haley *et al.*, 2005; Boland *et al.*, 2008) and seventy-two hours (Veissier and Le Neindre, 1989a) after weaning, indicating a peak in the animals' motivation to reunite.

Studies examining the effects of alternative weaning methods have generally implied that these methods serve to reduce the distress associated with weaning as measured by the reduced behavioural responses compared with abruptly weaned beef calves (Haley *et al.*, 2005; Price *et al.*, 2003; Boland *et al.*, 2008). However, a recent study has questioned the benefits of two-stage weaning with nose-clips and fence-line weaning on calf welfare, concluding that despite the reduction in vocalisations, walking and seeking behaviours observed, calves displayed behaviours synonymous with frustration, resultant from failed suckling attempts distress responses (Enriquez *et al.*, 2010). These authors also showed that there was no overall reduction to distress behaviours for calves weaned by two-stage methods with nose-clips, but rather redistribution over two periods (i.e. in the days post-attachment of nose-clip and days post-weaning).

The differences in behavioural responses in beef calves at abrupt weaning in relation to milk yield availability from their dams have recently been examined and confirm that dietary intake of milk is a strong component of weaning stress (Ungerfeld *et al.*, 2009). Calves reared on dams producing high milk yields had greater frequency

of grazing behaviours post-weaning compared with calves reared on high milk-yielding calves.

The extent to which weaning perturbs the behavioural activity in cattle provides evidence that this husbandry practice is stressful. Behaviour does not, however, inform us of the entire physiological consequence of stress experienced by animals (Rutherford *et al.*, 2006; Øverli *et al.*, 2007). Increased behavioural activity that characterises an animal's response to weaning is associated with physiological changes that are also indicative of animals under stress (Lefcourt and Elsasser, 1995; Broom, 2006), and it is these physiological changes that will become the focus of the remaining sections of this literature review.

Table 1.7. Changes in behavioural responses following weaning or temporary cow-calf separation in livestock.

Animals	Treatment	Results	Reference
Salers heifer calves, 7 mo, (n = 30)	3 treatments: AW-abruptly weaned, CO-controls not weaned, and NS-restricted from suckling, cloth place over dams' udders.	Weaning resulted in increased social interactions among calves. Increased aggressive events were recorded in calves that were restricted from suckling.	Veissier and Le Neindre, 1989a
Salers calves, 8 mo, (n = 67)	Measurement of attraction of calf to dam using choice tests based on time after weaning (0.5, 2, 7, 20 d) and (1, 9, 16, 24, and 35 d).	Weaned calves chose to remain closest to dam up to 24 d post-weaning, though motivation to suckle had decreased. Compared with female calves, male calves showed weaker preference to their dam compared with another cow.	Veissier *et al.*, 1990a
Crossbred beef calves (n = 139)	2 treatments: C-contact weaned, calves were penned adjacent to dams with which they had visual, olfactory and auditory contact. R-remote weaned-abruptly weaned and penned.	Treatment differences were greatest up to 48 h post-weaning. R vocalised and walked more and spent less time lying than C.	Stookey *et al.*, 1997
Angus × Hereford calves, mean age (s.d.) 220 (35) d, (n = 100)	5 treatments: CP-weaned controls at pasture, FP-fenceline contact at pasture following weaning, SP-abruptly weaned at pasture, SDP-abruptly weaned in drylot and preconditioned, SDNP-abruptly weaned, drylot, not preconditioned.	Treatment differences were greatest 20 – 30 h post-weaning. SP spent more time walking than other treatments. FP and CP spent more time eating than SP and SDNP and FP vocalised less than SP and SDNP. SP spent less time lying than other treatments.	Price *et al.*, 2003
Beef calves, mean (s.d.) age 184 (13.3) d, (n = 152)	2 treatments: 2-stage weaning (with nose-clips) vs. abrupt weaning (control)	Treatment differences were greatest 48 h post-weaning. Calves weaned using 2-stage weaning vocalised 97 % less and spent 79 % less time walking, 23 % time eating and 24 % more time resting than abruptly weaned calves. On d 1, abruptly weaned calves walked 17 km/d, whereas 2-stage weaned calves walked 5.2 km/d.	Haley *et al.*, 2005

Table 1.7. Contd.

Animals	Treatment	Results	Reference
Zebu (*Bos indicus*) male calves, 90 d, (n = 27)	3 treatments: FC-partially separated from dam with fenceline contact, NC-abruptly separated from dam with no contact, CO-controls remained with dam. Separation lasted 72 h.	Treatment differences were greatest 48 h post-separation. Increased vocalisations, walking, butting, and urinating evident in FC and NC compared with CO. Grooming and lying behaviour reduced in FC but not in NC.	Solano *et al.*, 2007
Swedish Red and White and Swedish Holstein calves, age range 20 h – 7 d, (n = 46)	6 treatments: Separated at 1, 4, or 7 d of age with or without visual and auditory (but not physical) contact of the dam in adjacent pen.	After separation, older calves moved more and displayed more explorative behaviours (sniffing walls, bedding, and licking walls) than calves separated at 1d.	Stěhulová *et al.*, 2008
Crosbred Angus calves, mean (s.e) age 224 (15) d, (n = 162)	3 treatments: FL: fenceline contact post-weaning, NC: nose-clip pre-weaning, and C: controls, abruptly weaned	C spent more time idling and walked more and less time eating compared with FL and NC post-weaning. Pre-weaning, NC spent less time eating, more time idling and walked more compared with FL and C.	Boland *et al.*, 2008

1.6.4. Physiological responses to weaning stress

1.6.4.1. Endocrine responses

Studies have shown that hormonal indicators of stress are evident in beef calves post-weaning. Following ACTH-challenge, cortisol concentration increased dramatically post-weaning in beef calves and was similar to the increase observed in beef calves post-transport (Zavy *et al.*, 1992). Crookshank *et al.* (1979) reported minor increases in concentration of cortisol in beef calves on d 1 post-weaning, which had returned to pre-weaning baseline by d 2 post-weaning. In contrast, Hickey *et al.* (2003a) reported that social group disruption resulted in elevated concentrations of cortisol up to 7 d post-weaning. Concentration of cortisol ranged from 7.5 ng/mL to 16.2 ng/mL at -168 h pre-weaning and 48 h post-weaning, respectively (Hickey *et al.*, 2003a). Additionally, the findings of Blanco *et al.* (2009) suggest that cortisol remained elevated beyond 7 d post-weaning in beef calves and is not affected by age (abrupt weaning at 90 d of age versus 150 d of age). However, contrary to the findings of Hickey *et al.* (2003a), concentrations did not exceed 8 ng/mL at maximum response (Blanco *et al.* (2009). There was no effect of breed with Pirenaica calves having higher concentrations of cortisol than Parada de Montana calves, post-weaning (Blanco *et al.*, 2009). Breed has been shown to have a significant effect on baseline cortisol levels in beef calves (Zavy *et al.* 1992). Concentration of cortisol transiently increased from 7.6 ng/mL at the time of calf removal to 11.9 ng/mL at 9 to 12 h post-weaning in beef cows, following which it did not differ from baseline (Whisnant *et al.*, 1985).

In terms of catecholamine response to weaning, there is less congruency in the literature on the effects of weaning on these hormones. Findings pertaining to the adrenaline response are equivocal, with increases and no change reported in beef calves post-weaning (Lefcourt and Elsasser, 1995; Hickey *et al.*, 2003a).

Noradrenaline increased immediately (45 min) post-weaning in beef calves (Lefcourt and Elsasser, 1995), and was found to be influenced by a weaning × sex interaction, with bull calves having increased concentrations of noradrenaline whereas, it decreased in heifer calves post-weaning (Hickey *et al.*, 2003a). With regard to the beef cows, adrenaline increased, whereas noradrenaline was unchanged post-weaning (Lefcourt and Elsasser, 1995).

Additionally, for other livestock species, weaning has been reported to increase the concentration of cortisol in piglets (Hay *et al.*, 2001; Le Dividich and Seve, 2002; Mason *et al.*, 2003; Kojima *et al.*, 2008), foals (Moons *et al.*, 2005; Fazio *et al.*, 2009) and lambs (Rhind *et al.*, 1998).

1.6.4.2. Immune cell distribution and function

As outlined in Section 1.4.and 1.5.3, extensive studies have demonstrated that stress affects immune cell distribution and functions. Total leukocyte number was not altered following abrupt weaning in beef calves that were weaned at 150 d of age, whereas, increased total leukocyte numbers were reported in calves that were weaned at 90 d of age (Blanco *et al.*, 2009). Hickey *et al.* (2003a) reported increased total leukocyte numbers following social disruption of the herd (facilitated by the removal of half the herd; these animals were allocated to abrupt weaning treatment), however post-weaning, there was no difference in total leukocyte number in abrupt weaned and non-weaned (control) calves. In contrast, Phillips *et al.* (1989) reported decreased total leukocyte number in calves following abrupt weaning. Increased percentage neutrophil and concurrent decreased lymphocytes have been reported in beef calves (Hickey *et al.*, 2003a; Blanco *et al.*, 2009) and red deer (Church and Hudson, 1999). Weaning age (90 d versus 150 d of age) and breed do not affect the

neutrophil:lymphocyte ratio in beef calves (Blanco *et al.*, 2009), however abrupt weaning results in greater increase in neutrophil:lymphocyte ratio compared with gradual weaning in foals (Turner *et al.*, 2003) and wapiti deer (Church and Hudson, 1999). The effects of weaning on other cell types such as monocytes, eosinophils, and basophils in beef calves have not been examined.

Few studies have examined leukocyte function following weaning. Hickey *et al.* (2003a) reported that *in vitro* production of IFN-γ was attenuated in weaned beef calves compared with non-weaned calves. A transient reduction in neutrophil phagocytosis has been documented in weaned foals (Sarwar *et al.*, 2008).

1.6.4.3. Acute phase protein response

Findings pertaining to the effects of weaning acute phase protein response in beef calves are not consistent. Although some studies have shown that this response is activated through the measurement of fibrinogen, ceruloplasmin and SAA (Arthington *et al.*, 2003; 2008; Blanco *et al.*, 2009; Carroll *et al.*, 2009), others have reported that concentrations of fibrinogen and haptoglobin are not affected by weaning (Hickey *et al.*, 2003a). Further research is needed to identify the most commonly affected acute phase protein in response to weaning stress which would greatly enhance the ability to identify stress susceptible individuals that may require veterinary intervention during periods of stress.

1.6.4.4. Metabolites

Biomarkers of altered nutritional status have been examined during weaning stress in cattle. Altered protein metabolism is evidenced by changes in circulating concentrations of total protein, and its constituents, albumin and globulin, and

concentration of urea. Following weaning, concentration of total protein and urea increased in calves and rapidly returned to pre-weaning concentrations (Phillips *et al.*, 1989). Crookshank *et al.* (1979) reported no difference in urea concentrations post-weaning. Increased energy metabolism, a hallmark of the stress response as the body diverts energy from processes of a lesser priority to processes required to react to the stressor (Campbell *et al.*, 1999), is evidenced by alterations to concentrations of glucose, NEFA and βHB. Conflicting data exists for the effect of weaning on concentration of glucose, with increases (Phillips *et al.*, 1989) and no differences observed in *Bos taurus* calves post-weaning (Crookshank *et al.*, 1979; Boland *et al.*, 2008). The serum concentration of glucose did not differ post-weaning compared with pre-weaning concentrations in abruptly weaned *Bos indicius* calves (Phillips *et al.*, 1989; Coppo *et al.*, 2001). Elevated concentrations of NEFA were found on d 1 post-weaning in abruptly weaned, fence-line contact weaned and nose-clip weaned calves, however baseline concentrations were restored by d 7 (Boland *et al.*, 2008).

1.7. Hypotheses and objectives of this thesis

The advancement of the scientific assessment of stress in cattle is fundamental to improving animal health and welfare. Many advances have been achieved through multidisciplinary research incorporating physiology, immunology, and behavioural studies of cattle, and by the exploitation cellular and molecular technologies which have utilised novel approaches such as flow cytometric analysis of cell phenotypes and functions, transcriptome-wide investigations and functional genomics.

The overall hypothesis of this thesis was that weaning, a significant stressor in the lifetime of the calf, would alter the physiological and immunological responses in beef calves under different management conditions. Additionally, it was hypothesised that weaning would also constitute a stressful event for the beef cow, and thus, alter the physiological and immunological responses in beef cows that were abruptly separated from their calves. In order to test these hypotheses, four studies were undertaken with the following objectives:

1. to (i) investigate the physiological, immunological and metabolic responses of previously grazed, abruptly weaned beef calves that were a) housed and offered a new diet of grass silage *ad libitum* plus concentrates (H) or b) returned to familiar pasture (P), and secondly (ii) to examine, the effect of subsequent housing on these responses in P compared with previously housed calves (Chapter 3).
2. to characterise the extended physiological, immunological and metabolic in beef cows to (i) abrupt weaning and (ii) subsequent housing (Chapter 4).
3. to examine the effect of abrupt weaning at housing on peripheral leukocyte and lymphocyte subset distribution, neutrophil functional

activity, and the acute phase protein response in abruptly weaned beef calves compared with non-weaned (control) calves (Chapter 5).

4. to examine the effect of concentrate supplementation for 26 d prior to abrupt weaning on (i) the peripheral distribution of leukocytes, (ii) functional activity of neutrophils, (iii) the acute phase protein, (iv) metabolic, and (v) behavioural responses, and (vi) the performance of beef calves compared with non-supplemented calves (Chapter 6).

Collectively, from these studies, a series of physiological and immunological profiles may be identified that can help to further describe the responses of beef cattle to weaning stress. From these profiles, potential biomarkers of weaning stress may be characterised that could be used in the future to identify cattle that are susceptible to weaning stress and thus, more likely to succumb to infection post-weaning.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Experimental subjects

The experimental subjects used in each chapter are described in Table 2.1. The experimental design for each chapter is described in Section 2.2.2.

Table 2.1. Description of the experimental subjects used in this thesis

Chapter	n	Sex (n)	Breed (n)	Date of birth[1]	Age at weaning[1]	Weight at weaning[2]
Calves						
Chapter 3	36	Male: 18 Female:18	LM[3] × HF[4]: 18 SM[5] × HF: 18	19 March (25.5) d	212 (24.5) d	279 (38.0) kg
Chapter 5	16	Male: 16	LM × HF: 8 SM × HF: 8	23 March (18.2) d	235 (18.2) d	310 (31.1) kg
Chapter 6	20	Male: 10 Female:10	SM: n = 20	23 March (12.7) d	201 (12.9) d	257 (19.9) kg

Chapter	n	Parity	Breed	Calving date[1]	Age at calving[1]
Cows					
Chapter 4	36	First	LM × HF: 18 SM × HF: 18	19 March (25.5) d	756 (24.3) d

[1]Mean (s.d.), d, [2]Mean (s.d.), kg, [3]LM = Limousin, [4]HF = Holstein-Friesian, [5]SM = Simmental.

2.1.2. Reagents for Physiological Measurements

2.1.2.1. Nanopure water

Nanopure water (Barnstead NANOpure Water Purification Systems, Thermo Scientific, UK), was used as required to make solutions and reconstitute reagents. This water was deionised, removed from organic compounds, treated with dual band UV light and passed through a 0.2 μm filter.

2.1.2.2. Cortisol Assay Kit and Reagents

A competitive immunoassay (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 901-701) was used for the quantitative determination of cortisol in plasma (Section 2.2.3.2.2.). All the reagents were supplied in the kit.

2.1.2.2.1. Cortisol Assay Buffer

Tris buffered saline containing sodium azide as a preservative was diluted 1/10 with Nanopure water and stored at room temperature (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0010).

2.1.2.2.2. Cortisol Standards

Prior to dilution, the Cortisol standard solution (100,000 pg/mL, Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0677) was allowed to equilibrate to room temperature. In tubes 1 to 7, 1000 µL and 500 µL of assay buffer was aliquoted into tube 1 and tubes 2 to 7, respectively. From tube 1, 100 µL was removed and 100 µL of 100,000 pg/mL standard was added. Following thorough vortexing, 500 µL of solution from tube 1 was aliquoted into tube 2. Following thorough vortexing, 500 µL from tube 2 was aliquoted into tube 3. This was repeated for tube 4 up to tube 7 yielding a set of 7 standards. The concentration of these standards was 10,000 pg/mL, 5000 pg/mL, 2500 pg/mL, 1250 pg/mL, 625 pg/mL, 313 pg/mL, and 156 pg/mL, respectively. Standards were used within 1 h of preparation.

2.1.2.2.3. Cortisol EIA Conjugate

A solution of alkaline phosphatase conjugated with cortisol (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0680).

2.1.2.2.4. Cortisol EIA Antibody

Solution of mouse monoclonal antibody to cortisol (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0678).

2.1.2.2.5. Cortisol Wash Buffer

Tris buffered saline containing detergents was diluted 1/20 with Nanopure water and stored at room temperature (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-1286).

2.1.2.2.6. P-Nitrophenyl phosphate substrate for Cortisol assay

Solution of p-nitrophenyl phosphate (pNpp) in diethanolamine buffer (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0075).

2.1.2.2.7. Cortisol Stop solution

A solution of trisodium phosphate in water to stop the enzymatic reaction (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0247).

2.1.2.3. Dehydroepiandrosterone Assay Reagents

A competitive immunoassay (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 901-093) was used for the quantitative determination of

dehydroepiandrosterone (DHEA) in plasma (Section 2.2.3.2.3.). All reagents were supplied in the kit.

2.1.2.3.1. DHEA Assay Buffer

Tris buffered saline containing sodium azide as a preservative was diluted 1/10 with Nanopure water and stored at room temperature (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-1591).

2.1.2.3.2. DHEA Standards

Prior to dilution, the DHEA standard solution (500,000 pg/mL, Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0980) was allowed to equilibrate to room temperature. In tubes 1 to 7, 1000 µL and 750 µL of assay buffer was aliquoted into tube 1 and tubes 2 to 7, respectively. From tube 1, 100 µL was removed and 100 µL of 500,000 pg/mL standard was added. Following thorough vortexing, 250 µL of solution from tube 1 was aliquoted into tube 2. Following thorough vortexing, 250 µL from tube 2 was aliquoted into tube 3. This was repeated for tube 4 up to tube 7 yielding a set of 7 standards. The concentration of these standards was 50,000 pg/mL, 12,500 pg/mL, 3,125 pg/mL, 781.25 pg/mL, 195.3 pg/mL, 48.8 pg/mL, and 12.2 pg/mL, respectively. Standards were used within 1 h of preparation.

2.1.2.3.3. DHEA EIA Conjugate

The DHEA EIA Conjugate was prepared by adding 50 µL of supplied conjugate, a solution (blue) of alkaline phosphatase conjugated with DHEA, to 450 µL of DHEA

Assay Buffer and was used within 3 h of preparation (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0978).

2.1.2.3.4. DHEA EIA Antibody

A solution of rabbit polyclonal antibody to DHEA (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0976).

2.1.2.3.5. DHEA Wash Buffer

Tris buffered saline containing detergents was diluted 1/20 with Nanopure water and stored at room temperature (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-1286).

2.1.2.3.6. P-Nitrophenyl phosphate substrate for DHEA assay

A solution of p-nitrophenyl phosphate (pNpp) in diethanolamine buffer (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0075).

2.1.2.3.7. DHEA Stop Solution

A solution of trisodium phosphate in water (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA, catalogue number 80-0247).

2.1.2.4. Clinical biochemistry

2.1.2.4.1. Total Protein

The concentration of total protein in plasma was determined on an automatic clinical analyser (Olympus AU400 Clinical Analyser, Tokyo, Japan) using the reagents

supplied by Olympus (catalogue number OSR6132). The composition of the reagents (at final concentration) required for this test were; sodium hydroxide (200 mmol/L), potassium sodium tartrate (32 mmol/L), copper sulphate (18.8 mmol/L) and potassium iodide (30 mmol/L), and following gently inversion the reagents were ready to use directly from the kit.

2.1.2.4.2. Albumin

The concentration of albumin was determined on an automatic clinical analyser (Olympus AU400 Clinical Analyser, Tokyo, Japan) using the reagents supplied by Olympus (catalogue number OSR6102). The composition of the reagents (at final concentration) required for this test were; succinate buffer (pH 4.2) (100 mmol/L) and bromocresol green (0.2 mmol/L), and following gently inversion the reagents were ready to use directly from the kit.

2.1.2.4.3. Creatine kinase

The activity of creatine kinase was determined on an automatic clinical analyser (Olympus AU400 Clinical Analyser, Tokyo, Japan) using the reagents supplied by Olympus (catalogue number OSR6179). The composition of the reagents (at final concentrations of reactive ingredients) required for this test were; imidazole (pH 6.5) (100 mmol/L), NADP (2 mmol/L), ADP (2 mmol/L), AMP (5 mmol/L), EDTA (2 mmol/L), glucose (20 mmol/L), creatine phosphate (30 mmol/L), n-acetlycysteine (0.2 mmol/L), activator (26 mmol/L), Mg^{2+} (10 mmol/L), diadenosine pentaphosphate (0.01 mmol/L), hexokinase (> 4 kU/L), and glucose-6-phosate dehydrogenase (> 2.8 kU/L). Prior to the placement of the reagents on board the instrument, the entire contents of R1-2 was transferred to the entire contents of R1-1 and mixed by gentle inversion.

The second reagent bottle (R2) was ready to use from the kit and was placed directly on board the instrument.

2.1.2.4.4. Glucose

The concentration of glucose was determined on an automatic clinical analyser (Olympus AU400 Clinical Analyser, Tokyo, Japan) using the reagents supplied by Olympus (catalogue number OSR6121). The composition of the reagents (at final concentration of reactive ingredients) required for this test were; piperazine-N,N'-bis(ethanesulfonic acid (PIPES) buffer (pH 7.6) (24 mmol/L), ATP (≥ 2 mmol/L), NAD^+ (≥ 1.32 mmol/L), Mg^{2+} (2.37 mmol/L), hexokinase (≥ 0.59 mmol/L), G6P-DH (≥ 0.59 mmol/L), and required no preparation prior to placement on board the instrument.

2.1.2.4.5. Non-esterified fatty acids

The concentration of non-esterified fatty acids (NEFA) was determined on an automatic clinical analyser (Olympus AU400 Clinical Analyser, Tokyo, Japan) using the reagents supplied by Randox Laboratories (catalogue number FA115). The kit comprised of 5 reagents, and the composition of these reagents is described in Table 2.2. The Buffer (R1a) and Enzyme diluent (R2a) were ready to use from the kit. The Enzyme/coenzymes (R1b) were reconstituted with 10 mL of Buffer (R1a) and mixed by gently swirling. Maleimide (R2b) was reconstituted with Enzyme diluent (R2a) and was inverted several times to ensure that maleimide was completely dissolved. This was then used immediately to reconstitute Enzyme reagent (R2c) which was protected from light and placed on board the instrument for analysis. All reagents were kept at 4 °C prior and after preparation.

2.1.2.4.6. β-hydroxybutyrate

The concentration of β-hydroxybutyrate (βHB) was determined on an automatic clinical analyser (Olympus AU400 Clinical Analyser, Tokyo, Japan) using the reagents supplied by Randox Laboratories (catalogue number RB 1007). The kit comprised of 5 reagents, and the composition of these reagents is described in Table 2.3. The Buffer (R1a) was ready to use from the kit following gently inversion and was used to reconstitute the Enzyme/coenzymes (R1b). Reagents were held at 4°C prior and after preparation.

Table 2.2. Reagent composition for quantitative NEFA determination

Reagent		Initial concentration of solutions
R1a	**Buffer**	
	Phosphate buffer (pH 6.9)	0.04 mmol/L
	Magnesium chloride	3 mmol/L
	Surfactant	
R1b	**Enzyme/Coenzyme**	
	Acyl coenzyme A synthesase	≥ 0.3 U/mL
	Ascorbate oxidase	≥ 1.5 U/mL
	Coenzyme A	0.9 mmol/L
	ATP	5 mmol/L
	4-aminoantipryine	1.5 mmol/L
R2a	**Enzyme diluent**	
	Phenoxyethanol	0.3 % (w/v)
	Surfactant	
R2b	**Malemide**	10.6 mmol/L
R2c	**Enzyme Reagent**	
	Acyl coenzyme A oxidase	≥ 10 U/mL
	Peroxidase	7.5 U/mL
	TOOS (N-ethyl-N-(2hydroxy-3-sulphorpropl)m-toluidine)	1.2 mmol/L

Table 2.3. Reagent composition for quantitative β-hydroxybutyrate determination

Reagent		Initial concentration of solutions
R1a	**Buffer**	
	Tris buffer (pH 8.5)	100 mmol/L
	EDTA	2 mmol/L
	Oxalic acid	20 mmol/L
R1b	**Enzyme/Coenzyme**	
	NAD^+	2.5 U/mL
	3-hydroxybutyrate dehydrogenase	0.12 U/mL

2.1.2.4.7. Urea

The concentration of urea was determined on an automatic clinical analyser (Olympus AU400 Clinical Analyser, Tokyo, Japan) using the reagents supplied by Olympus (catalogue number OSR6134). All reagents were stored at 4 °C. The composition of the reagents (at final concentration of reactive ingredients) required for this test were; Tris buffer (100 mmol/L), NADH (≥ 0.26 mmol/L), tetra-sodium disulphate (10 mmol/L), EDTA (2.65 mmol/L), 2-oxoglutarate (≥ 9.8 mmol/L), Urease (≥ 17.76 kU/L), ADP (≥ 2.6 mmol/L), glutamate dehydrogenase (GLDH) (≥ 0.16 kU/L), and required no preparation prior to placement on board the instrument.

2.1.2.5. Haematology

2.1.2.5.1. Reagents for ADVIA 2120 haematology analyser

Isotonic, balanced electrolye sheath fluid supplied by Siemens, UK.

2.1.2.5.2. Peripheral blood smear solutions

A series of three solutions; methanol, buffered eosin, and buffered azur 1 methylene blue (Speedy-Diff Complete Clin-tech Ltd, Surrey, UK) were used to fix and stain peripheral blood smears.

2.1.3. Reagents for Immunological Measurements

2.1.3.1. Immunostaining

2.1.3.1.1. BD FACS lysing solution

A 10 X buffered solution containing < 15 % (v/v) formaldehyde and < 50 % (v/v) diethylene glycol (BD FACS lysing solution, BD Biosciences, Oxford, UK) was diluted 1/10 with Nanopure water and stored at room temperature.

2.1.3.1.2. Coulter Isoton II diluent

A balanced electrolyte solution (Beckman Coulter UK Ltd., High Wycombe, UK).

2.1.3.1.3. Antibodies for selected surface antigens

The sources, specificities, isotypes and working solutions of primary and secondary antibodies are described in Table 2.4. Working solutions were prepared using PBS (pH 7.2) (Section 2.1.3.1.4., GibcoBRL, Life Technologies Ltd., Paisley, Scotland, UK).

Table 2.4. Antibodies used in the immunostaining of leukocyte surface markers.

Specificity	Cell types	Clone	Isotype	Working solution	Source[1]
CD4	T-helper/ inducer cells	CC8	IgG_2a	7 µL/mL	Serotec
CD8	T-cytotoxic/ suppressor cells	CC63	IgG_2a	7 µL/mL	Serotec
WC1	Subset of γδ T cells	CC15	IgG_2a	7 µL/mL	Serotec
MHC class II	Antigen presenting cells (B cells, activated T cells)	H42A	IgG_2a	7 µL/mL	VMRD
CD62L	L-selectin	BAQ92A	IgG_1	14 µL/mL	VMRD
G1	Neutrophils (and eosinophils)	MM20A	IgG_1	14 µL/mL	VMRD
CD45	All leukocytes (pan marker)[2]	CC1	IgG_1	3.5 µL/mL	Serotec
IgG_1 isotype	Mouse IgG_1a negative control	-	-	7 µL/mL	Serotec
IgG_2a isotype	Mouse IgG_2a negative control	-	-	7 µL/mL	Serotec

[1]Serotec (Oxford, UK), VMRD (Pullman, WA, USA).

[2]Used to differentiate leukocyte populations and exclude debris (gate analysis).

2.1.3.1.4. Phosphate-Buffered Saline (PBS)

A solution of 0.01 M PBS (pH 7.2) (GibcoBRL, Life Technologies Ltd., Paisley, Scotland, UK).

2.1.3.1.5. PBS -1 % (w/v) % BSA (pH 7.4).

One pre-weighed pouched (Sigma-Aldrich Chemical Co. Ltd., UK) of powered PBS with 1 g bovine serum albumin (BSA) was dissolved in 1 L of Nanopure water and stored at 4 °C.

2.1.3.1.6. Paraformaldehyde 1 % (v/v)

In a fume hood, 1 g of paraformaldehyde (Sigma-Aldrich Chemical Co. Ltd., UK) was dissolved in 80 mL of PBS using a magnetic stirrer while heating on a heat/stir plate.

Caution was taken not heat the solution above 58 °C. Approximately 5 drops of 1 M NaOH were added slowly to fully dissolve the paraformaldehyde into solution. When cooled, the solution was light protected by wrapping in tinfoil and stored at 4 °C for maximum of 5 d.

2.1.3.2. Neutrophil phagocytosis activity assay

The Phagotest assay kit (Orpegen Pharma, Heidelberg, Germany) was used to determine phagocytic activity of bovine neutrophils. All reagents necessary for this assay were supplied in the kit and are described briefly below.

2.1.3.2.1. Opsonised FITC-labelled *Escherichia coli (E. coli)* suspension (Reagent B)

The ready to use 1 X solution contained approximately 2×10^9 *E. coli* bacteria/ mL. According to the manufacturer, cells were opsonised with immunoglobulin (Fc) and complement (C3) of pooled sera. Cells were maintained in the dark at 4 ºC.

2.1.3.2.2. Quenching solution (Reagent C)

Dark blue quenching solution was used to suppress the fluorescence signal of *E. coli* cells attached to the surface of neutrophils.

2.1.3.2.3. Phagotest Wash Solution (1 X) (Reagent A)

1 bottle of Instamed-Salts (containing chloroacetamide and EDTA) was reconstituted in 1000 µL of Nanopure water and stored at room temperature.

2.1.3.2.4. Lysing Solution (Reagent D)

The erythrocyte Lysing solution was prepared after a 1/10 dilution of Lysing Solution (10 X) with distilled Nanopure water. Fresh lysing solution was prepared prior to each test and stored at 4 ºC. The constituents of this solution, diethylene glycol and formaldehyde, caused the lysis of the erythrocyte cell population within the whole blood sample and allowed for the simultaneous fixing of leukocytes.

2.1.3.2.5. DNA staining Solution (Reagent E)

1 X DNA staining solution for cytometric discrimination of bacteria cells during leukocyte analysis.

2.1.3.3. Neutrophil oxidative burst activity assay

The Phagoburst assay kit (Orpegen Pharma, Heidelberg, Germany) was used to examine the oxidative burst activity of bovine neutrophils. The kit contained all the reagents necessary to carry out the functional tests and are described below.

2.1.3.3.1. Opsonised *Escherichia coli (E. coli)* suspension (Reagent B)

The 1 X solution contained approximately 2×10^9 *E. coli* bacteria/ mL. According to the manufacturer, cells were opsonised with immunoglobulin (Fc) and complement (C3) of pooled sera, and were not labelled with flurochromes. Cells were maintained at 4 ºC.

2.1.3.3.2. Substrate Solution (Reagent C)

Dihydrorhodamine 123 was reconstituted following the addition of 1 mL of wash solution to the substrate disk. Substrate was prepared immediately prior to addition to cells (20 - 25 min max).

2.1.3.3.3. Lysing Solution (Reagent D)

The erythrocyte Lysing solution was prepared after a 1:10 dilution of stock solution Lysing Solution (10 X) with Nanopure water. Fresh lysing solution was prepared prior to each test and stored at 4 ºC. The constituents of this solution were diethylene glycol and formaldehyde.

2.1.3.3.4. Phagoburst Wash solution (1 X) (Reagent A)

1 bottle of Instamed-Salts (containing chloroacetamide and EDTA sodium salt) was reconstituted in 1000 µL of Nanopure water.

2.1.3.3.5. DNA staining Solution (Reagent E)

1 X DNA staining solution for cytometric discrimination of bacteria during leukocyte analysis.

2.1.3.4. Acute phase protein assays

2.1.3.4.1. Haptoglobin

A colorimetric assay (Phase™ Range Haptoglobin Assay, Tridelta Development Ltd., Co. Kildare, Ireland) was used to determine concentration of haptoglobin. The kit contained all necessary reagents and the method based on Eckersall *et al.* (1999).

2.1.3.4.1.1. Haemaglobin (Reagent 1)

Equal volumes of haemoglobin and haemoglobin diluent were mixed by inversion prior to placement on board the instrument.

2.1.3.4.1.2. Chromogen/substrate (Reagent 2)

Chromogen and substrate were mixed in a 9:5 ratio immediately prior to placement on board the instrument.

2.1.3.4.2. Fibrinogen reagents

2.1.3.4.2.1. Atroxin

Purified extract from *Bothrops atroz* venom, stabilised in buffered bovine albumin, 5 µg/ vial (pH 7.5) (11335, Fluka, Sigma-Aldrich, Dublin, Ireland).

2.1.3.5. Interferon-γ production reagents

2.1.3.5.1. Concanavalin A

Concanavalin A from *Canavalia ensiformis* (Jack bean) (Sigma-Aldrich Chemical Co. Ltd. UK) was reconstituted with PBS (Section 2.1.3.1.4.) to final concentration of 1 mg/mL.

2.1.3.5.2. Phytohaemagglutinin

Phytohaemagglutinin A extracted from *Phaseolus vulgaris* (red kidney bean) (Sigma-Aldrich Chemical Co. Ltd. UK) was reconstituted with PBS (Section 2.1.3.1.4.) to final concentration of 1 mg/mL.

2.1.3.5.3. Bovine INF-γ assay

A qualitative bovine IFN-γ assay was used to determine the in vitro production of INF-γ (BOVIGAM, Biocor, Prionics AG, Switzerland). All of the required reagents were supplied in the kit.

2.1.3.5.3.1. Assay Diluent

Stored at 4 °C. Prior to use, controls were brought to room temperature and mixed thoroughly by vortexing.

2.1.3.5.3.2. Bovine INF-γ positive and negative controls

The positive and negative controls were reconstituted with 1 mL of Nanopure water and stored at 4 °C. Prior to use, controls were brought to room temperature and mixed thoroughly by vortexing.

2.1.3.5.3.3. Wash buffer

Wash buffer was prepared by diluting 20 X Concentrate Wash Buffer solution 1/19 with Nanopure water and was stored at room temperature.

2.1.3.5.3.4. Conjugate

Horseradish peroxidase labelled anti-bovine IFN-γ conjugate (100 X) was reconstituted with 1.5 mL of Nanopure water and stored at 4 °C. After allowing the Conjugate Buffer Diluent to reach room temperature, it was diluted 1/4 with Nanopure water. For each plate, 120 µL of 100 X conjugate was mixed with 12 mL Conjugate Buffer Diluent and used within five minutes of preparation.

2.1.3.5.3.5. Enzyme substrate solution

Enzyme substrate solution was prepared immediately prior to the enzyme substrate addition step of the ELISA by mixing 120 μL of 100 X Chromogen Solution to 12 mL of Enzyme Substrate Buffer for each plate.

2.1.3.5.3.6. Stop Solution

Enzyme reaction stopping solution of 0.5 M H_2SO_4.

2.1.4. Behavioural Measures

2.1.4.1. IceTag pedometers

Wireless motion sensors (IceRobotics, Midlothian, Scotland, UK) were used to monitor standing, lying activity and to record the number of steps taken by the calves.

2.2 Methods

2.2.1. Experimental licence

All animal procedures performed in this study were conducted under experimental licence from the Irish Department of Health and Children in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulation 2002 and 2005. Licence number: B100/2869 (Bernadette Earley, Ph.D Supervisor, Teagasc).

2.2.2 Experimental design

The experimental design including management of animals, rectal body temperature recording and blood sampling schedule for Chapters 3 to 6 are described separately below.

2.2.2.1 Experimental design and animal management (Chapter 3)

Thirty-six spring-born (mean date of birth (s.d.) March, 19 (25.5 d)) single-suckled (without concentrate creep feed) progeny of first parity Limousin × Holstein-Friesian (n = 18) and Simmental × Holstein-Friesian (n = 18) dams, and Simmental and Limousin sires, respectively, were used (Section 2.1.1). Cows and calves were rotationally grazed, in four groups that were balanced for genotype and birth date, on a predominantly perennial ryegrass (*Lolium perenne*)-based sward from early April until weaning on 17 October. Male calves were castrated 28 d prior to weaning.

On the day of weaning (d 0), cows and calves were moved to a handling yard and calves received a Rispoval-3 vaccination (Pfizer Healthcare, Ireland) against bovine respiratory syncytial virus, parainfluenza-3 virus and bovine viral diarrhoea. A

secondary dose of Rispoval-3 vaccine was administered to all calves on d 28. Calves (mean age (s.d.) 212 (24.5) d; mean weight (s.d.) 278.8 (38.0) kg) were abruptly separated from their dams and assigned to one of two treatments, either a) a housed treatment (H: n = 18) or b) a pasture treatment (P: n = 18) (Figure 2.1). Treatments were balanced for gender, birth date, and genotype (10 males and 8 females per treatment). Allocation to treatment resulted in calves from each grazing group being mixed, similar to social disruption experienced by weaned calves in practice. Following abrupt weaning, H calves were immediately housed in a slatted floor shed equipped with automatic drinkers. Calves were loose-housed in 4 pens (space allowance 2.5m^2/calf) containing either 5 male or 4 female calves, and offered a new diet of grass silage (mean dry matter digestibility (DMD) (s.d.) 713 (19.5) g/kg; mean crude protein (CP) concentration (s.d.) 151 (12.5) g/kg dry matter (DM)) *ad libitum* and were supplemented with 0.25 kg of concentrate (935 g/kg barley, 50 g/kg molasses and 15 g/kg minerals and vitamins (mean DMD (s.d.) 830 (8.0) g/kg, mean CP (s.d.) 103 (9.7) g/kg DM, mean neutral detergent fibre (NDF) (s.d.) 190 (23.7) g/kg DM) per animal daily. Concentrate allowance was increased in daily increments of 0.25 kg over 4 d until an allowance of 1.0 kg per animal per day was reached. Following abrupt weaning, P calves were returned to pasture, similar in nutritive quality and quantity to that grazed prior to weaning, and were not supplemented with concentrates. After 35 d at pasture, P calves were housed and offered the same diet and supplementary concentrates as previously described for H calves.

2.2.2.1.1. Sample collection method and schedule (Chapter 3)

Blood samples were collected into test tubes (Vacuette, Cruinn Diagnostics, Ireland) containing the appropriate anticoagulant via direct jugular venipuncture to investigate

the effect of post-weaning management relative to (i) weaning; sampling occurred on d 0 (weaning), 2, 7, 14, 21, 28 and 35 (d 0 to 35), and relative to (ii) subsequent housing of P; sampling occurred at d 0 (housing), 2, 7, 14 and 21 (d 0 - 21) (Figure 2.1.). Animals were moved to a handling chute and mild restraint was used to obtain blood samples. All samples were transported to the laboratory at ambient temperature and processed within 2 h of blood collection.

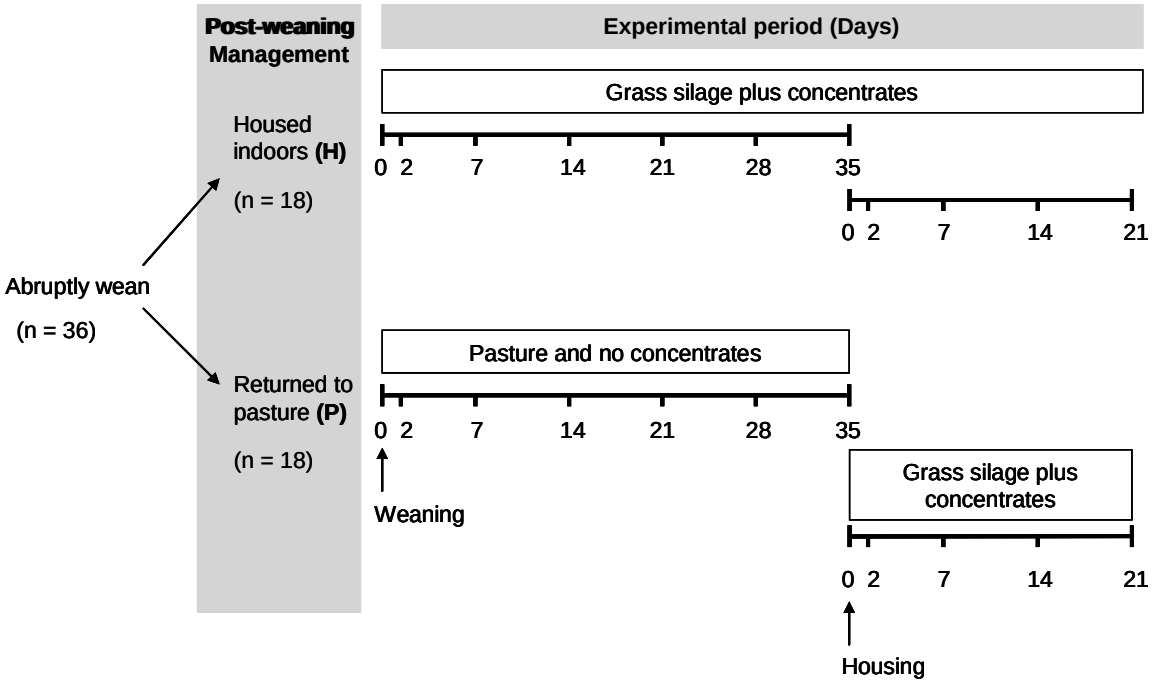

Figure 2.1. Management and blood sampling schedule of beef calves that were abruptly weaned and either (i) housed (H) in a slatted floor shed and offered grass silage *ad libitum* plus supplementary concentrates or (ii) returned to pasture (P) and not supplemented with concentrates.

After 35 d, P calves were housed and offered grass silage *ad libitum* plus supplementary concentrates. Blood sampling and rectal body temperature recording occurred on days outlined in the schedule.

2.2.2.2 Experimental design and animal management (Chapter 4)

Thirty-six first parity, spring-calving (mean date of calving (s.d) March, 19 (25.5) d), single-suckled Limousin × Holstein-Friesian (n = 18) and Simmental × Holstein-Friesian (n = 18) cows were used (Section 2.1.1). Cows and their progeny were rotationally grazed on a predominantly perennial ryegrass (*Lolium perenne*)-based sward from early April until weaning on 17 October. The pasture had a mean (s.d.) dry matter digestibility (DMD) of 750 (22.1) g/kg and mean (s.d.) crude protein (CP) concentration of 229 (8.7) g/kg DM. On the day of weaning (d 0), cows and calves were moved to a handling yard where the cows were abruptly separated from their calves (mean age (s.d.) 212 (24.5) d; mean weight (s.d.) 279 (38.0) kg) and were returned to the grazing area for a 35 d period (Figure 2.2.). The grazing area was located at a sufficient distance away from the handling yard, thus vocalisations between the cows and calves could not be heard. On d 35, the cows were housed indoors in a slatted floor shed in 3 pens with equal space allowance (n = 6 cows per pen). Each pen was equipped with automatic water drinkers and cows were offered grass silage *ad libitium* (mean (s.d) DMD 714 (17.7) g/kg; CP 144 (11.3) g/kg DM) plus 60 g of a mineral vitamin supplement daily.

2.2.2.2.1 Sample collection method and schedule (Chapter 4)

Blood samples were collected into test tubes (Vacuette, Cruinn Diagnostics, Ireland) containing the appropriate anticoagulant via direct jugular venipuncture to evaluate (i) the effects of weaning (d 0 (pre-weaning baseline), 2, 7, 14, 21, 28, and 35) and (ii) the effects of housing (d 0 (pre-housing baseline), 2, 7, 14 and 21) (Figure 2.2.). All samples were transported to the laboratory at ambient temperature and processed within 2 h of collection.

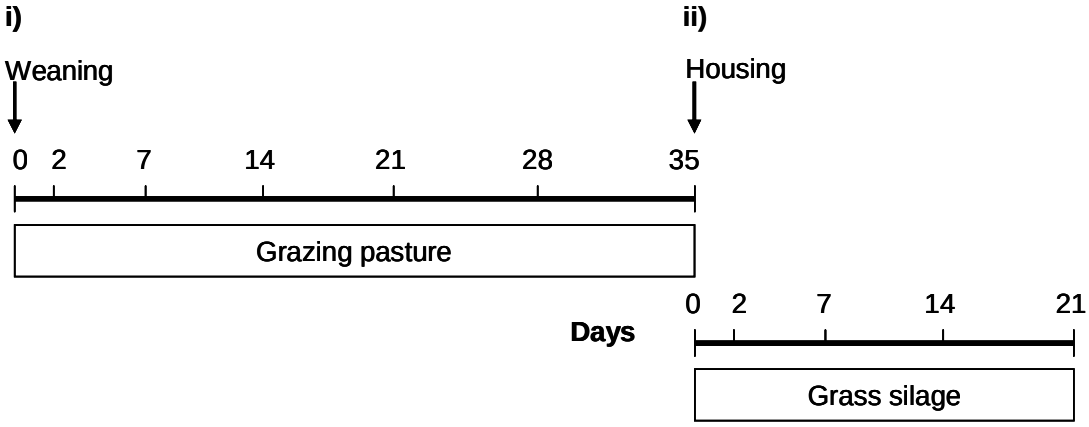

Figure 2.2. Management and blood sampling schedule for beef cows that were abruptly separated from their calves and (i) returned to pasture on d 0, and (ii) subsequently housed in a slatted floor shed on d 35.

Sampling occurred at 7 d intervals except for immediately post-weaning and post-housing where a 2 d interval was allowed for sampling.

2.2.2.3. Experimental design and animal management (Chapter 5)

Sixteen, spring-born (mean date of birth (s.d); 23 March (18.2) d) single-suckled, castrated, male calves of Limousin × Holstein-Friesian and Simmental × Holstein-Friesian dams and Simmental and Limousin sires, respectively, were used in this study. Cows and calves were rotationally grazed together on a predominantly perennial ryegrass-based sward from April until housing in a slatted floor shed at the end of the grazing season (13 November). On the day of housing (d 0), calves (mean age (s.d.) 235 (18.2) d; mean weight (s.d.) 310 (31.1) kg) were randomly assigned by genotype, age, and weight to one of two treatments (i) abruptly weaned (W; n = 8) or (ii) non-weaned (control) (C; n = 8) (Figure 2.3). Weaned calves were housed in pens without their dams (4 calves per pen), whereas non-weaned (control calves) were housed with their dams (2 cow-calf pairs per pen). Space allowance within the pens was equal for animals. Pens were equipped with automatic drinkers and animals were offered grass silage *ad libitum*. Cows that were separated from their calves were housed and had no auditory or visual contact with their calves.

2.2.2.3.1. Sample collection method and schedule (Chapter 5)

Blood was collected into vacutainers (Vacuette, Cruinn Diagnostics, Ireland), containing the appropriate anticoagulant for subsequent haematological and flow cytometric analysis via direct jugular venipuncture using mild restraint in a holding chute at d -7, 0 (housing), 2, 7 and 14 (Figure 2.3). Blood samples were transported to the laboratory, stored at ambient temperature and processed within 3.5 h.

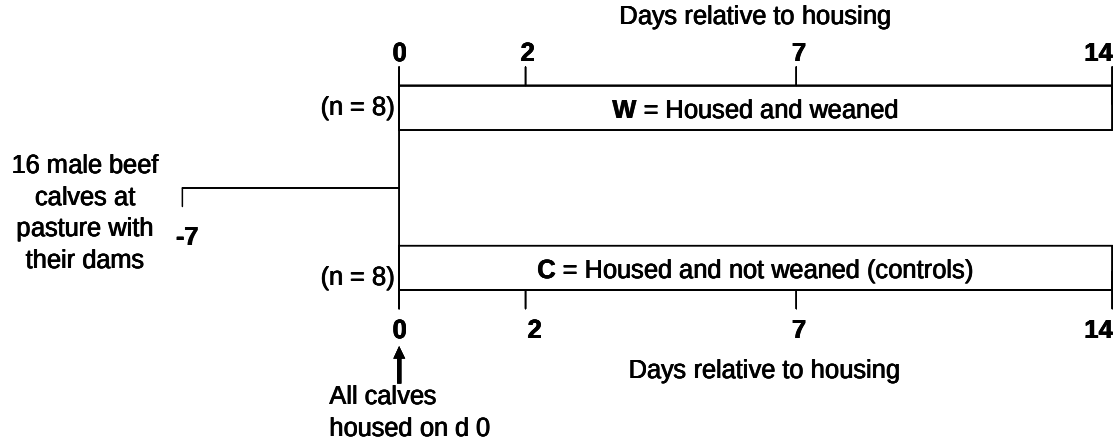

Figure 2.3. Experimental design and rectal body temperature recording and blood sampling schedule for beef calves that were housed on d 0, and either (i) weaned (W: n = 8), or (ii) not weaned (controls) (C: n = 8).

Rectal body temperatures were recorded and blood samples were collected on d -7, 0, 2, 7, and 14 relative to housing on d 0. On d 0, samples were obtained prior to housing.

2.2.2.4. Experimental design and animal management (Chapter 6)

Twenty, spring-born (mean date of birth (s.d.) 23 March (12.7) d), singled suckled, pure-bred Simmental male (non-castrated, n = 10) and female (n = 10) calves were used in this study. Calves and their dams were rotationally grazed together on a predominantly perennial ryegrass (*Lolium perenne*)-based sward from April until housing in a slatted floor shed at the end of the grazing season (4 November). Twenty-six days prior to housing (d -26), calves were weighed, vaccinated subcutaneously against bovine respiratory syncytial virus, bovine parainfluenza-3 virus, bovine viral diarrhea virus, and infectious bovine rhinotracheitis virus using Rispoval-3 and Rispoval IBR (Pfizer Animal Health, Ireland), and were then randomly assigned age, weight and sex to one of two treatments (i) concentrate supplementation offered (CS: n = 10, mean age (s.d.) 201 (12.8) d, mean weight (s.d.) 258 (20.2) kg, 5 males and 5 females) or (ii) no concentrate supplementation offered (controls) (NCS: n = 10, mean age (s.d.) 201 (13.4) d, mean weight (s.d.) 257 (19.6) kg, 5 male and 5 female) during a pre-weaning period (d -26 to 0) and returned to one of two adjacent paddocks of similar herbage quality and quantity. During the pre-weaning period (d -26 to d 0), CS calves were offered concentrates in a metal feeding trough once daily which increased in increments of 0.25 kg until 1.0 kg of concentrates per animal daily was reached, whereas NCS calves received no concentrate supplementation. On d 0, calves were abruptly weaned from their dams and housed in pens (n = 4) in a slatted floor shed and offered grass silage *ad libitum* plus concentrates. Concentrate supplemented calves continued to receive 1.0 kg of concentrates per animal daily, whereas NCS calves were now offered 0.25 kg of concentrates until 1.0 kg of concentrates per animal daily was reached (Figure 2.4.). Cows were relocated with no audio or visual contact with their

calves. Each pen held 5 male or 5 female calves and space allowance within pens was equal for all calves.

2.2.2.4.1. Sample collection method and schedule (Chapter 6)

Blood was collected into vacutainers (Vacuette, Cruinn Diagnostics, Ireland) containing the appropriate anticoagulant via direct jugular venipuncture using mild restraint in a holding chute at d -7, 0 (weaning), 2, 7 and 14 (Figure 2.4). Additional rectal body temperatures and blood samples were collected on d 1 and 3 for haematological analysis. Blood samples were transported to the laboratory, stored at ambient temperature and processed within 3.5 h.

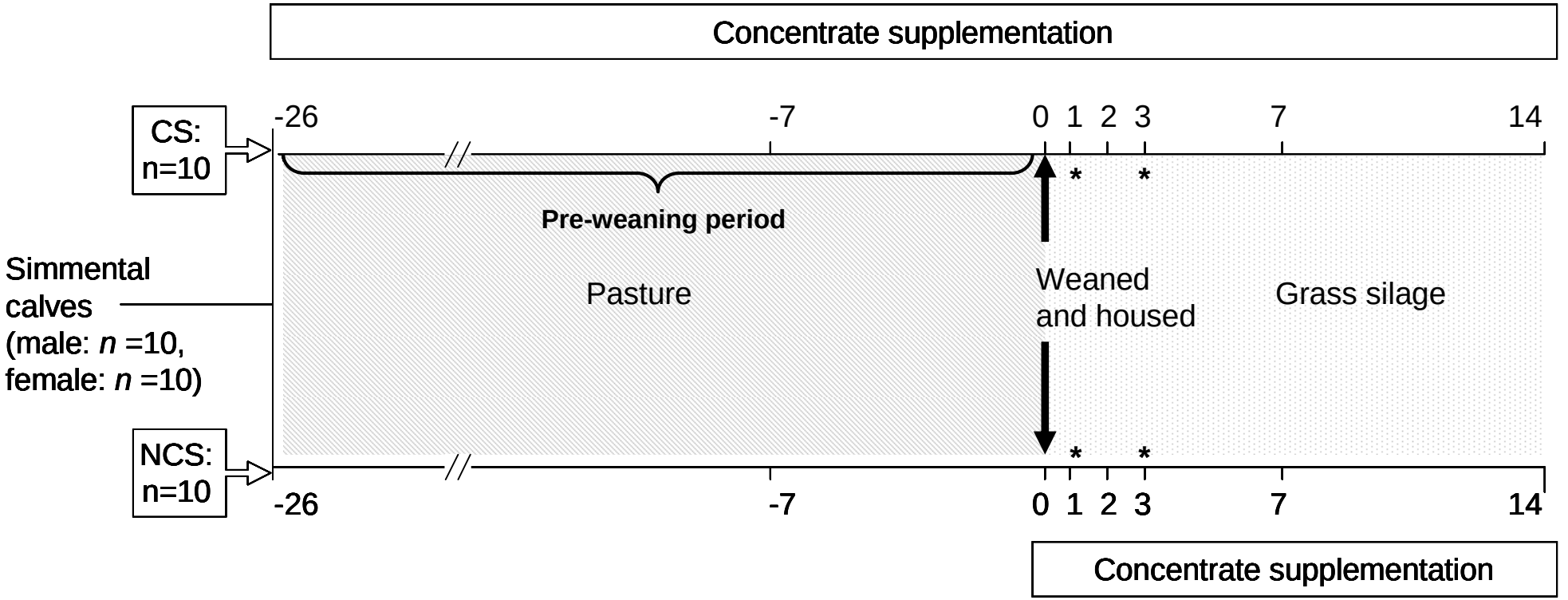

Figure 2.4. Experimental design and sample collection schedule for beef calves that were supplemented with concentrates for 26 d prior to abrupt weaning (d 0) and subsequently offered grass silage plus concentrates at housing.

* denotes additional blood collection time-points for haematological analysis and rectal body temperature recordings. Concentrate supplemented (CS) calves were offered concentrates for 26 d prior to weaning, whereas non supplemented (NCS) calves were not offered concentrates during the pre-weaning period (d -26 to d 0). On d 0, all calves were abruptly weaned, housed and offered grass silage *ad libitium* plus concentrates.

2.2.3. Physiological Measures

2.2.3.1. Rectal body temperature

Rectal body temperature was recorded to one decimal place prior to blood sampling, as previously described (Sections 2.2.2.1.1., 2.2.2.2.1., 2.2.2.3.1., 2.2.2.4.1.), using a digital degrees centigrade (^{o}C) thermometer (Jørgen Kruuse A/S model VT-801 BWC, Marslev, Denmark; catalogue No. 0701).

2.2.3.2. Steroidal analysis

2.2.3.2.1. Sample collection

Blood (9 mL) was collected via direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) that were spray coated with lithium heparin as anticoagulant. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until assayed.

2.2.3.2.2. Cortisol Assay

A competitive immunoassay (Correlate-EIA Cortisol, Assay Designs, Ann Harbor, MI, USA (Section 2.1.2.2.) was used for the quantitative determination of cortisol in plasma following the manufacturers instructions. Briefly, 100 µL of assay buffer (Section 2.1.2.2.1.) was aliquoted into the non-specific binding (NSB) and the Blank only (Bo) (0 pg/mL Standard) wells. One hundred microliters of each of the 7 standards (10,000 pg/mL - 156 pg/mL cortisol, Section 2.1.2.2.2.) was added to appropriate wells. For each sample, 100 µL of plasma was added to appropriate wells and 50 µL of assay buffer was added to NSB wells. Conjugate (50 µL, Section 2.1.2.2.3.) was added to each well with the exception of Total Activity (TA) and Bo

wells. Following this, 50 µL of antibody (Section 2.1.2.2.4.) was added into each well, except TA, Bo and NSB wells. Plates were then sealed, incubated at room temperature on a plate shaker for 2 h at 500 rpm. Following incubation, contents of each well was emptied and washed using 400 µL of wash solution (Section 2.1.2.2.5.). Wash step was repeated 2 more times (total of 3 wash steps). After the final wash, the contents were carefully emptied from the wells and plate was firmly tapped on filter paper to remove residual wash buffer. Conjugate (5 µL, Section 2.1.2.2.3.) was then added to TA wells only. P-nitrophenyl phosphate (pNpp) substrate (200µL, Section 2.1.2.2.6.) was added to all wells and was allowed to incubate at room temperature for 1 h without shaking. Following incubation, the reaction was stopped by adding 50 µL of Stop Solution (Section 2.1.2.2.7.) of every well and the plate was read immediately using a plate reader (TECAN SUNRISE Absorbance Reader, TECAN Austria GmbH, Austria) at 405 nm optical density (OD). The plate reader was blanked against the Bo wells.

2.2.3.2.3. Dehydroepiandrosterone Assay

A competitive immunoassay (Correlate-EIA DHEA, Assay Designs, Ann Harbor, MI, USA (Section 2.1.3.2.) was used for the quantitative determination of DHEA in plasma following the manufacturer's instructions. Briefly, 100 µL of assay buffer (Section 2.1.2.3.1.) was aliquoted into the non-specific binding (NSB) and the Blank only (Bo) (0 pg/mL Standard) wells. One hundred microliters of each of the 7 standards (50,000 pg/mL - 12.2 pg/mL DHEA, Section 2.1.2.3.2.) was added to appropriate wells. For each sample, 100 µL of plasma was added to appropriate wells and 50 µL of assay buffer (Section 2.1.2.3.1.) was added to NSB wells. Conjugate (50 µL, Section 2.1.2.3.3.) was added to each well with the exception of Total Activity (TA) and Bo

wells. Following this, 50 µL of antibody (Section 2.1.2.3.4.) was added into each well, except TA, Bo and NSB wells. Plates were then sealed, incubated at room temperature on a plate shaker for 2 h at 500 rpm. Following incubation, contents of each well was emptied and washed using 400 µL of wash solution (Section 2.1.3.1.5.). The wash step was repeated 2 more times (total of 3 wash steps). After the final wash, the contents were carefully emptied from the wells and plate was firmly tapped on lint free paper to remove residual wash buffer. Conjugate (5 µL, Section 2.1.2.3.3.) was then added to TA wells only. Two hundred microliters of pNpp substrate (Section 2.1.2.3.6.) was added to each well. The plate was sealed and incubated at 37 °C for 3 h without shaking. Following incubation, the reaction was stopped by adding 50 µL of Stop Solution (Section 2.1.2.3.7.) to every well and the plate was read immediately using a plate reader (TECAN SUNRISE Absorbance Reader, TECAN Austria GmbH, Austria) at 405 nm optical density (OD). The plate reader was blanked against the Bo wells.

2.2.3.2.4. Calculation of concentration of Cortisol and DHEA

The average net OD bound for each standard and sample was calculated by subtracting the average NSB OD from the overage OD bound. The binding of each pair of standards was calculated as a percentage of the maximum binding wells (Bo). Percent bound = [Net OD ÷ Net Bo OD] × 100. Percentage bound versus the concentration of cortisol or DHEA for the standards was plotted on a logarithmic scale and a straight line was approximated using a logarithmic trendline and concentration of cortisol or DHEA in each sample was determined by linear interpolation.

2.2.3.3. Clinical chemistry

2.2.3.3.1. Total protein, albumin and globulin

Blood samples were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing lithium heparin as anticoagulant. Tubes were inverted gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until analysed. Concentration of plasma total protein and albumin was determined on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan) using a photometric colour test method (Biuret reaction) and the reagents supplied by Olympus (0SR6132 (Section 2.1.2.4.1.) and OSR6102 (Section 2.1.2.4.2.), respectively, Olympus Life and Material Science Europa GmbH (Irish Branch), Co. Clare, Ireland). Briefly, cupric ions in an alkaline solution react with proteins and polypeptides containing at least two peptide bonds to produce a violet coloured complex, and the absorbance of this complex at 540/660 nm is directly proportional to the concentration of total protein in the sample. The concentration of albumin is determined by the addition of bromocresol green to the sample which reacts with albumin and forms a coloured complex. The absorbance of this BCG-albumin complex at 600/800 nm is directly proportionally to the concentration of albumin in the sample. Concentration of plasma globulin was calculated by subtracting the concentration of albumin from concentration of total protein for each sample.

2.2.3.3.2. Creatine kinase

Blood samples were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing lithium heparin as anticoagulant. Tubes were inverted

gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until analysed. The activity of creatine kinase was determined on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan) using a kinetic UV test method and the reagents supplied by Olympus (OSR6179 (Section 2.1.2.4.3.), Olympus Life and Material Science Europa GmbH (Irish Branch), Co. Clare, Ireland). The activity of creatine kinase in the sample is based on the assumption that creatine kinase reversibly catalyses the transfer of a phosphate group from creatine phosphate to adenosine diphosphate (ADP) to give creatine and adenosine triphosphate (ATP) as products. The ATP formed is catalysed by hexokinase to produce glucose-6- phosphate and ADP from glucose. The resulting glucose-6-phosphate is oxidised by glucose-6-phospahte dehydrogenase (G6PDH) which stimulates the reduction in nicotinamide adenine dinucleotide (NADP) to give NADPH and 6-phosphogluconate. The rate of increase in absorbance at 340/660 nm due to formation of NADPH is directly proportional to the activity of creatine kinase in the sample.

2.2.3.3.3. Glucose

Blood samples (4 mL) were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing sodium fluoride/potassium oxalate as anticoagulant. Tubes were inverted gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until analysed. Concentration of glucose was determined on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan) using a enzymatic (hexokinase) UV test method and the reagents supplied by Olympus (OSR6179 (Section 2.1.2.4.4.), Olympus Life and Material Science Europa GmbH (Irish Branch),

Co. Clare, Ireland). Briefly, glucose is phosphorylated by hexokinase in the presence of ATP and magnesium to produce glucose-6-phosphate and ADP. Glucose-6-phosphate dehydrogenase (G6PDH) specifically oxidises glucose-6-phosphate to gluconate-6-phosphate with concurrent reduction of NAD^+ to NADH. The increase in absorbance at 340 nm is proportional to the concentration of glucose in the sample.

2.2.3.3.4. Non-esterified fatty acids

Blood samples were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing sodium citrate as anticoagulant. Tubes were inverted gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until analysed. Concentration of NEFA was determined on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan) using an enzymatic colorimetric method and reagents supplied by Randox Laboratories (catalogue number FA115 (Section 2.1.2.4.5.), Randox Laboratories, Co. Antrim, UK). Briefly, NEFA in the sample is converted to Acyl-CoA, adenosine monophosphate (AMP) and pyrophosphoric acid by Acyl-CoA synthetase in the presence of coenzyme A and ATP. Oxidation by Acyl-CoA oxidase forms 2, 3,-trans-Enoyl-CoA and hydrogen peroxide, and in the presence of peroxidase, hydrogen peroxide forms a blue purple pigment by oxidative condensation with N-ethyl-N-(2hydroxy-3-sulphopropyl)m-toluidine and 4-aminiantipyrine. The absorbance of the blue purple pigment at 550 nm is proportional to the concentration of NEFA.

2.2.3.3.5. Beta-hydroxybutyrate

Blood samples were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing lithium heparin as anticoagulant. Tubes were inverted gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until analysed. Concentration of βHB was determined on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan) a kinetic enzymatic method and reagents supplied by Randox Laboratories (catalogue number RB 1007 (Section 2.1.2.4.6.), Randox Laboratories, Co. Antrim, UK). This method is based on the oxidation of hydroxybutyrate to acetoacetate by the enzyme 3-hydroxybutyrate dehydrogenase. Concomitant with this oxidation, the cofactor NAD^+ is reduced to NADH, and its associated change in absorbance at 340 nm is directly correlated to concentration of β-hydroxybutyrate.

2.2.3.3.6. Urea

Blood samples were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing lithium heparin as anticoagulant. Tubes were inverted gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until analysed. Concentration of urea was determined on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan) using a kinetic UV test method and the reagents supplied by Olympus (OSR6134 (Section 2.1.2.4.7) Olympus Life and Material Science Europa GmbH (Irish Branch), Co. Clare, Ireland). Briefly, urea, hydrolysed in the presence of water and urease produces ammonia and carbon dioxide. Ammonia combines with 2-oxoglutarate and NADH in the presence of glutamate-dehydrogenase to yield

glutamate and NAD^+. The decrease in NADH absorbance at 340/660 nm per unit time is proportional to the concentration of urea.

2.2.3.4. Haematology

2.2.3.4.1. Sample collection for complete blood count

Blood samples were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) spray dried with K_3EDTA as anticoagulant. Tubes were inverted gently several times to prevent clot formation and were processed within 3 h of collection.

2.2.3.4.2. Total leukocyte and differential count

Total leukocyte and differential count was determined using an automatic haematology analyser (ADVIA 2120 Haematology System, Siemens, UK). The flow cytometry-based system identifies cells according to their light scattering properties as they pass through a laser beam. The cell types counted include; total leukocytes, neutrophils, lymphocytes, monocytes, eosinophil and basophils.

2.2.3.4.2. Blood smears and Giemsa staining

To verify differential counts from the automatic haematological analyser, peripheral blood smears were prepared and differential cell morphology was examined under a microscope following Giemsa staining. Briefly, using a glass capillary tube, a drop of K_3EDTA anti-coagulated venous blood, approximately 2 - 3 mm in diameter was transferred onto a grease proof microscope slide. A second slide, placed directly in front of the drop at a 30 ° angle, was then drawn towards the blood allowing it to

spread evenly along the edge of the spreading slide and was then pushed forward in one smooth, quick motion forming a smear that is thick in appearance at the starting point and progressively thinner towards the opposite end of the slide. Smears were allowed to air-dry for 10 min prior to staining. Following this, blood smears were stained using a three-step method whereby smears were firstly fixed by immersion in methanol for 5 to 7 s. Excess fixative was drained from the slides before immersion into the initial staining solution, buffered Eosin (Section 2.1.2.5.2.), for 5 s. Excess stain was briefly drained from the slides and residual stain was removed using gently rinsing with Nanopure water. Blood smears were then immersed into the final staining solution containing buffered Azur 1 and methylene blue for 5 s, rinsed as previously stated, and allowed to air-dry. One hundred leukocytes in three microscopic fields within the zone of differentiation were counted to determine mean percentage of each cell type. Differential number was determined by multiplying the mean percentage of each cell type by the total leukocyte number for each sample.

2.2.3.4.3. Red blood cells and associated variables

Red blood cell number and associated variables determined using an automatic haematology analyser (ADVIA 2120 Haematology System, Siemens, UK). Variables include: haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelet number. Calculations for the aforementioned variables are described in Appendix 2.

2.2.4. Immunological Measurements

2.2.4.1. Flow cytometric analysis

2.2.4.1.1. Immunophenotyping

2.2.4.1.1.1. Sample collection for immunophenotyping

Blood samples (8 mL) were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing acid citrate dextrose (ACD) as anticoagulant. Tubes were inverted gently several times to prevent clot formation and were processed within 3 h of collection.

2.2.4.1.1.2. Immunostaining

Immunostaining was carried out using a whole blood assay described by Weber *et al.* (2001) for bovine samples. Briefly, 1 mL aliquot of whole blood was transferred to a 5 mL test tube (Sarstedt, Nümbreacht, Germany) and tubes were centrifuged at 250 × *g* for 5 min at 4 °C. After aspiration of supernatants, 3 mL of BD FACS lysing solution (Section 2.1.3.1.1., BD Biosciences, Oxford, UK) was added for 10 min at room temperature to lyse erythrocytes. Remaining leukocytes were re-suspended in 1.5mL of sheath fluid (Section 2.1.3.1.2., Coulter Isoton II Diluent (Beckman Coulter UK Ltd., High Wycombe, UK)) and counted using a Z1 Coulter Particle Counter (LABPLAN Ltd., Galway, Ireland). One hundred microliter aliquots of cell suspension (1×10^6 cells) were seeded into a series of wells on a 96-well microtiter plate followed by 100 µL of primary antibody. Plates were then sealed, and shaken at a low speed for 30 seconds on a plate shaker, and then incubated for 30 min at 4 ° C in

the dark. Following primary incubation, 100 µL PBS-1 (w/v) % BSA (pH 7.2) (Section 2.1.3.1.5.) was added to each well to stop the reaction, plates were centrifuged 250 × g for 5 min at 4 ° C, and the supernatant was carefully aspirated. The secondary antibody, FITC-labelled goat anti-mouse IgG F(ab')2 (Section 2.1.3.1.3), was added to each well (100 µL), plates were sealed and shaken at a low speed for 30 s, and then incubated for 15 min at 4 ° C in the dark. Following final incubation, plates were centrifuged as described above and supernatant was carefully aspirated and discarded. Stained leukocytes were fixed with 200 µl of 1 % (v/v) paraformaldehyde (Section 2.1.3.1.6.) and transferred to 12 × 75 mm flow cytometry tubes. Sample volume was increased by the addition of 800 µL of sheath fluid to allow for sample acquisition. Cells were kept at 4 °C in the dark prior to acquisition.

2.2.4.1.1.3. Immunophenotype data acquisition

A Partec SL flow cytometer (Partec, Münster, Germany) equipped with a 488 nm argon-ion laser (blue-green excitation light) and standard filter configuration (525 nm bandpass, 630 nm longpass was used for data acquisition. A minimum of 30,000 events were acquired and analysed using FloMax software (Partec GmbH, Münster, Germany). Lymphocytes and neutrophils were gated from all other leukocyte populations based on their forward and side scatter characteristics on dot plots and were confirmed using CD45 (pan leukocyte) staining (Pelan-Mattocks *et al.*, 2001). The percentage lymphocytes positive for CD4, CD8, WC1, and MHC class II, and percentage and the mean fluorescence intensity (MFI) of neutrophils positive for G1 was recorded. Surface expression of CD62L was recorded as MFI of CD62L positive neutrophils. A threshold for positive staining cells was set using histograms

identifying irrelevant isotype controls, secondary antibody alone and PBS-1 (w/v) % BSA only treated leukocytes.

2.2.4.1.2. Phagocytic activity of neutrophils

2.2.4.1.2.1. Sample collection for functional assays

Blood samples (8 mL) were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing lithium heparin. Tubes were inverted gently several times to prevent clot formation and were processed within 3 h of collection.

2.2.4.1.2.2. Neutrophil phagocytosis assay

The *in vitro* phagocytic activity of neutrophils was determined as per manufacturer's instructions (Orpegen Pharma, Heidelberg, Germany) with minor modifications as follows. Heparinised whole blood, stored on ice for 10 min, was gently mixed using a gentle rolling motion and 100 µL of blood per sample, in duplicate, was aliquoted to the bottom of a 5 ml test tube. Following vortexing to disaggregate *E. coli* cells, 20 µL of opsonised FITC-labelled *E. coli* (Section 2.1.3.2.1.) was added to each test sample. A third aliquot for each test sample received 20 µL of PBS and remained unstimulated serving as a negative control. After vortexing at an intermediate speed for 5 s, test samples were incubated at 38.5 ºC in a water bath and the controls were placed on ice for 10 min. At the end of the incubation, all samples where simultaneously placed on ice to stop phagocytosis. To eliminate the fluorescence of non-phagocytosed *E. coli* cells, 100 µL of quenching solution (Section 2.1.3.2.2.) was added to all samples and mixed well. Cells were washed twice using 3 mL of washing solution (Section 2.1.3.2.3.) and pelleted by centrifugation (250 g, for 5 min at 4 ºC). Following careful

aspiration of supernatants, cells were resuspended in 2 mL of lysing solution (Section 2.1.3.2.4.) for 20 min at room temperature to allow for the lysis of erythrocytes and fixation of phagocytes. After centrifugation, cells were recovered by resuspension in 3 mL of washing solution, and centrifuged at 250 g for 5 min at 4ºC. Supernatants were carefully aspirated and 200 µL of DNA staining solution (Section 2.1.3.2.5.) was added to all samples which were mixed well and incubated on ice for 10 min in the dark.

2.2.4.1.2.3. Data acquisition for phagocytosis assay

A Partec SL flow cytometer equipped with a 488 nm argon-ion laser (blue-green excitation light) and standard filter configuration (525 nm bandpass, 630 nm longpass was used for data acquisition. During data acquisition, a 'live gate' was set in red (630 nm) fluorescence histogram (FL2 channel) for those events having at least the same DNA content as diploid cells to exclude cell debris and bacterial aggregates. For all samples, > 10,000 events were acquired. A gate identifying the neutrophil population was set using the FSC and SSC properties of these cells and corresponding green fluorescence histogram (FL1) was analysed. The control sample was used to set a threshold for fluorescence so that only 1-3 % of the acquired events are positive. The number of events above this threshold were counted as percentage of neutrophils actively carrying out phagocytosis and the mean fluorescence intensity correlates to the number of bacteria phagocytosed by each cell.

2.2.4.1.3. Oxidative burst activity of neutrophils

2.2.4.1.3.1. Sample collection for functional assays

Blood samples (8 mL) were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing lithium heparin. Tubes were inverted gently several times to prevent clot formation and were processed within 3 h of collection.

2.2.4.1.3.2. Neutrophil oxidative burst activity assay

The *in vitro* oxidative burst activity of neutrophils was determined as per manufacturer's instructions (Orpegen Pharma, Heidelberg, Germany) with minor modifications as follows. Heparinised whole blood, stored on ice for 10 min, was gently mixed using a slow forward and back rolling motion and 100 μL of blood was aliquoted to the bottom of three 5 ml test tubes. Following vortex mixing to disaggregate *E. coli* cells, 20 μL of opsonised *E. coli* (Section 2.1.3.3.1.) was added to samples in duplicate. The addition of 20 μL of wash solution (Section 2.1.3.3.4) to a third aliquot served as negative control. Following gently vortexing, cells were incubated in a water bath at 38.5 ºC for 10 min. Dihydrorhodamine 123 substrate (Section 2.1.3.3.2.) was then added (20 μL) and tubes were mixed thoroughly before incubating for a further 10 min in a water bath at 37 ºC. The reaction was stopped and cells were simultaneously fixed using 2 mL pre-warmed (room temperature) lysing solution (Section 2.1.3.3.3.). Following short vortexing, cells were incubated at room temperature for 20 min. Cells were then pelleted following centrifugation (250 × g, 5 min, 4 ºC), and supernatant containing erythrocyte debris was discarded. Leukocytes were then resuspended with 3 mL of washing solution (Section 2.1.3.3.4.), and washed twice as previously stated. Supernatant was carefully aspirated between washes and

discarded. After the final wash step, cells were stained using DNA staining solution (Section 2.1.3.3.5.), mixed gently, and incubated on ice for 10 min in the dark. Samples were then immediately acquired by flow cytometry.

2.2.4.1.3.3. Data acquisition for oxidative burst assay

A Partec SL flow cytometer equipped with a 488 nm argon-ion laser (blue-green excitation light) and standard filter configuration (525 nm bandpass, 630 nm longpass was used for data acquisition following Phagotest and Bursttest procedures. During data acquisition, a 'live gate' was set in red (630 nm) fluorescence histogram (FL2 channel) for those events having at least the same DNA content as diploid cells to exclude cell debris and bacterial aggregates. For all samples, > 10,000 events were acquired. A gate identifying the neutrophil population was set using the FSC and SSC properties of these cells and corresponding green fluorescence histogram (FL1) was analysed. The control sample was used to set a threshold for fluorescence so that only 1-3 % of the acquired events are positive. The number of events above this threshold was counted as percentage of neutrophils actively carrying out oxidative burst activity and the mean fluorescence intensity correlates to the oxidative quantity per neutrophil.

2.2.4.2. Acute phase proteins

2.2.4.2.1. Haptoglobin

Blood samples (8 mL) were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing lithium heparin as anticoagulant. Tubes were inverted gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until

analysed. A colorimetric assay (Section 2.1.3.4.1., Eckersall *et al.*, 1999, Phase™ Range Haptoglobin Assay, Tridelta Development Ltd., Co. Kildare, Ireland) was used to determine concentration of haptoglobin on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan). Briefly, haptoglobin binds to haemoglobin in the sample, and at low pH, the peroxidase activity of haemoglobin bound to haptoglobin is preserved. Preservation of the peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin in the sample.

2.2.4.2.2. Fibrinogen

Blood samples (4.5 mL) were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing sodium citrate as anticoagulant. Tubes were inverted gently several times to prevent clot formation. Plasma was harvested following centrifugation at 2000 × *g* at 4 °C for 10 min and stored at -20 °C until analysed. A photometric assay (Section 2.1.3.4.2.) based on the method described by Becker *et al.* (1984) was carried out on an automatic analyser (spACE, Alfa Wassermann, Inc., West Caldwell, NJ, USA) to determine concentration of fibrinogen. Briefly, clotting velocity is a function of fibrinogen concentration in a sample, therefore by the addition of the enzyme Atroxin (Section 2.1.3.4.2.1.), the sample is digested and the rate of fibrinogen formation is recorded turbidimetrically.

2.2.4.3. Interferon-γ production assay

Blood samples (4.5 mL) were collected by direct venipuncture into test tubes (Vacuette, Cruinn, Dublin, Ireland) containing sodium citrate as anticoagulant. Tubes were inverted gently several times to prevent clot formation. A whole blood culture procedure, described by Wood *et al.* (1990) was used to determine the *in vitro*

lymphocyte production of IFN- γ. Duplicate 1.48 mL aliquots of blood were cultured in sterile 24 well flat culture plates (Sarstedt Ltd., Drinagh, Wexford, Ireland) with 20 μL of phosphate buffer saline (PBS) (GibcoBRL, Life Technologies Ltd., Paisley, Scotland, UK) containing 1.0 mg/mL of concanavalin A (Con A) (Section 2.1.3.5., Sigma-Aldrich, Inc., UK), 1.0 mg/mL phytohaemagglutinin (PHA) (Section 2.1.3.5.2., Sigma-Aldrich, Inc., UK) or no additive, for 24 h at 37 ºC and in an atmosphere of 5 % (v/v) CO_2. The culture plates were then centrifuged at 500 × g at 4 °C for 20 min, supernatant harvested and frozen at -20 ºC until assayed. A qualitative commercial bovine ELISA (Section 2.1.3.5.3., BOVIGAM, Biocor, Prionics AG, Switzerland) was used to determine in vitro production of IFN-γ. Using a 96-well plate, 50 μL of diluent (Section 2.1.3.5.4.) was added to the appropriate wells, followed by 50 μL of each sample and positive and negative controls (Section 2.1.3.5.5.) in duplicate to the appropriate wells. The plate was sealed, contents of the wells were mixed for 1 min at an intermediate speed on a plateshaker, and plate was incubated for 1 hr at room temperature. Contents of the wells were discarded and 400 μL of wash buffer (Section 2.1.3.5.5.) was added to each well. This wash step was carefully repeated 5 times. After the sixth wash step, the plate was allowed to drain face down on clean filter paper. One hundred microliters of conjugate (Section 2.1.3.5.6.) was added to the wells and mixed as before. Plates were incubated at room temperature for 1hr. Wells were then washed as before, 100 μL of freshly prepared enzyme substrate (Section 2.1.3.5.7.) was added and plates were sealed, mixed and incubated at room temperature for 30 min whilst wrapped in tinfoil to protect from sunlight. After incubation, 50 μL of enzyme stopping solution (Section 2.1.3.5.8) was added to each well. The absorbance of each well at 450 nm was recorded immediately using a plate reader (TECAN SUNRISE Absorbance Reader, TECAN Austria GmbH, Austria). The Con

A or PHA stimulated production of IFN-γ was calculated by subtracting the absorbance at 450 nm of wells that received PBS alone from wells that received Con A or PHA, respectively.

2.2.5. Behavioural Analysis

Lying and walking behaviour was monitored using IceTag motion sensor pedometers (IceTag 2.004, IceRobotics, Midlothian, Scotland, UK) attached to the front left leg of each calf with a Velcro strap on d 0 (weaning and housing), and were removed on d 14. Data from IceTag pedometer was downloaded onto a PC with the software IceTag Analyser^{TM} (2.009), and steps per day, percentage of time spent lying, standing or active were registered. Seven complete days of data were used in the analysis. Feeding behaviour was monitored using 24 h continuous camera recordings of each pen. Cameras were connected to a DVD recorder via a multi-vision system (Robot, duplex multiplexer), which allowed pictures from all cameras to be viewed on one screen at one time, and were calibrated with time and date settings. For each pen, behavioural observations were recorded on d 0 (weaning and housing), 1, 2, 3, 7 and 14. The calves were observed by instantaneous scan sampling and the interval between scans was 10 min. Each calf was observed for eating concentrates (head in the concentrate trough), eating silage at the feed face and drinking water. A count of the total number of occurrences of each behaviour was made for each scan time point. For presentation purposes, the percentage time values were calculated from the total count data for eating and drinking behaviours. As the animals were subjected to continuous recordings, the count data was expressed as percentage time.

2.2.6. Statistical Analysis

The statistical analysis for chapters 3 to 6 are described separately below. All statistical analysis was performed using SAS/STAT Version 9.1. for Windows (SAS Institute, Cary, NC, USA, 2003).

2.2.6.1. Statistical analysis (Chapter 3)

All data were tested for normality using the PROC UNIVARIATE procedure of SAS. Data that was not normally distributed were transformed by a square root transformation prior to statistical analysis. Data were analysed as repeated measures using the PROC MIXED procedure of SAS with an unstructured covariance matrix within animal. Two datasets were analysed, i) the onset of weaning, d 0 to d 35, and ii) the onset of subsequent housing of P, d 0 to d 21. The effects of sampling time, treatment, genotype and gender and all possible interactions were listed in the model statement. Genotype and gender were not significant and were not included in the final model. Least squares means were estimated, differences between least squares means were determined using a Tukey-Kramer adjustment for multiple comparisons, and the associated *P*-values presented were derived from the statistical analysis of appropriately transformed data using the model described above. A probability of $P < 0.05$ was selected as the level of significance.

2.2.6.2. Statistical analysis (Chapter 4)

All data were tested for normality using the PROC UNIVARIATE procedure of SAS. Data was analysed as 2 separate datasets relative to management practice: i.) weaning (d 0 to d 35), and ii.) subsequent housing (d 0 to d 21). Each dataset was analysed as repeated measures using the PROC MIXED procedure of SAS with an unstructured

covariance matrix within animal. Breed (Limousin × Holstein-Friesian and Simmental × Holstein-Friesian) and sampling time were listed in the model statement. Breed was not significant and was excluded from the final model. Least squares means were estimated and differences between least squares means were tested using the PDIFF option in SAS. The PDIFF option calculates a separate probability value for each pair of means being compared. Means were considered significantly different at the $P < 0.05$ probability level.

2.2.6.3. Statistical analysis (Chapter 5)

All statistical analysis was performed using SAS/STAT for Windows [62]. Data were tested for normality using PROC UNIVARIATE, and data (neutrophil G1 data only) that did not meet parametric assumptions were transformed by a square root transformation prior to statistical analysis. Data were then analyzed as repeated measures using the PROC MIXED procedure of SAS with an unstructured covariance matrix within animal. The effects of treatment, sampling time, genotype and all possible interactions were listed in the model statement. Genotype was not significant ($P > 0.05$) and was not included in the model. Day -7 was included as a covariate but this was not significant ($P > 0.05$) and was omitted from the model. Baseline is defined as day 0 for each variable in the description of the results and representation of the data. Least squares means were estimated, differences between least squares means were determined using the Tukey-Kramer test for multiple comparisons, and the associated *P*-values presented were derived from the statistical analysis of the data using the model described above. A probability of $P < 0.05$ was selected as the level of significance.

2.2.6.4. Statistical analysis (Chapter 6)

All statistical analysis was performed using SAS/STAT for Windows (SAS, 2003). Data were tested for normality using PROC UNIVARIATE, and data (eosinophil and basophil number, phagocytosis positive neutrophils MFI, oxidative burst positive neutrophils MFI, βHB, creatine kinase and urea) that did not meet parametric assumptions were log transformed prior to statistical analysis. Data were then analysed as repeated measures using the PROC MIXED procedure of SAS with an unstructured covariance matrix within animal. The effects of treatment, sampling time and treatment × sampling time interactions were listed in the model statement and day -7 was included as a covariate. For behavioural data, no covariate was included in the model which listed the effects of treatment, sampling time and treatment × sampling time interaction. Least square means were estimated and differences between least square means were determined using Tukey Kramer Adjustment for multiple comparisons. A probability of $P < 0.05$ was selected as the level of significance.

Chapter 3

Effect of post-weaning management practices on physiological and immunological responses of newly weaned beef calves

3.1 Introduction

Seasonal, grassland-based suckler beef production systems typically comprise a grazing season followed by an indoor winter period. The majority of calves are spring-born and allowed to continually nurse the dam at pasture until the end of the grazing season in autumn following which, they are weaned and generally housed indoors for a period up to five months (Drennan and McGee, 2009). In non-integrated systems, housing of weaned calves is preceded by transport and mixing with unfamiliar calves at livestock markets. Alterations in immune function and hormonal mediators of stress are evident in beef calves 7 d after abrupt weaning (Hickey *et al.*, 2003a). Bovine respiratory disease is a major animal welfare and economic concern for the beef cattle industry, particularly with newly weaned and feedlot cattle (Duff and Galyean, 2007). Consequently, there is a need to better understand and mitigate weaning associated stress, which may otherwise predispose calves to infection.

Weaning is a necessary husbandry practice in which nutritional, social, physical and psychological stressors are imposed on the calf. Research to date has examined the effect of breed and age at weaning (Blanco *et al.*, 2009), and the benefit of management strategies designed to mitigate stress and improve performance, namely two-stage weaning with nose-flaps (Haley *et al.*, 2005) and fence-line contact post-weaning (Price *et al.*, 2003; Boland *et al.*, 2008). Distress behaviours (e.g. increased vocalisations and activity) are most prominent immediately after weaning and progressively decline over 7 d post-weaning (Price *et al.*, 2003; Boland *et al.*, 2008). There is, however, a paucity of literature examining the effect of post-weaning management practices on the physiological responses in the beef calf, particularly over an extended period (> 7 d) post-weaning.

The objectives of this study were to (i) investigate the physiological, haematological and immunological responses of previously grazed, abruptly weaned beef calves that were (a) housed (H) and offered a new diet of grass silage *ad libitum* plus concentrates or (b) returned to familiar pasture (P), and (ii) to examine, the effect of subsequent housing on these responses in P compared with previously housed (H) calves. The experimental design is described in Section 2.2.2.1.

3.2 Effect of post-weaning management practices on physiological responses of weaned beef calves

3.2.1 Rectal body temperature

There was a treatment × time interaction ($P < 0.05$) for rectal body temperature post-weaning and post-housing. Rectal body temperature increased in H ($P < 0.001$, mean (s.e.m.) 39.4 (0.10) °C) and P ($P < 0.05$, mean (s.e.m.) 39.4 (0.12) °C) on d 2 and returned to pre-weaning baseline (d 0 at weaning, H: mean (s.e.m.) 38.9 (0.11) °C, P: mean (s.e.m.) 39.1 (0.10) °C)) on d 7 (Figure 3.1a). On d 7, rectal body temperature was higher ($P < 0.05$) in H (mean (s.e.m.) 39.1 (0.11) °C) than in P (mean (s.e.m.) 38.7 (0.12) °C), following which they did not differ ($P > 0.05$). On d 2 post-housing, rectal body temperature was higher ($P < 0.01$) in P (mean (s.e.m.) 39.5 (0.10) °C) and was unchanged in H (mean (s.e.m.) 38.9 (0.12) °C). Rectal body temperature returned to baseline (day 0 at housing) on d 7 in P (Figure 3.1b).

3.2.2 Cortisol and dehydroepiandrosterone (DHEA)

There was no effect of treatment or treatment × time interactions ($P > 0.05$) for concentration of cortisol and DHEA post-weaning or post-housing. Concentration of cortisol increased ($P < 0.05$) from mean (s.e.m.) 9.15 (0.66) ng/mL to mean (s.e.m.) 9.9 (0.69) ng/mL on d 2 to d 21 in H and P and returned to pre-weaning baseline on d 28 (Table 3.1). Post-housing, there was no effect of treatment or treatment × time interactions ($P > 0.05$) for cortisol and DHEA (Table 3.1).

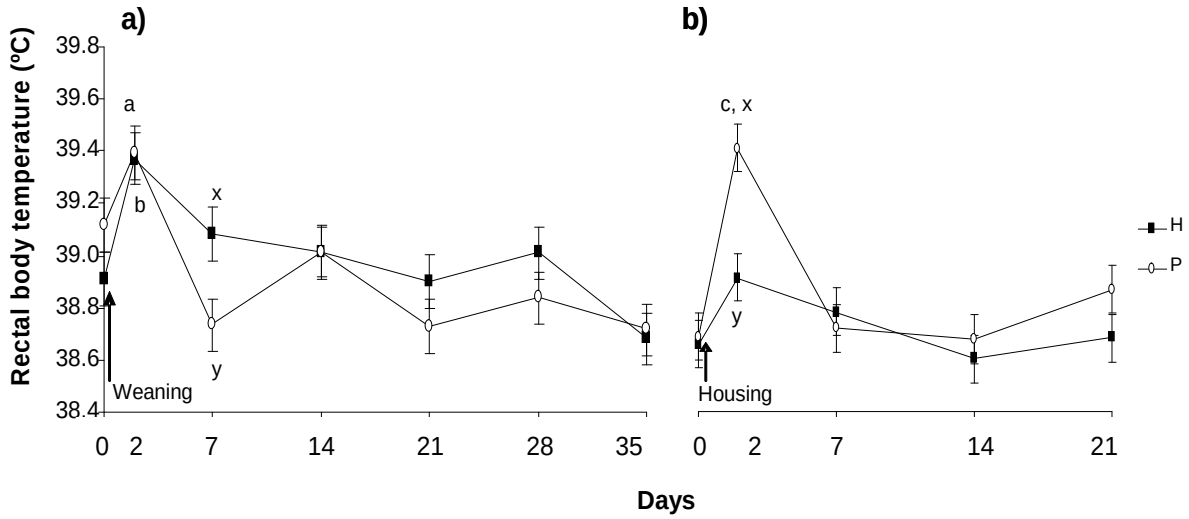

Figure 3.1a and b: Effect of post-weaning management practice on rectal body temperature in beef calves that were abruptly weaned and either housed (H: -■-, n = 18) and offered new diet or returned to pasture (P: -○-, n = 18) for 35 days, deferring the onset of housing and subsequently comparing the response of P to housing with calves habituated to housing (H).

Post-weaning and post-housing changes for H and P calves are shown on a and b, respectively. [a]Lsmeans for P calves differ from pre-weaning baseline (d 0) by $P < 0.05$, [b]Lsmeans for H calves differ from pre-weaning baseline (d 0) by $P < 0.001$, [c]Lsmeans for P calves differ from pre-housing baseline (d 0) by $P < 0.001$, [x,y]Treatment Lsmeans that do not have a common superscript differ by $P < 0.05$.

Table 3.1 Effect of post-weaning management practice on concentrations of cortisol and dehydroepiandrosterone (DHEA) in weaned beef calves[1,2]

		Pre-weaning	Days post-weaning							*P*-values[3]		Pre-housing	Days post-housing				
Variable		**0**	**2**	**7**	**14**	**21**	**28**	**35**	**s.e.m.**	**T**	**S**	**0**	**2**	**7**	**14**	**21**	**s.e.m.**
Cortisol	**H**	7.2	9.2[b]	9.7[c]	9.9[c]	10.4[c]	6.0	6.4	0.65	NS	***	6.4	6.2	6.8	5.9	6.6	0.51
(ng/mL)	**P**	5.5	9.1[c]	8.0[b]	10.1[c]	9.4[c]	7.0	6.3	0.67			6.3	6.8	6.3	6.3	6.7	0.54
DHEA	**H**	1.6	2.4	2.4	4.8	5.0	0.9	1.8	0.89	NS	**	1.8	2.2	3.0	2.7	1.4	0.62
(ng/mL)	**P**	8.8	0.8	0.9	3.7	1.8	0.6	3.2	0.89			3.2	2.6	1.3	1.8	1.8	0.61

[1]There were no treatment × sampling time interactions for concentration of cortisol or DHEA post-weaning therefore only the main effects are shown. [2]Treatment, sampling time and their interaction were not significant post-housing.

[3]T = treatment, S = sampling time, NS = not significant, $P > 0.05$. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

[a,b,c] Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

[x,y] Between rows for each variable, treatment Lsmeans differ by $P < 0.05$.

n = 18 calves per treatment.

3.2.3 Total protein, albumin and globulin

Post-weaning, there was a treatment × sampling time interaction ($P < 0.05$) for total protein, whereby on d 35, it increased ($P < 0.01$) in P (mean (s.e.m.) 78.0 (1.04) g/dL) compared with pre-weaning baseline (mean (s.e.m.) 74.7 (0.9) g/dL), and was higher ($P < 0.05$) than H (mean (s.e.m.) 72.2 (1.04) g/dL) (Table 3.2). There was a treatment × sampling time interaction ($P < 0.05$) for albumin and globulin concentration post-weaning. Concentration of albumin decreased ($P < 0.05$) in H and P on d 14 to d 35. On d 35, H (mean (s.e.m.) 31.9 (0.45) g/dL) was lower ($P < 0.05$) than P (mean (s.e.m.) 34.0 (0.45) g/dL). Concentration of globulin decreased ($P < 0.05$) on d 14 and d 21 to d 35 in H and P, respectively, and it was higher ($P < 0.05$) in P (mean (s.e.m.) 43.9 (1.14) g/L) on d 35 compared with H (mean (s.e.m.) 40.3 (1.13) g/L) (Table 3.2).

Post-housing, there was a treatment × sampling time interaction ($P < 0.05$) for total protein, whereby at pre-housing baseline the concentration of total protein was higher ($P < 0.05$) in P than H, and it subsequently decreased ($P < 0.05$) in P on d 7 (mean (s.e.m.) 73.9 (1.24) g/dL) and 21 (mean (s.e.m.) 73.0 (1.28) g/dL) and did not differ ($P > 0.05$) from H. There were no effects of treatment or treatment × sampling time interactions ($P > 0.05$) for concentration of albumin and globulin post-housing. Concentration of albumin decreased ($P < 0.001$) on d 7 (mean (s.e.m.) 32.0 (0.46) g/dL) to 21 (mean (s.e.m.) 30.2 (0.50) g/dL) in P, and decreased ($P < 0.05$) in H on d 21 (mean (s.e.m.) 30.7 (0.50) g/dL) compared with pre-housing baselines (P: mean (s.e.m.) 34.0 (0.45) g/dL; H: mean (s.e) 31.9 (0.45) g/dL) (Table 3.2).

3.2.4 Creatine kinase

There was no effect of treatment or treatment × sampling time interaction ($P > 0.05$) for creatine kinase activity post-weaning (Table 3.2). On d 2, it increased ($P < 0.05$) in P compared with pre-weaning baseline (mean (s.e.m.) 582 (37.6) U/L versus mean (s.e.m.) 407 (29.0) U/L), and subsequently did not differ ($P > 0.05$) from pre-weaning baseline for the remainder of the experimental period. On d 14 to 35, creatine kinase activity in H decreased ($P < 0.01$; mean (s.e.m.) 228 (29.5) U/L on d 14 and mean (s.e.m.) 197 (29.2) U/L on d 35) compared with pre-weaning baseline (mean (s.e.m.) 465 (29.0) U/L).

Post-housing, there was no effect of treatment or treatment × time interaction ($P > 0.05$) for creatine kinase activity. It decreased ($P < 0.05$) on d 14 (mean (s.e.m.) 194 (16.4) U/L) and 21 (mean (s.e.m.) 199 (18.6) U/L) in P compared with pre-housing baseline (Table 3.2).

3.2.5 Glucose, β-hydroxybutyrate (βHB) non-esterified fatty acid (NEFA)

Post-weaning, there was a treatment × sampling time interaction ($P < 0.001$) for glucose whereby the concentration of glucose in P (mean (s.e.m.) 4.3 (0.11) mmol/L) was lower ($P < 0.05$) than that of H (mean (s.e.m.) 4.9 (0.12) mmol/L) on d 2 and continued to decrease ($P < 0.001$) up to 35 (mean (s.e.m.) 4.4 (0.11) mmol/L over this period) compared with pre-weaning baseline (mean (s.e.m.) 4.8 (0.09) mmol/L), whereas there was no difference ($P > 0.05$) in concentration from d 2 to 35 (mean (s.e.m.) 4.8 (10.6) mmol/L) in H compared with pre-weaning baseline (mean (s.e.m.) 4.8 (0.09) mmol/L) (Table 3.2). There was a treatment × sampling time interaction ($P < 0.001$) for NEFA concentration post-weaning, whereby on d 2 the concentration of NEFA was increased ($P < 0.01$) in H and P compared with pre-weaning baseline (H:

mean (s.e.m.) 0.15 (0.014) mmol/L; P: mean (s.e.m.) 0.13 (0.014) mmol/L) and was higher ($P < 0.05$) in P (mean (s.e.m.) 0.37 (0.013) mmol/L) compared with H (mean (s.e.m.) 0.27 (0.013) mmol/L) (Table 3.2). On d 14 to 35, concentration of NEFA was higher ($P < 0.05$) in P (range (min – max) over this period, mean (s.e.m.) 16-45 (0.016) mmol/L) compared with H (mean (s.e.m.) 0.08 (0.017) mmol/L). For concentration of βHB, there was also a treatment × sampling time interaction ($P < 0.001$) on d 2 to 35, whereby it increased ($P < 0.001$) in H and P compared with pre-weaning baseline (H: mean (s.e.m.) 0.17 (0.015) mmol/L; P: mean (s.e.m.) 0.14 (0.015) mmol/L), and the increase was greater ($P < 0.05$) in P on d 2 (mean (s.e.m.) 0.41 (0.020) mmol/L) to 14 (mean (s.e.m.) 0.35 (0.012) mmol/L) compared with H (d 2: mean (s.e.m.) 0.32 (0.020) mmol/L; d 14 mean (s.e.m.) 0.27 (0.015) mmol/L). There was no difference ($P > 0.05$) between H and P on d 35 (Table 3.2).

Post-housing, there was a treatment × sampling time interaction ($P < 0.001$) for NEFA whereby the concentration of NEFA in P decreased ($P < 0.001$) on d 2 (mean (s.e.m.) 0.11 (0.009) mmol/L) to 35 (mean (s.e.m.) 0.07 (0.005) mmol/L), whereas it was unchanged ($P > 0.05$) in H compared with pre-housing baseline (P: mean (s.e.m.) 0.45 (0.029) mmol/L, H: mean (s.e) 0.08 (0.029) mmol/L). On d 14, concentration of NEFA was higher ($P < 0.05$) in P (mean (s.e.m.) 0.13 (0.014) mmol/L) compared with H (mean (s.e.m.) 0.07 (0.009) mmol/L). There were no effects of treatment or treatment × sampling time interactions ($P > 0.05$) for glucose or βHB (Table 3.2).

Table 3.2. Effect of post-weaning management practice on concentrations of blood metabolites in weaned beef calves.

		Pre-weaning	Days post-weaning							*P*-values[1]			Pre-housing	Days post-housing					*P*-values[1]		
Variable		**0**	**2**	**7**	**14**	**21**	**28**	**35**	**s.e.m.**	**T**	**S**	**T×S**	**0**	**2**	**7**	**14**	**21**	**s.e.m.**	**T**	**S**	**T×S**
TP[2]	**H**	74.0	74.7	74.2	73.4	73.3	72.2	72.2^{x}	0.99	NS	NS	*	72.2^{x}	72.6	73.1	72.3	69.9	1.01	*	**	*
(g/dL)	**P**	74.7	75.2	73.6	75.1	74.5	75.1	$78.0^{b,y}$	1.04				78.0^{y}	75.6	73.9^{d}	75.6	73.0^{d}	1.21			
Albumin	**H**	36.0	36.0	35.6	34.0^{c}	32.9^{c}	$31.9^{c,x}$	$31.9^{c,x}$	0.33	NS	***	*	31.9	32.1	31.7	31.2	30.7^{d}	0.46	NS	***	NS
(g/dL)	**P**	35.8	36.3	35.7	34.4^{a}	33.8^{c}	$33.4^{c,y}$	$34.0^{a,y}$	0.32				34.0	33.5	32.0^{f}	31.4^{f}	30.2^{f}	0.48			
Globulin	**H**	38.0	38.7	38.6	39.4	40.5^{a}	40.3^{a}	$40.^{3a,x}$	1.08	NS	***	*	40.3	40.4	41.3	41.1	38.9	1.24	NS	NS	NS
(g/dL)	**P**	38.9	38.9	37.9	40.7^{a}	40.7^{a}	41.6^{a}	$43.9^{c,y}$	1.02				43.9	42.0	41.9	44.3	42.8	1.29			
CK[3]	**H**	465	457	294	228^{b}	208^{b}	236^{b}	197^{b}	29.0	NS	***	NS	197	205	192	321	195	44.0	NS	NS	NS
(U/L)	**P**	407	582^{a}	388	316	298	346	340	27.9				340	253	275	194^{a}	199^{a}	46.0			
Glucose	**H**	4.8	4.9^{x}	4.8	5.0^{x}	4.7	4.8	4.7	0.09	**	***	***	4.7	4.7	4.6	4.7	4.2^{f}	0.12	NS	***	NS
(mmol/L)	**P**	4.8	$4.3^{c,y}$	4.4^{a}	$4.3^{c,y}$	4.4^{a}	4.5^{a}	4.3^{b}	0.11				4.3	4.2	4.7^{f}	4.5	4.3	0.14			
NEFA[4]	**H**	0.15	$0.27^{b,x}$	0.14	0.08^{x}	0.08^{x}	0.08^{x}	0.08^{x}	0.015	***	***	***	0.08^{x}	0.09	0.09	0.07^{x}	0.07	0.011	***	***	***
(mmol/L)	**P**	0.13	$0.37^{c,y}$	0.11	0.18^{y}	0.16^{y}	$0.25^{c,y}$	$0.45^{c,y}$	0.013				0.45^{y}	0.11^{f}	0.13^{f}	$0.13^{f,y}$	0.07^{f}	0.013			
βHB[5]	**H**	0.17	$0.32^{c,x}$	$0.25^{c,x}$	$0.27^{c,x}$	0.28^{c}	$0.38^{c,x}$	0.29^{c}	0.014	NS	***	***	0.29	0.30	0.30	0.28	0.32	0.0013	NS	**	NS
(mmol/L)	**P**	0.14	$0.41^{c,y}$	$0.35^{c,y}$	$0.35^{c,y}$	0.29^{c}	$0.27^{c,y}$	0.26^{c}	0.015				0.26	0.33^{e}	0.28	0.25	0.33^{e}	0.0013			

[1]T = treatment, S = sampling time, T × S treatment × sampling time interaction, NS = not significant, $P > 0.05$. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

[2]TP = Total protein, [3]CK= creatine kinase activity, [4]NEFA = non-esterified fatty acids, [5]βHB = β-hydroxybutyrate.

[a,b,c] Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

[d,e,f] Within rows, Lsmeans differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

[x,y] Between rows for each variable, treatment Lsmeans differ by $P < 0.05$. n = 18 calves per treatment.

3.2.6 Urea

Post-weaning, there was a treatment × sampling time interaction ($P < 0.001$) whereby the concentration of urea increased ($P < 0.001$) in P and decreased ($P < 0.001$) in H on d 2 to 35 compared with pre-weaning baseline (P: mean (s.e.m.) 5.6 (0.23) mmol/L; H: mean (s.e.m.) 6.0 (0.23) mmol/L) with a significant difference ($P < 0.001$) between H and P over this period (Figure 3.2a).

Post-housing, concentration of urea decreased ($P < 0.001$) in P from mean (s.e.m.) 7.1 (0.22) mmol/L at pre-housing baseline to mean (s.e.m.) 2.8 (0.15) mmol/L on d 21 (Figure 3.2.b).

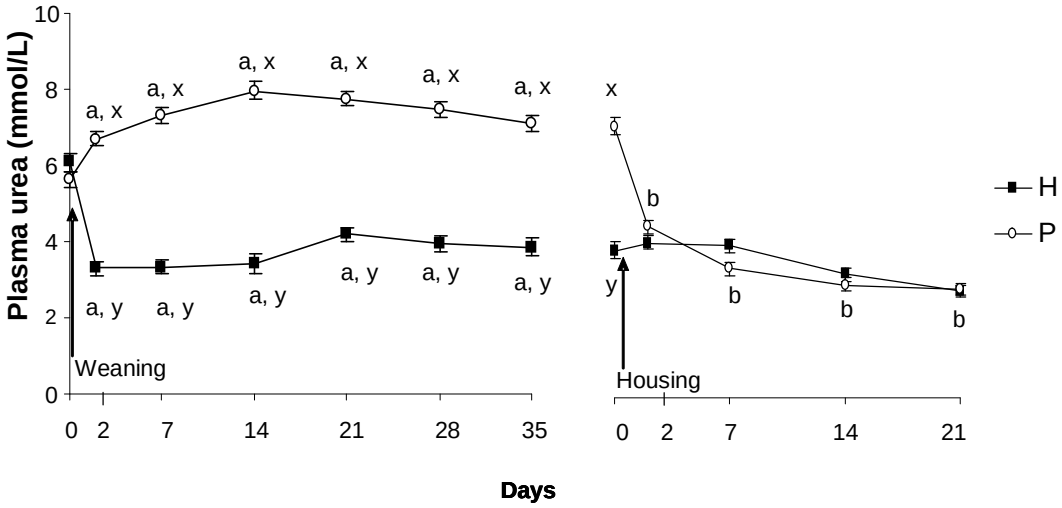

Figure 3.2a and b: Effect of post-weaning management practice on concentration of plasma urea in beef calves that were abruptly weaned and either housed (H, n = 18) and offered new diet or returned to pasture (P, n = 18) for 35 days, thus deferring the onset of housing and subsequently comparing the response of P to housing with calves habituated to housing (H).

Post-weaning and post-housing changes for H and P calves are shown on a and b, respectively. [a]Lsmeans differ from pre-weaning baseline (day 0) by $P < 0.001$. [b]Lsmeans differ from pre-housing baseline (day 0) by $P < 0.001$. [x,y]Treatment Lsmeans that do not have common superscript differ by $P < 0.05$. There was a treatment × sampling time ($P < 0.001$) for urea post-weaning and no effect of treatment or treatment × sampling time interaction ($P > 0.05$) post-housing.

3.3 Effect of post-weaning management practices on immunological responses of weaned beef calves

3.3.1 Total leukocytes and differential populations

Post-weaning, there was a treatment × time interaction ($P < 0.01$) for total leukocyte, neutrophil, lymphocyte number and N:L ratio. Total leukocyte number increased ($P < 0.05$) on d 2 (mean (s.e.m.) 14.0 (0.56) $\times 10^3$ cells/ μL) and d 7 (mean (s.e.m.) 13.7 (0.62) $\times 10^3$ cells/ μL) in P and decreased ($P < 0.05$) on d 14 (mean (s.e.m.) 10.8 (0.51) $\times 10^3$ cells/ μL) to d 35 (mean (s.e.m.) 10.9 (0.50) $\times 10^3$ cells/ μL) in H compared with pre-weaning baseline (P: mean (s.e.m.) 12.5 (0.71) $\times 10^3$ cells/ μL, H: mean (s.e.m.) 13.5 (0.72) $\times 10^3$ cells/ μL) (Figure 3.2b). Neutrophil number increased ($P < 0.01$) on d 2 (mean (s.e.m.) 3.6 (0.30) $\times 10^3$ cells/ μL) and 7 (mean (s.e.m.) 3.8 (0.35) $\times 10^3$ cells/ μL) in P, whereas it decreased ($P < 0.05$) on d 7 (mean (s.e.m.) 3.1 (0.35) $\times 10^3$ cells/ μL) to 35 (mean (s.e.m.) 2.3 (0.34) $\times 10^3$ cells/ μL) in H compared with pre-weaning baseline (Table 3.3). Lymphocyte number decreased ($P < 0.05$) on d 2 (mean (s.e.m.) 8.0 (0.45) $\times 10^3$ cells/ μL) to 28 (mean (s.e.m.) 7.7 (0.53) $\times 10^3$ cells/ μL) in H and increased on d 2 in P (mean (s.e.m.) 9.3 (0.45) $\times 10^3$ cells/ μL) compared with pre-weaning baseline (H: mean (s.e.m.) 8.6 (0.48) $\times 10^3$ cells/ μL, P: mean (s.e.m.) 8.5 (0.48) $\times 10^3$ cells/ μL) (Table 3.3). Neutrophil: lymphocyte ratio increased on d 2 (mean (s.e.m.) 0.41 (0.041)) and 7 (mean (s.e.m.) 0.43 (0.040)) in P and decreased ($P < 0.01$) on d 14 (mean (s.e.m.) 0.33 (0.033)) to 35 (mean (s.e.m.) 0.29 (0.047)) in H compared with pre-weaning baselines (H: mean (s.e.m.) 0.45 (0.046), P: mean (s.e.m.) 0.33 (0.046)) (Table 3.3). There was no effect of treatment or treatment × sampling time interaction ($P > 0.05$) for monocyte and eosinophil number post-weaning. Monocyte number did not differ ($P > 0.05$) from pre-weaning baseline in H (mean

(s.e.m.) 0.52 (0.08) × 10^3 cells/ μL) and P (mean (s.e.m.) 0.51 (0.08) × 10^3 cells/ μL) post-weaning, except on d 28 when monocyte number in P increased ($P < 0.05$, mean (s.e.m.) 0.74 (0.05) × 10^3 cells/ μL). Eosinophil number increased ($P < 0.05$) in H and P on d 2 (H: mean (s.e.m.) 0.46 (0.06) × 10^3 cells/ μL, P: mean (s.e.m.) 0.48 (0.08) × 10^3 cells/ μL), and subsequently decreased ($P < 0.05$) on d 21 (H: mean (s.e.m.) 0.13 (0.03) × 10^3 cells/ μL P: mean (s.e.m.) 0.19 (0.03) × 10^3 cells/ μL) to 35 (H: mean (s.e.m.) 0.10 (0.02) × 10^3 cells/ μL P: mean (s.e.m.) 0.18 (0.02) × 10^3 cells/ μL) compared with pre-weaning baseline (H: mean (s.e.m.) 0.27 (0.09) × 10^3 cells/ μL, P: mean (s.e.m.) 0.29 (0.09) × 10^3 cells/ μL). There was a treatment × time interaction ($P < 0.01$) for basophil number whereby basophil number decreased ($P < 0.001$) in H on d 7 (mean (s.e.m.) 0.12 (0.009) × 10^3 cells/ μL) and 14 (mean (s.e.m.) 0.12 (0.008) × 10^3 cells/ μL) and increased ($P < 0.05$) in P on d 21 (mean (s.e.m.) 0.18 (0.010) × 10^3 cells/ μL) to 35 (mean (s.e) 0.18 (0.009) × 10^3 cells/ μL) compared with pre-weaning baseline (H: mean (s.e.m.) 0.17 (0.015) × 10^3 cells/ μL; P: mean (s.e.m.) 0.14 (0.015) × 10^3 cells/ μL). Basophil number was higher ($P < 0.05$) in P than in H from d 14 to d 35 (Table 3.3).

Post-housing, there was a treatment × time interaction ($P < 0.05$) for total leukocyte, neutrophil, and lymphocyte number and N:L ratio. Total leukocyte number decreased ($P < 0.05$) in P on d 7 (mean (s.e.m.) 11.0 (0.47) ×10^3 cells/ μL) to 21 (mean (s.e.m.) 11.5 (0.45) ×10^3 cells/ μL) and was unchanged ($P > 0.05$) in H compared with pre-housing baseline (mean (s.e.m.) 12.8 (0.49) ×10^3 cells/ μL) (Figure 3.2b). Neutrophil number in P increased ($P < 0.05$) on d 2 (mean (s.e) 3.7 (0.26) ×10^3 cells/μL) and subsequently decreased ($P < 0.001$) on d 7 (mean (s.e.m.) 2.3 (0.25) ×10^3 cells/ μL) and 14 (mean (s.e.m.) 2.4 (0.30) ×10^3 cells/ μL) compared with pre-housing baseline (mean (s.e.m.) 3.1 (0.29) ×10^3 cells/ μL), whereas neutrophil number in H did

not differ ($P > 0.05$) compared with pre-housing baseline (mean (s.e.m.) 2.3 (0.28) $\times 10^3$ cells/ μL) (Table 3.3). On d 2, lymphocyte number decreased ($P < 0.05$) in P (mean (s.e.m.) 7.8 (0.32) $\times 10^3$ cells/ μL), whereas it was unchanged ($P > 0.05$) in H (mean (s.e.m.) 7.8 (0.31) $\times 10^3$ cells/ μL) compared with pre-housing baseline (P: mean (s.e.m.) 8.6 (0.34) $\times 10^3$ cells/ μL; H: mean (s.e.m.) 7.9 (0.33) $\times 10^3$ cells/ μL). The N:L ratio increased ($P < 0.05$) on d 2 (mean (s.e.m.) 0.50 (0.041)), and subsequently decreased ($P < 0.01$) on d 7 (mean (s.e.m.) 0.29 (0.035)) to 21 (mean (s.e.m.) 0.33 (0.029)) in P, whereas it was unchanged in H (mean (s.e.m.) 0.29 (0.035)) compared with pre-housing baseline (P: mean (s.e) 0.40 (0.039); H: mean (s.e) 0.29 (0.038)) (Table 3.3). There were no effects of treatment or treatment × sampling time interactions ($P > 0.05$) for monocyte, eosinophil and basophil number post-housing. Monocyte number decreased ($P < 0.001$) in P on d 14 (mean (s.e.m.) 0.47 (0.03) $\times 10^3$ cells/ μL) and 21 (mean (s.e.m.) mean (s.e.m.) 0.37 (0.03) $\times 10^3$ cells/ μL) compared with pre-housing baseline (mean (s.e.m.) 0.65 (0.04) $\times 10^3$ cells/ μL) (Table 3.3).

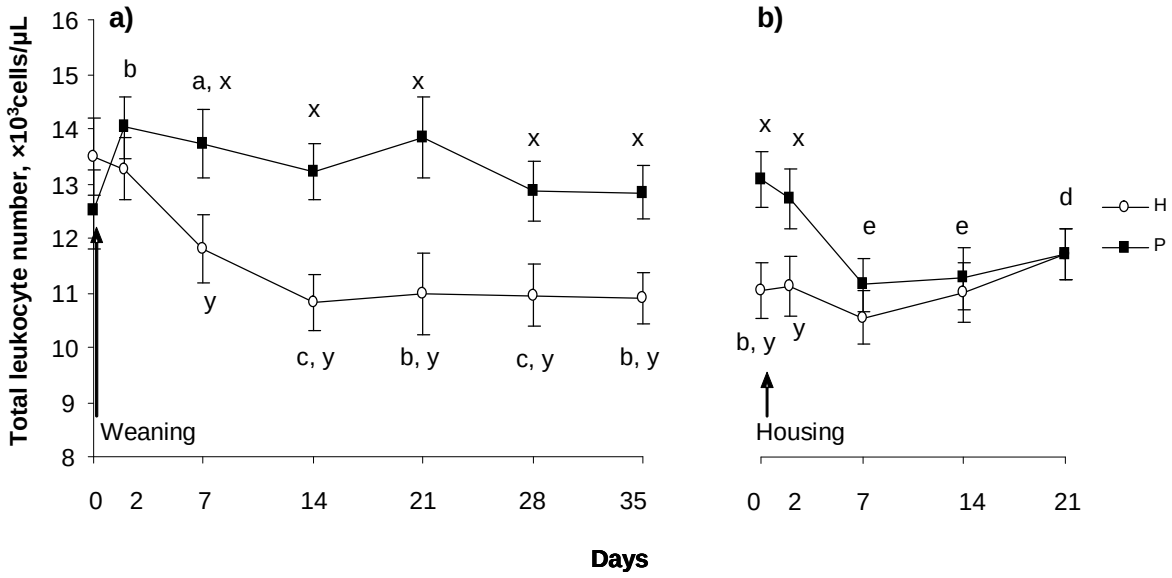

Figure 3.3a and b: Effect of post-weaning management practice on total leukocyte number in beef calves that were abruptly weaned and either housed (H:-■-, n = 18) and offered new diet or returned to pasture (P: -○-, n = 18) for 35 days, deferring the onset of housing and subsequently comparing the response of P to housing with calves habituated to housing (H).

Post-weaning and post-housing changes for H and P calves are shown on a and b, respectively. [a,b,c]Lsmeans differ from pre-weaning baseline (day 0) by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. [d,e]Lsmeans differ from pre-housing baseline (day 0) by $P < 0.05$ and $P < 0.01$, respectively. [x,y]Treatment Lsmeans that do not have common superscript differ by $P < 0.05$. There was a treatment × sampling time interaction post-weaning ($P < 0.01$) and post-housing ($P < 0.05$).

Table 3.3 Effect of post-weaning management practice on leukocyte differential populations and neutrophil: lymphocyte ratio in weaned beef calves.

		Pre-weaning	Days post-housing							*P*-values[1]			Pre-housing	Days post-housing					*P*-values[1]		
Variable		**0**	**2**	**7**	**14**	**21**	**28**	**35**	**s.e.m.**	**T**	**S**	**T×S**	**0**	**2**	**7**	**14**	**21**	**s.e.m.**	**T**	**S**	**T×S**
Neu[2]	**H**	3.9^{x}	4.1	3.1^{a}	2.4^{c}	2.5^{x}	2.4^{c}	2.3^{c,x}	0.34	NS	***	***	2.3^{x}	2.3^{x}	1.8	1.7	2.3	0.28	*	*	NS
(×10^{3}/μL)	**P**	2.7^{y}	3.6^{b}	3.8^{b}	3.1	2.8^{y}	2.5	3.1^{y}	0.32				3.1^{y}	3.7^{d,y}	2.3^{f}	2.4^{f}	2.6	0.26			
Lym[3]	**H**	8.6	8.0^{a,x}	7.6^{c,x}	7.5^{c,x}	7.7^{b,x}	7.7^{a}	7.9	0.49	†	NS	***	7.9	7.8	7.9	8.5	8.5	0.32	NS	*	**
(×10^{3}/μL)	**P**	8.5	9.3^{b,y}	9.0y	9.0^{y}	9.0^{y}	9.0	8.6	0.51				8.6	7.8^{d}	7.8^{a}	7.9	8.2	0.35			
N:L[4]	**H**	0.45^{x}	0.52	0.40	0.33^{b}	0.31^{b,x}	0.31^{b}	0.29^{b,x}	0.027	NS	***	**	0.29^{x}	0.31	0.26	0.20	0.27	0.035	**	**	NS
ratio	**P**	0.33^{y}	0.41^{a}	0.43^{a}	0.36	0.44^{y}	0.31	0.40^{a,y}	0.025				0.40^{y}	0.50^{d}	0.29^{f}	0.29^{f}	0.33^{e}	0.039			
Mon[5]	**H**	0.52	0.51	0.54	0.48	0.51	0.54	0.52	0.059	NS	*	NS	0.52	0.47	0.43	0.42	0.43	0.042	NS	*	NS
(×10^{3}/μL)	**P**	0.51	0.54	0.53	0.67	0.53	0.74^{a}	0.65	0.054				0.65	0.54	0.51	0.47^{f}	0.37^{f}	0.046			
Eos[6]	**H**	0.27	0.46^{a}	0.27	0.17	0.13^{a}	0.09^{a}	0.10^{a}	0.063	NS	*	NS	0.10	0.08	0.08	0.09	0.09	0.025	*	NS	NS
(×10^{3}/μL)	**P**	0.29	0.48^{a}	0.21	0.17	0.19^{a}	0.14^{a}	0.18^{a}	0.064				0.18	0.21	0.21	0.13	0.18	0.021			
Bas[7]	**H**	0.17	0.15	0.12^{c}	0.12^{c,x}	0.13^{x}	0.14^{x}	0.13^{x}	0.012	*	*	**	0.13^{x}	0.13	0.12	0.13	0.14	0.009	**	*	NS
(×10^{3}/μL)	**P**	0.14	0.15	0.16	0.15^{y}	0.18^{a,y}	0.21^{b,y}	0.18^{a,y}	0.013				0.18^{y}	0.15	0.15	0.17	0.17	0.009			

[1]T = treatment, S = sampling time, T×S = treatment × sampling time interaction, NS = not significant, $P > 0.05$. * = $P < 0.05$, † = $P = 0.06$, ** = $P < 0.01$, *** = $P < 0.001$.
[2]neutrophil number, [3]lymphocyte number, [4]neutrophil: lymphocyte ratio, [5]monocyte number, [6]eosinophil number, [7]basophil number
[a,b,c] Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.
[d,e,f] Within rows, Lsmeans differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.
[x,y] Between rows for each variable, treatment Lsmeans differ by $P < 0.05$. n = 18 calves per treatment.

3.3.2. Red blood cell (RBC) number and associated haematological variables

Post-weaning, there were no effect of treatment or treatment × time interaction ($P > 0.05$) for RBC number and concentration of HGB. Red blood cell number and concentration of HGB decreased ($P < 0.05$) on d 2 to d 35 in H and P compared with pre-weaning baseline (Table 3.4). There was a treatment × time interaction ($P < 0.05$) for HCT %, whereby on d 2 to d 35, it decreased ($P < 0.05$) in H (d 2: mean (s.e.m.) 36.2 (0.52) %, d 35: mean (s.e.m.) 33.1 (0.53) %) and P (d 2: mean (s.e.m.) 35.9 (0.56) %, d 35: mean (s.e.m.) 33.4 (0.51) %) compared with pre-weaning baseline (H: mean (s.e.m.) 37.7 (0.58) %, P: mean (s.e.m.) 37.1 (0.56) %) (Table 3.4). There was a treatment × sampling time interaction ($P < 0.05$) for MCHC whereby on d 7, MCHC % was higher ($P < 0.05$) in P (mean (s.e.m.) 39.6 (0.16) %) than H (mean (s.e.m.) 39.0 (0.16) %), and both H and P increased ($P < 0.001$) on d 7 to 35 (H: mean (s.e.m.) 39.6 (0.18) %; P: mean (s.e.m.) 39.8 (0.19) %) compared with pre-weaning baseline (H: mean (s.e.m.) 38.1 (0.17) %; P: mean (s.e.m.) 38.1 (0.17) %). There were no effects of treatment or treatment × sampling time interactions ($P > 0.05$) for MCV and MCH post-weaning (Table 3.4).

Post-housing, there was a treatment × time interaction for RBC number ($P < 0.01$) and HGB concentration ($P < 0.05$), whereby RBC number and HGB concentration decreased ($P < 0.05$) in H and P on d 14 to 21 (RBC: mean (s.e.m.) 10.0 (0.22) $\times 10^6$ cells/ μL; HGB: mean (s.e.m.) 12.6 (0.24) g/dL), and d 7 to 21 (RBC: mean (s.e.m.) 10.1 (0.20) $\times 10^6$ cells/ μL; HGB: mean (s.e.m.) 12.5 (0.24) g/dL), respectively, compared with pre-housing baseline (RBC: H: mean (s.e.m.) 10.5 (0.21) $\times 10^6$ cells/ μL, P: mean (s.e.m.) 10.8 (0.21) $\times 10^6$ cells/ μL; HGB: H: mean (s.e.m.) 13.1 (0.22) g/dL, P: mean (s.e.m.) 13.3 (0.22) g/dL) (Table 3.4). There was no effect

($P > 0.05$) of treatment or treatment × time interaction for HCT, MCV, MCH, and MCHC post-housing (Table 3.4).

Table 3.4. Effect of post-weaning management practice on haematological variables in weaned beef calves and subsequent housing

		Pre-weaning	Days post-housing							*P*-values[1]			Pre-housing	Days post-housing					*P*-values[1]		
Variable		**0**	**2**	**7**	**14**	**21**	**28**	**35**	**s.e.m.**	**T**	**S**	**T×S**	**0**	**2**	**7**	**14**	**21**	**s.e.m.**	**T**	**S**	**T×S**
RBC[2]	**H**	11.9	11.4[a]	11.3[b]	11.0[c]	10.5[c]	10.2[c]	10.5[c]	0.23	NS	***	NS	10.5	10.5	10.3	10.0[e]	10.0[e]	0.20	NS	***	**
(×10^6/ µL)	**P**	11.9	11.4[a]	11.1[c]	10.8[c]	10.6[c]	10.6[c]	10.8[c]	0.22				10.8	10.7	10.0[f]	10.3[e]	10.0[f]	0.21			
HGB[3]	**H**	14.4	13.9[b]	13.2[c]	13.8[b]	13.1[c]	12.7[c]	13.1[c]	0.24	NS	***	NS	13.1	13.2	12.9	12.7[d]	12.6[d]	0.24	NS	***	*
(g/dL)	**P**	14.1	13.8[a]	13.7[a]	13.5[b]	13.1[c]	13.1[c]	13.3[c]	0.23				13.3	13.1	12.5[e]	12.7[d]	12.5[e]	0.25			
HCT[4]	**H**	37.7	36.2[a]	36.3[a]	35.0[c]	33.3[c]	32.1[c]	33.1[c]	0.56	NS	***	*	33.1	32.8	32.4	30.6[e]	31.1[d]	0.56	NS	***	NS
(%)	**P**	37.1	35.9[a]	34.7[c]	34.0[c]	33.1[c]	32.9[c]	33.4[c]	0.54				33.4	32.9	32.0[d]	31.0[d]	30.3[e]	0.54			
MCV[5]	**H**	31.8	31.9	32.2[a]	32.0	31.7	31.4	31.5	0.81	NS	*	NS	31.5	31.5	31.7	30.7	31.1	0.61	NS	***	NS
(pg)	**P**	31.5	31.5	31.5	31.6	31.2	31.3	31.1	0.83				31.1	30.9	31.6	30.3	30.5	0.61			
MCH[6]	**H**	12.1	12.2	12.5	12.6	12.5	12.4	12.5	0.29	NS	***	NS	12.5	12.6	12.5	12.7	12.8[d]	0.22	NS	***	NS
(fL)	**P**	12.0	12.2	12.5	12.5	12.4	12.5	12.3	0.30				12.3	12.2	12.4	12.5	12.5	0.21			
MCHC[7]	**H**	38.1	38.4	39.0[c,x]	39.4[c]	39.3[c]	39.6[c]	39.6[c]	0.23	NS	***	*	39.6	40.1	39.6	41.4	41.2	0.18	NS	*	NS
(%)	**P**	38.1	38.6	39.6[c,y]	39.6[c]	39.7[c]	39.8[c]	39.8[c]	0.24				39.8	39.7	39.3	41.1	41.1	0.19			

[1]T = treatment, S = sampling time, T×S = treatment × sampling time interaction, NS = not significant, $P > 0.05$. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.
[4]red blood cell number, [5]haemaglobin concentration, [6]haematocrit percentage, [5]mean corpuscular volume, [6]mean corpuscular haemoglobin, [7]mean corpuscular haemoglobin concentration.
[a,b,c] Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.
[d,e,f] Within rows, Lsmeans differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.
[x,y] Between rows for each variable, treatment Lsmeans differ by $P < 0.05$. n = 18 calves per treatment.

3.3.3. In vitro lymphocyte production of interferon-γ (IFN-γ)

Post-weaning, there was a treatment × time interaction ($P < 0.01$) for the PHA A-induced *in vitro* IFN-γ production (Table 3.5), with H having decreased ($P < 0.05$) production on d 7 (mean (s.e.m.) 0.16 (0.034) OD at 450 nm) compared with pre-weaning baseline (mean (s.e.m.) 0.32 (0.065) at 450 nm) and no change ($P > 0.05$) in P. There was no effect of treatment or treatment × time interaction ($P > 0.05$) for Con A-induced *in vitro* IFN-γ production post-weaning. On d 2 and 7, production decreased ($P < 0.05$) in P (mean (s.e.m.) 0.20 (0.126) at 450 nm) and H (mean (s.e.m.) 0.34 (0.132) at 450 nm), respectively, compared with pre-weaning baseline (P: mean (s.e.m.) 0.61 (0.143) at 450 nm; H: mean (s.e.m.) 0.86 (0.147) at 450 nm) (Table 3.5).

Post-housing, there was no effect of treatment or treatment × time interactions ($P > 0.05$) for Con A- or PHA-induced IFN-γ production. On d 2, 7 and 21, Con A-induced IFN-γ production decreased ($P < 0.05$) in H (mean (s.e.m.) 0.38 (0.137) at 450 nm) compared with pre-housing baseline (mean (s.e.m.) 0.68 (0.138) at 450 nm) (Table 3.5).

3.3.4. Acute phase proteins

Post-weaning, there was a treatment × time interaction for fibrinogen ($P < 0.01$) and haptoglobin ($P < 0.05$), whereby the concentration of fibrinogen increased ($P < 0.01$) on d 2 to d 21 and on d 2 to 35, and haptoglobin increased ($P < 0.05$) on d 2 to 35 and on d 7 to 35 in H and P, respectively, compared with pre-weaning baseline (Table 3.5 and Figure 3.3A). On 7, concentration of fibrinogen was higher ($P < 0.05$) in H (mean (s.e.m.) 548 (28.4) mg/dL) than in P (mean (s.e.m.) 462 (16.8) mg/dL) (Figure 3.3A and Table 3.5).

Post-housing, there was no effect of treatment or treatment × time interactions ($P > 0.05$) for concentrations of fibrinogen or haptoglobin. Concentration of fibrinogen decreased ($P < 0.05$) in P on d 2 (mean (s.e.m.) 402 (20.1) mg/dL) and 21 (mean (s.e.m.) 353 (20.4) mg/dL) compared with pre-housing baseline (mean (s.e.m.) 519 (20.1) mg/dL) (Figure 3.3B), whereas haptoglobin concentration did not differ ($P > 0.05$) from pre-housing baseline in P (mean (s.e.m.) 0.72 (0.062) mg/dL) and H (mean (s.e.m.) 0.52 (0.061) post-housing (Table 3.5).

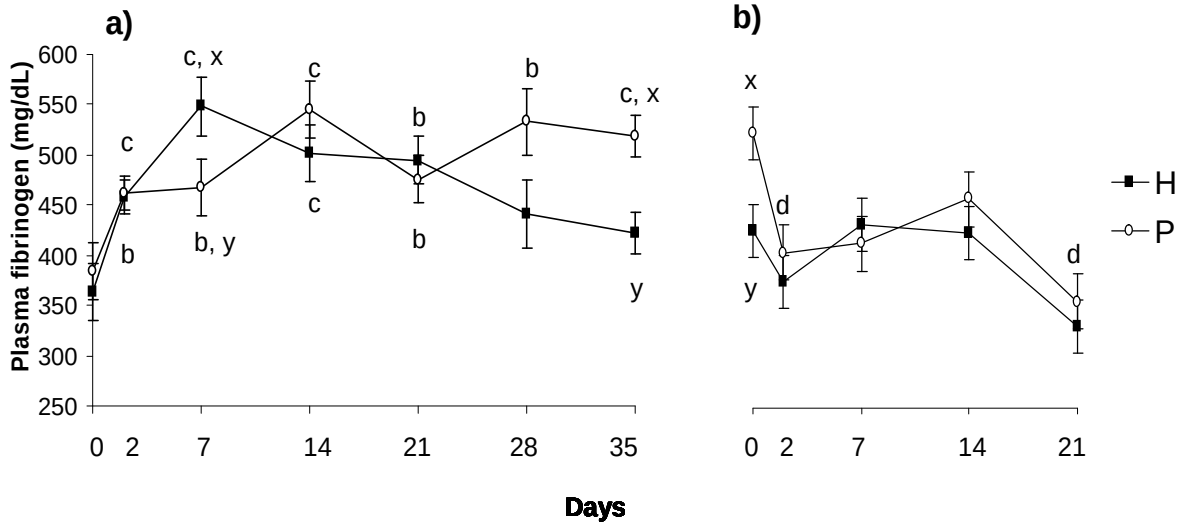

Figure 3.4a and b. Effect of post-weaning management practice on concentration of fibrinogen in beef calves that were abruptly weaned and either housed (H: -■-, n = 18) and offered a new diet or returned to pasture (P: -○-, n = 18) for 35 days, deferring the onset of housing and subsequently comparing the response of P to housing with calves habituated to housing (H).

Post-weaning and post-housing changes for H and P calves are shown on a and b, respectively. [a,b,c]Lsmeans differ from pre-weaning baseline (day 0) by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. [d]Lsmeans differ from pre-housing baseline (day 0) by $P < 0.05$. [x,y]Treatment Lsmeans that do not have common superscript differ by $P < 0.05$. There was a treatment × sampling time interaction ($P < 0.01$) post-weaning and there was no effect of treatment or treatment × sampling time interaction ($P < 0.05$) post-housing.

Table 3.5. Effect of post-weaning management practice on concanavalin (Con A) - induced and phytohaemagglutinin (PHA) -induced *in vitro* production of interferon gamma (INF-γ) and concentration of haptoglobin in weaned beef calves.

		Pre-weaning	Days post-weaning							*P*-values[1]			Pre-housing	Days post-housing					*P*-values[1]		
Variable		**0**	**2**	**7**	**14**	**21**	**28**	**35**	**s.e.m.**	**T**	**S**	**T×S**	**0**	**2**	**7**	**14**	**21**	**s.e.m.**	**T**	**S**	**T×S**
Con A[2]	**H**	0.86	0.71	0.34^{b}	0.55	0.97	0.55	0.68	0.158	NS	**	NS	0.68	0.31^{c}	0.29^{c}	0.50	0.42^{a}	0.137	NS	*	NS
	P	0.61	0.20^{a}	0.55	0.84	0.61	0.90	0.36	0.164				0.36	0.40	0.30	0.31	0.60	0.145			
PHA[2]	**H**	0.32	0.21	0.16^{b}	0.20	0.58	0.28	0.15	0.084	NS	**	**	0.15	0.17	0.20	0.26^{x}	0.30	0.088	NS	*	NS
	P	0.16	0.03	0.12	0.18	0.25	0.62^{a}	0.29	0.086				0.29	0.10	0.22	0.09^{y}	0.35	0.083			
Hp[3,4]	**H**	0.36	0.53^{c}	0.63^{c}	0.65^{c}	0.53^{b}	0.53^{b}	0.52^{a,x}	0.053	*	***	*	0.52^{x}	0.55	0.59	0.48	0.44	0.054	NS	***	NS
	P	0.41	0.45	0.65^{b}	0.81^{c}	0.55^{a}	0.65^{b}	0.72^{c,y}	0.051				0.72^{y}	0.67	0.67	0.64	0.51^{d}	0.049			

[1]T = treatment, S = sampling time, T×S = treatment × sampling time interaction, NS = not significant, $P > 0.05$. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

[2]Absorbance at 450nm, [3] haptoglobin, mg/dL

[a,b,c] Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

[d,e,f] Within rows, Lsmeans differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

[x,y] Between rows for each variable, treatment Lsmeans differ by $P < 0.05$. n = 18 calves per treatment.

3.4. Chapter Summary and Discussion

3.4.1. Summary of the main findings

The effect of post-weaning management practice was determined in previously grazed beef calves that were (i) abruptly weaned, and (a) housed (H) and offered a new diet of grass silage *ad libitum* plus concentrates or (b) returned to familiar pasture (P), and also (ii) the effect of subsequent housing of calves in P compared with previously housed calves in H using physiological, haematological and immunological stress related variables.

The main findings of this chapter are summarised as follows. Rectal body temperature and concentration of cortisol increased ($P < 0.05$) in H and P post-weaning, whereas dehydroepiandrosterone was unchanged in H and P compared with pre-weaning and pre-housing baselines. Post-weaning, there was a treatment × time interaction ($P < 0.05$) for total leukocyte and lymphocyte number, and interferon-γ production with H having lower total leukocyte and lymphocyte number on d 7 to 35 and d 2 to 28, respectively, and decreased interferon-γ production on d 7, compared with P. Neutrophil number increased ($P < 0.01$) in P on d 2 and 7 and decreased in H on d 7 to 35 compared with pre-weaning baseline. Concentrations of fibrinogen and haptoglobin increased ($P < 0.05$) in P and H up to d 21 and 35 post-weaning, respectively. There was a treatment × time interaction ($P < 0.05$) for fibrinogen with H having higher concentration of fibrinogen on d 7 compared with P. Post-housing, total leukocyte and neutrophil number increased ($P < 0.05$), whereas lymphocyte number decreased on d 2 compared with pre-housing baseline. There was no effect ($P > 0.05$) of treatment or treatment × time interaction for interferon-γ production post-housing.

3.4.2. Discussion

The multifaceted nature of weaning exposes the beef calf to physical, physiological and psychological stressors. The ability of the calf to adapt to these stressors may affect their subsequent health and performance. Consequently, reducing the negative impact of weaning stress is an important factor in achieving an effective herd health management plan.

In the present study, calves from differing grazing groups were mixed post-weaning in order to mimic social disruption that is typically experienced by beef calves at weaning. Social group disruption has been shown to be stressful to cattle (Gupta *et al.*, 2005) and is an important factor involved in the weaning process (Veissier and Le Neindre, 1989a). Elimination of milk from the diet and introduction to an entirely solid and often novel diet is a major adaptation required by beef calves at weaning. Moreover, low feed intake is associated with stressed calves (Duff and Galyean, 2007). In the present study, milk would still constitute an significant proportion of the calves total diet as the milk yield of the cow breed type (beef × dairy) used remains relatively high at weaning (McGee *et al.*, 2005b). Although both post-weaning management practices resulted in cessation of milk from the calves' diet, calves that were housed were required to adapt to a new diet of grass silage and supplementary concentrates, whereas calves that were returned to pasture they had grazed previously were not supplemented. Additionally, the indoor slatted accommodation provided a new physical environment for housed calves.

In line with previous research (Lefcourt and Elsasser, 1995; Hickey *et al.*, 2003a; Blanco *et al.*, 2009), a transient increase in cortisol concentration was associated with weaning in the present study, irrespective of post-weaning management practice. Deferral of housing failed to produce a measurable difference in

cortisol concentration compared with the traditional practice of weaning and simultaneous housing. The increases (< 4 ng/mL) in cortisol concentration measured post-weaning were similar to those reported by Hickey *et al.* (2003a) but greater than those reported by Blanco *et al.* (2009). Disparity between these findings may have been influenced by calf management. In the present study, calves were allowed continuous, unlimited nursing and contact with their dam for approximately 7 months prior to weaning, whereas in the latter study calves were housed in adjacent pens to their dams for 90 or 150 days prior to weaning and were subjected to restricted suckling of two 30 minute sessions daily. Limited contact and lower nursing frequency from birth may result in a less intense maternal-offspring bond. Indoor housing in slatted floor pens did not result in increased concentration of cortisol in pasture calves suggesting that the change in environment and diet alone were insufficient to elicit an increase in this steroidal hormone. Similarly, no change in concentration of cortisol was found in previously grazed beef cows that were housed indoors in slatted floor pens (Lynch *et al.*, 2008a; Chapter 4).

Decreased concentration of DHEA in combination with increased cortisol has been implicated in the stress response (Zinder and Dar, 1999), whereby this androgen antagonises the immunosuppressive actions of cortisol via its anti-inflammatory and immunoprotective properties (Saccó *et al.*, 2002; Straub *et al.*, 2002). Concentration of DHEA was unchanged post-weaning, which is in accord with the findings of Lynch *et al.* (2008a) in newly separated (weaned) beef cows. The unaltered concentration of DHEA in calves returned to pasture post-housing is contrary to the findings of Lynch *et al.* (2008a; Chapter 4), who reported decreased concentration of DHEA in beef cows following housing. The utility of DHEA, a measure of the shift in the steroidogenic pathway from the precursor DHEA towards cortisol, as a potential biomarker,

specifically related to management-associated stressors in the bovine, therefore requires more research.

Alterations in the levels of blood cell constituents are indicative of an attempt to restore homeostasis when abrupt physical conditions are encountered by an organism and thus, blood cells are very sensitive indicators of the patho-physiological responses in an animal (Jones and Allison, 2007). In the present study, calves that were simultaneously weaned and housed had lower leukocyte numbers up to d 35 post-weaning compared with calves that were weaned and returned to pasture. These findings are contrary to Blanco *et al.* (2009) and Hickey *et al.* (2003a) who reported that leukocyte number was unchanged post-weaning. The changes reported in our study may be a consequence of decreased lymphocyte and neutrophil numbers observed in housed calves post-weaning. Decreased lymphocyte number has been reported in weaned calves that were housed (Hickey *et al.*, 2003a; Blanco *et al.*, 2009) and may be due to a redistribution of lymphocytes from the peripheral circulation, in response to increased endogenous glucocorticoids and catecholamines, to tissues and organs (Nonnecke *et al.*, 1997; Dhabhar, 2002). An opposing lymphocyte profile was evident in pasture calves immediately post-weaning, however, this response was short lived. Often measured as a conjunct to lymphocyte number, neutrophil number was found to increase in calves returned to pasture post-weaning, whereas this was not the case for housed calves, in the present study. The lack of neutrophilia in housed calves is contrary to the findings of Hickey *et al.* (2003a) and Blanco *et al.* (2009), who reported neutrophilia in calves that were housed post-weaning. The high neutrophil number observed for housed calves pre-weaning likely contributes to the disparity between the present and the aforementioned studies. Following the subsequent housing of calves, neutrophilia and lymphopenia were evident in the present study

which is in agreement with Lynch *et al.* (2008a; Chapter 4) who reported similar findings in previously grazed beef cows that were housed.

Erythrocytosis, often associated with acute stress, was not evident in the present study, which concurs with the findings of Blanco *et al.* (2009). Negligible changes in concentration of haemoglobin and haematocrit percentage and a small increase in rectal body temperature post-weaning and post-housing suggests that animal health was not compromised in the present study.

Lymphocyte functional assays in terms of PHA-induced and Con A-induced IFN-γ production were used to assess cell-mediated immune function in the present study. Induction of a proliferative response induced by antigen *in vitro* has been shown to be representative of cellular immunocompetence (Kristensen *et al.*, 1982; Earley *et al.*, 2002; Ting *et al.*, 2003). The secretion of IFN-γ by lymphocytes is critically important in orchestrating an effective immune response, especially cellular immunity (Rothel *et al.*, 1990), and the induction of IFN-γ by Con A and PHA lectins corresponds to the degree of blastogenesis in cattle (Ishikawa *et al.*, 1992). In the present study, weaning and simultaneous housing suppressed Con A- and PHA-induced IFN-γ production. Although similar to the findings of Hickey *et al.* (2003a) who reported suppressed IFN-γ production up to d 7 post-weaning, reduced IFN-γ production was only evident on d 7 in H in the present study. An immediate and short-lived reduction in Con A-induced INF-γ production was observed for pasture calves on d 2 and despite there being no change in PHA-induced IFN-γ production, there was a significant difference between post-weaning management practices at this time. These findings suggest that deferral of housing at weaning may prove a more beneficial management practice to calves in terms of immune function. Housing of previously weaned calves did not effect the *in vitro* production of IFN-γ, which supports the

findings of Lynch *et al.* (2008a; Chapter 4) who reported that housing did not compromise *in vitro* production of IFN-γ in beef cows.

The acute phase protein response has been used to support clinical and observational measurements to describe animal health and well-being (Earley and Crowe, 2002; Arthington *et al.*, 2003; Gånheim *et al.*, 2007). Studies examining the effect of weaning on the acute phase protein response in beef calves did not examine the response beyond a 7 d window post-weaning (Hickey *et al.*, 2003a; Blanco *et al.*, 2009), however, the findings of the present study have shown that the acute phase protein response is still evident up to 35 d post-weaning. Moreover, the greater increase in fibrinogen concentration found in housed calves compared with pasture calves post-weaning, suggests that deferral of housing may be more advantageous rather than imposing both practices on the calf simultaneously. The absence of an acute phase protein response following housing of pasture calves, suggests that housing alone was not sufficient to elicit a stress response.

Contrary to the findings of Phillips *et al.* (1987), an increase in concentration of total protein was not found immediately post-weaning in the present study. These authors, however, only measured total protein concentration immediately prior to- and 24 h post-weaning, and moreover, between these two time-points, calves were fasted, having no access to feed or water. Dehydration, may have contributed to the elevated total protein concentration reported by these authors. Thus, the disparity between the post-weaning management practices of the present and the aforementioned study account for the conflicting findings. Concentration of albumin increased, whereas globulin decreased post-weaning, similar to the findings in reported for beef cows (Chapter 4).

Behavioural observations of weaned beef calves have shown a period of increased locomotor activity immediately post-weaning which declines over 4 – 7 d period following the event (Price *et al.*, 2003; Boland *et al.*, 2008). Elevated creatine kinase activity is associated with increased or unaccustomed exercise (Berg and Haralambie, 1978). An increase of approximately 40 % was evident for creatine kinase activity in pasture calves immediately post-weaning, suggesting a greater period of physical activity. Concordant with these findings, McVeigh *et al.* (1982) reported bulls that were stressed following mixing with an established herd of bulls with which they had no previous contact, had elevated creatine kinase activity that declined slowly over a 7 d recovery period. These authors confirmed by visual observation that mixing of unfamiliar animals had a high degree of physical activity, consisting of aggressive butting and pushing, and frequent mounting. Such behaviours are associated with re-establishment of social hierarchy (Gupta *et al.*, 2005). Converse to the profile observed when calves were returned to pasture, creatine kinase activity decreased in housed calves on d 14 to 35 compared with pre-weaning baseline, and d 14 and 21 in pasture compared with pre-housing baseline, reflecting the restricted movement of the calves within the pens of the indoor housing.

Maintaining homeostasis is an energy-dependent process that is tightly regulated, with stressful events associated with a greater demand of energy in order to temporarily prioritise specific biological functions which may challenge the stability of other physiological functions (Moberg and Mench, 2000). A stress-induced increase in glucose was not found in the post-weaning management practices in the present study, and is in accordance with the findings of Crookshank *et al.* (1979). Phillips *et al.* (1987) concluded that the elevated concentration of glucose evident in fasted bulls 24 h post-weaning was a consequence of disruption to feeding pattern, diet, and

activation of the endocrine system. The animals used in the present study were not fasted or restricted from accessing feed or water post-weaning. Calves that were returned to pasture following weaning were not supplemented with concentrates, unlike those that were housed, and this dissimilarity in diet may account for the varying glucose profiles. Elevated concentrations of NEFA and βHB, associated with a shift in energy balance in the ruminant, were evident in both post-weaning management practices. A short-lived increased in concentration in NEFA was found in bulls following mixing with unfamiliar peers (McVeigh *et al.*, 1982). Elevated concentrations of NEFA and βHB in peripheral blood impairs the chemotactic functions of bovine leukocytes *in vitro* (Suriyasathaporn *et al.*, 1999), suppresses bovine lymphocyte blastogenesis (Sato *et al.*, 1995), and inhibit the respiratory burst capabilities of bovine neutrophils to some degree (Hoeben *et al.*, 1997). However, the concentrations of plasma βHB observed in this study did not attain the levels at which βHB is reported to have an inhibitory affect on immune cell function, hence it is unlikely that these functional capacities were impaired under the conditions of the present study.

The differing crude protein concentration of the grass silage diet and the grass pasture diet is reflected in the contrasting urea profiles for each post-weaning management practice, whereby calves offered grass silage *ad libitum* had lower concentrations of plasma urea compared with those at pasture. When milk was eliminated from the diet post-weaning, the concentration of urea increased in calves that were returned to pasture compared with pre-weaning baseline, indicating that their intake of crude protein in the form of grass pasture may have increased post-weaning. Accordingly, when offered the lower crude protein content grass silage, the urea concentration in pasture calves decreased, compared with pre-housing baseline and

was comparable to that of housed calves who had been fed grass silage *ad libitum* for the previous 37 d. Low feed intake has been associated with stressed calves (Galyean *et al.*, 1999: Lofgreen, 1988), however without actual feed intake values, it cannot be categorically reported, on the basis of decreased concentrations of urea, a by-product of protein metabolism, that these calves had an overall lower dietary feed intake post-weaning and subsequently post-housing, rather a lower dietary intake of crude protein.

In summary, the main findings of this study show that compared with the traditional post-weaning management practice of weaning and simultaneous housing, deferring indoor housing and adaptation to a novel solid diet for 35 d post-weaning results in a less marked stress response. Calves that were weaned and housed simultaneously had lower circulating leukocyte number, suppressed *in vitro* lymphocyte production of IFN-γ and increased acute phase response. Weaning can elicit an extended acute phase protein response up to 35 d post-weaning which is longer than the 7 d window reported in other studies. Despite the differences observed between the post-weaning management practices for these variables, changes were minimal suggesting that the overall health and welfare of beef calves was not compromised by abrupt weaning and simultaneous housing. Subsequent housing resulted in altered leukocyte response and a transitory suppression IFN-γ production suggesting that housing can potentially elicit a stress response in calves but this response is short-lived and has no negative consequences for the calf.

In conclusion, reducing the cumulative effect of multiple stressors, by deferring housing at the time of weaning resulted in a less marked stress response in beef calves compared with the traditional, combined practice of weaning and simultaneous housing.

Chapter 4

Characterisation of physiological and immunological responses in beef cows to abrupt weaning and subsequent housing

4.1. Introduction

Within seasonal grassland-based, spring-calving suckler beef production systems calves are generally allowed continuous and unlimited nursing of the dam at pasture for approximately 6 to 8 months until weaning at the end of the grazing season. Husbandry management practices, including weaning and housing, form integral components of beef production systems, and often expose beef cattle to novel environmental, physical and psychological stressors. Research measuring stress related variables in cattle has focused on parturition (Burton *et al.*, 2005), routine handling (Grignard *et al.*, 2001; Breuer *et al.*, 2003), mixing with unfamiliar cattle (Gupta *et al.*, 2005; Raussi *et al.*, 2005), transportation (Marahrens *et al.*, 2003; Buckham Sporer *et al.*, 2007a), restrictive space allowance during indoor housing (Fisher *et al.*, 1997) and weaning on the calf (Hickey *et al.*, 2003a). However, very limited research has examined weaning stress in the cow. The effect of cow-calf separation on concentration of cortisol in the cow is equivocal with no change (Lefcourt and Elsasser, 1995) and an increase (Whisnant *et al.*, 1985) reported. Furthermore, these studies were limited up to 48 h and 6 d post-weaning, respectively. Extended effects of weaning on physiological, haematological and immunological responses were found in beef calves (Lynch *et al.*, 2008b; Chapter 3) but have not been examined in beef cows.

At the end of the grazing season in autumn, weaned cows are typically housed indoors over the winter period (Drennan and McGee, 2009). In beef cattle, housing research has focused on the effect of varying space allowance (Fisher *et al.*, 1997; Gupta *et al.*, 2007) and floor type (Lowe *et al.*, 2001; Platz *et al.*, 2007) on production and behavioural responses. There is little data available on the effects of moving animals from an outdoor grazing environment to indoor accommodation in slatted

floor sheds. Higashiyama *et al.* (2007) found that previously grazed cows that were tethered during indoor housing had increased concentration of cortisol for up to 7 days post-housing. Research on the extended effect of indoor housing on other stress related variables is warranted.

Therefore, the objectives of this study (Chapter 4) were to characterise the extended physiological (rectal body temperature, cortisol, dehydroepiandrosterone (DHEA), glucose, non-esterified fatty acids (NEFA), β-hydroxybutrate (β-HB), total protein, albumin, globulin, creatine kinase and urea), haematological (total leukocyte number and differentials, red blood cell number and associated variables), and immunological (interferon (IFN)-γ production and acute phase proteins) responses in beef cows to i.) abrupt weaning and ii.) subsequent housing. The experimental design is described in Section 2.2.2.2.

4.2 Characterisation of the physiological responses to abrupt weaning and subsequent housing in beef cows

4.2.1 Rectal body temperature

Rectal body temperature increased ($P < 0.01$) on d 2 (mean (s.e.m.) 38.7 (0.04) º C) compared with pre-weaning baseline (d 0 at weaning: mean (s.e.m.) 38.4 (0.05) º C). Rectal body temperature did not differ ($P > 0.05$) on d 7 to d 35 (mean (s.e.m.) over this sampling period was 38.5 (0.05) º C) compared with d 0. Post-housing, rectal body temperature did not differ ($P > 0.05$) from pre-housing baseline (d 0 at housing).

4.2.2 Cortisol and dehydroepiandrosterone (DHEA)

Concentration of cortisol was lower than 7.5 ng/mL in cows at all sampling points during the study. On d 2 post-weaning, concentration of cortisol (mean (s.e.m.) 4.4 (0.34) ng/ml) decreased ($P < 0.05$) compared with pre-weaning baseline (mean 5.8 (s.e.m.) 0.39 ng/ml). Concentrations of cortisol did not differ ($P > 0.05$) from pre-weaning and pre-housing baselines for the remainder of the study. Post-weaning, concentration of DHEA did not differ ($P > 0.05$) from pre-weaning baseline, whereas following housing, concentration of DHEA decreased ($P < 0.05$) on d 2 (mean (s.e.m.) 1.9 (0.55) ng/mL) to d 21 (mean (s.e.m.) 1.3 (0.31) ng/mL) compared with pre-housing baseline (mean (s.e.m.) 3.4 (0.65) ng/mL). The cortisol: DHEA ratio did not differ ($P > 0.05$) from pre-weaning (mean (s.e.m.) 0.9 (0.5)) and pre-housing baselines (mean (s.e.m.) 0.4 (0.3)) at any sampling time-points.

4.2.3 Glucose, β-hydroxybutyrate (βHB) non-esterified fatty acid (NEFA)

Post-weaning, concentration of glucose increased ($P < 0.001$) on d 2 to d 35 compared with pre-weaning baseline (mean (s.e.m.) 3.2 (0.03) mmol/L), with a maximum concentration of mean (s.e.m.) 3.8 (0.04) mmol/L recorded on d 7 and 21 (Table 4.1). Concentration of βHB increased ($P < 0.001$) on d 2 (mean (s.e.m.) 0.39 (0.012) mmol/L), followed by a decrease ($P < 0.001$) on d 7 (mean (s.e.m.) 0.23 (0.006) mmol/L) and returned to pre-weaning baseline (mean (s.e.m.) 0.29 (0.009) on d 14 (Table 4.1). Concentration of NEFA decreased ($P < 0.05$) on d 2 (mean (s.e.m.) 0.20 (0.09) mmol/L) to d 28 (mean (s.e.m.) 0.12 (0.007) mmol/L) compared with pre-weaning baseline (mean (s.e.m.) 0.37 (0.08) mmol/L). Post-housing, concentration of glucose did not differ ($P > 0.05$) from pre-housing baseline (mean (s.e.m.) 3.7 (0.06) mmol/L), whereas concentration of βHB decreased ($P < 0.001$) on d 7 (mean (s.e.m.) 0.20 (0.005) mmol/L) and 14 (mean (s.e.m.) 0.24 (0.008) mmol/L) and concentration of NEFA decreased ($P < 0.05$) on d 7 (mean (s.e.m.) 0.26 (0.007) mmol/L) to 14 (mean (s.e.m.) 0.24 (0.008) mmol/L) compared with pre-housing baseline (Table 4.1).

4.2.4 Total protein, albumin and globulin

Post-weaning, concentration of albumin decreased ($P < 0.001$) on d 14 to 21 (mean (s.e.m.) 34.3 (0.32) g/dL), whereas concentration of globulin increased ($P < 0.01$) on d 21 to 35 (mean (s.e) 46.6 (0.78) g/dL) compared with pre-weaning baseline, respectively (albumin: mean (s.e.m.) 35.7 (0.31), globulin: mean (s.e.m.) 42.5 (0.70). However, concentration of total protein (mean (s.e.m.) 79.5 (0.68) g/dL) did not differ ($P > 0.05$) from pre-weaning baseline (mean (s.e.m.) 78.2 (0.66) g/dL) (Table 4.2). Post-housing, concentration of total protein, albumin and globulin decreased ($P < 0.05$) on d 14 and 21 (total protein: mean (s.e.m.) 80.4 (0.73) g/dL, albumin: mean

(s.e.m.) 33.7 (0.37) g/dL, globulin 46.7 (0.84) g/dL) compared with pre-housing baseline (total protein: mean (s.e.m.) 84.4 (0.80) g/dL, albumin: mean (s.e.m.) 35.3 (0.42) g/dL, globulin 49.1 (0.74) g/dL) (Table 4.2)

4.2.5 Creatine kinase

Post-weaning, creatine kinase activity increased ($P < 0.01$) on d 2 (mean (s.e.m.) 281 (24.4) U/L) and 7 (mean (s.e.m.) 217 (12.9) U/L) compared with pre-weaning baseline (mean (s.e.m.) 161 (6.7) U/L). Post-housing, creatine kinase activity did not differ ($P > 0.05$) on d 2 (mean (s.e.m.) 205 (9.8) U/L) compared with pre-housing baseline (mean (s.e.m.) 177 (7.8) U/L), however on d 7 to 21 (mean (s.e.m.) 126 (5.2) U/L) creatine kinase activity was lower ($P < 0.001$) than pre-housing baseline (Table 4.2)

4.2.6 Urea

Concentration of urea increased ($P < 0.001$) on d 2 (mean (s.e.m.) 7.7 (0.19) mmol/L) and 14 (mean (s.e.m.) 8.1 (0.15) mmol/L) and decreased ($P < 0.05$) on d 35 (mean (s.e.m.) 6.0 (0.16) mmol/L) compared with pre-weaning baseline (mean (s.e.m.) 6.7 (0.13) mmol/L) (Table 4.2). Post-housing, concentration of urea decreased ($P < 0.001$) on d 2 (mean (s.e.m.) 3.6 (0.12) mmol/L) to 21 (mean (s.e.m.) 2.4 (0.12) mmol/L) compared with pre-housing baseline (mean (s.e.m.) 6.0 (0.16) mmol/L).

Table 4.1. Least squares means (s.e.m.) for plasma glucose, non-esterfied fatty acid (NEFA) and β-hydroxybutyrate (βHB) concentration in beef cows following abrupt weaning and subsequent housing compared with pre-weaning and pre-housing baselines, respectively

	Pre-weaning	Days post-weaning						Pre-housing	Days post-housing			
Variable	**0**	**2**	**7**	**14**	**21**	**28**	**35**	**0**	**2**	**7**	**14**	**21**
Glucose	3.2	3.7[c]	3.8[c]	3.6[c]	3.8[c]	3.7[c]	3.7[c]	3.7	3.7	3.6	3.8	3.8
(mmol/L)	(0.03)	(0.04)	(0.04)	(0.03)	(0.04)	(0.04)	(0.06)	(0.06)	(0.06)	(0.05)	(0.04)	(0.07)
NEFA	0.37	0.20[a]	0.24[a]	0.12[a]	0.10[a]	0.12[a]	0.41	0.41	0.38	0.26[d]	0.08[d]	0.10[d]
(mmol/L)	(0.08)	(0.09)	(0.08)	(0.008)	(0.006)	(0.007)	(0.08)	(0.008)	(0.008)	(0.007)	(0.006)	(0.008)
βHB	0.29	0.39[c]	0.23[c]	0.30	0.28	0.29	0.28	0.28	0.27	0.20[f]	0.24[e]	0.31
(mmol/L)	(0.009)	(0.012)	(0.006)	(0.010)	(0.009)	(0.006)	(0.008)	(0.018)	(0.010)	(0.005)	(0.008)	(0.009)

[a b,c]Within a row, means differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

[d,e,f]Within a row, means differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

n = 18 cows per treatment.

Table 4.2. Least squares means (s.e.m.) for plasma total protein, albumin, globulin, creatine kinase and urea concentration in beef cows following abrupt weaning and subsequent housing compared with pre-weaning and pre-housing baselines, respectively.

	Pre-weaning	Days post-weaning						Pre-housing	Days Post-housing			
Variable	**0**	**2**	**7**	**14**	**21**	**28**	**35**	**0**	**2**	**7**	**14**	**21**
Total protein (g/dL)	78.2 (0.66)	78.4 (0.68)	78.5 (0.66)	77.6 (0.58)	79.2 (0.64)	80.2 (0.74)	84.4 (0.80)	84.4 (0.80)	84.9 (0.75)	84.8 (0.78)	80.5[f] (0.75)	80.4[f] (0.71)
Albumin (g/dL)	35.7 (0.31)	35.9 (0.34)	35.4 (0.33)	34.3[c] (0.34)	34.1[c] (0.34)	34.5[c] (0.29)	35.3 (0.42)	35.3 (0.42)	34.9 (0.35)	34.9 (0.34)	33.9[f] (0.36)	33.5[f] (0.37)
Globulin (g/dL)	42.5 (0.70)	42.5 (0.74)	43.1 (0.77)	43.3 (0.75)	45.1[b] (0.78)	45.7[b] (0.83)	49.1[c] (0.74)	49.1 (0.74)	50.0 (0.84)	49.9 (0.88)	46.6[e] (0.84)	46.9[d] (0.83)
Creatine kinase (U/L)	161 (6.3)	281[c] (24.4)	217[b] (12.9)	175 (8.3)	159 (11.2)	178 (6.2)	177 (7.8)	177 (7.8)	205 (9.8)	145[f] (5.0)	130[f] (5.7)	105[f] (4.9)
Urea (mmol/L)	6.7 (0.13)	7.7[c] (0.19)	6.4 (0.12)	8.1[c] (0.15)	6.5 (0.11)	7.2 (0.10)	6.0[a] (0.16)	6.0 (0.16)	3.6[f] (0.12)	3.3[f] (0.14)	2.7[f] (0.08)	2.4[f] (0.12)

[a,b,c]Within a row, means differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

[d,e,f]Within a row, means differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

n = 18 cows per treatment.

4.3 Characterisation of the immunological responses to abrupt weaning and subsequent housing in beef cows

4.3.1 Total leukocyte number and differential populations

Post-weaning, there was an increase ($P < 0.001$) in neutrophil number (mean (s.e) 3.6 (0.21) × 10^3 cells/ μL vs. mean (s.e.m.) 2.6 (0.14) × 10^3 cells/ μL) with a concurrent decrease ($P < 0.05$) in lymphocyte number (mean (s.e.m.) 4.9 (0.18) × 10^3 cells/ μL vs. mean (s.e.m.) 5.4 (0.15) × 10^3 cells/ μL) which resulted in an increase ($P = 0.07$) in total circulating leukocyte number on d 2 (mean (s.e.m.) 9.6 (0.34) × 10^3 cells/ μL) compared with pre-weaning baseline (mean (s.e) 8.9 (0.26) × 10^3 cells/ μL) (Figure 4.1a). Subsequently on d 7, total leukocyte number decreased ($P < 0.05$) (mean (s.e.m.) 8.0 (0.23) × 10^3 cells/ μL) and returned to pre-weaning baseline by d 14 (mean (s.e.m.) 8.6 (0.23) × 10^3 cells/ μL). This decrease in total leukocyte number reflected the decrease ($P < 0.05$) in neutrophil number on d 7, 14 and 21 (mean (s.e.m.) over these time-points was 2.13 (0.12) × 10^3 cells/ μL) with a concurrent decrease ($P < 0.01$) in lymphocyte number on d 7 (mean (s.e.m.) 4.8 (0.16) × 10^3 cells/ μL) compared with pre-weaning baseline. The N:L ratio increased ($P < 0.001$) on d 2 (mean (s.e) 0.73 (0.04)) and returned to pre-weaning baseline (mean (s.e.m.) 0.50 (0.02)) on d 7 (mean (s.e.m.) 0.44 (0.03)) (Figure 4.2a). Monocyte number decreased ($P < 0.001$, mean (s.e.m.) 0.23 (0.012) × 10^3 cells/μL) on d 2, whereas eosinophil number increased ($P < 0.001$) on d 2 (mean (s.e.m.) 0.72 (0.051) × 10^3 cells/μL) and 7 (mean (s.e.m.) 0.63 (0.040) × 10^3 cells/μL) compared with pre-weaning baselines (monocyte: mean (s.e.m.) 0.30 (0.013) × 10^3 cells/μL, eosinophils: mean (s.e.m.) 0.47 (0.033) × 10^3 cells/μL) (Table 4.3). Post-weaning, on d 14 to 35, monocyte (range mean (s.e.m.) 0.41-0.46 (0.022) × 10^3 cells/μL) and eosinophil (range mean (s.e.m.) 0.62-0.81

(0.055) × 10^3 cells/μL) number increased ($P < 0.05$) and did not return to pre-weaning baselines. Basophil number did not differ ($P > 0.05$) from pre-weaning baseline at any time-point post-weaning.

Post-housing, neutrophil number increased ($P < 0.01$) (mean (s.e.m.) 3.2 (0.24) × 10^3 cells/ μL) and lymphocyte number decreased ($P < 0.05$) (mean (s.e.m.) 4.7 (0.16) × 10^3 cells/ μL) on d 2 and both returned to pre-housing baseline (neutrophil: mean (s.e.m.) 2.7 (0.14) × 10^3 cells/ μL, and lymphocyte: mean (s.e.m.) 5.1 (0.19) × 10^3 cells/ μL) by d 7 (neutrophil: mean (s.e.m.) 2.4 (0.13) × 10^3 cells/ μL, and lymphocyte: mean (s.e.m.) 5.3 (0.20) × 10^3 cells/ μL) (Figure 4.1b). Total leukocyte number did not differ ($P > 0.05$) post-housing (mean (s.e.m.) 9.0 (0.27) × 10^3 cells/ μL) compared with pre-housing baseline (mean (s.e.m.) 9.2 (0.36) × 10^3 cells/ μL). The N:L ratio increased ($P < 0.05$) to mean (s.e) 0.68 (0.05) on d 2 and returned to pre-housing baseline level of mean (s.e.m.) 0.56 (0.04) by d 7 (Figure 4.2b). Monocyte number decreased ($P < 0.001$) on 2 to 21 (range mean (s.e.m.) 0.31-0.25 (0.017) ×10^3cells/μL) compared with pre-housing baseline (mean (s.e.m.) 0.46 (0.021) ×10^3 cells/μL). An increase ($P < 0.01$) in eosinophil number (mean (s.e.m.) 0.99 (0.076) ×10^3 cells/μL) compared with pre-housing baseline (mean (s.e.m.) 0.81 (0.076) ×10^3 cells/μL) was observed on d 2 post-housing. There was no difference ($P > 0.05$) in basophil number post-housing (Table 4.3).

4.3.2 Red blood cell (RBC) number and associated variables

Red blood cell number and HCT percentage decreased ($P < 0.05$) on d 7 to 21 and on d 14 and d 21, respectively, compared with pre-weaning baseline (RBC: mean (s.e.m.) 7.4 (0.10) $\times 10^6$cells/μL, HCT: mean (s.e.m.) 31.6 (0.31) %). There was no change in HGB concentration post-weaning (Table 4.4.). On d 7 to 35, MCV, MCH, and MCHC increased ($P < 0.05$) compared with pre-weaning baseline (MCV: mean (s.e.m.) 42.6 (0.49) fL, MCH: mean (s.e.m.) 16.0 (0.18) pg, MCHC: 37.7 (0.07) g/dL).

Post-housing, RBC number was unchanged ($P > 0.05$), whereas HGB and HCT percentage were increased ($P < 0.05$) on d 7 to 21 compared with pre-housing baseline (RBC: mean (s.e.m.) 7.6 (0.11) $\times 10^6$cells/μL, HGB: mean (s.e.m.) 13.3 (0.16) g/dL, HCT: mean (s.e.m.) 34.1 (0.42) %). On d 7 to 21, MCV decreased ($P < 0.001$) whereas MCHC increased ($P < 0.001$) compared with pre-weaning baseline (MCV: mean (s.e.m.) 44.9 (0.48) fL, MCHC: mean (s.e.m.) 38.9 (0.08) g/dL).

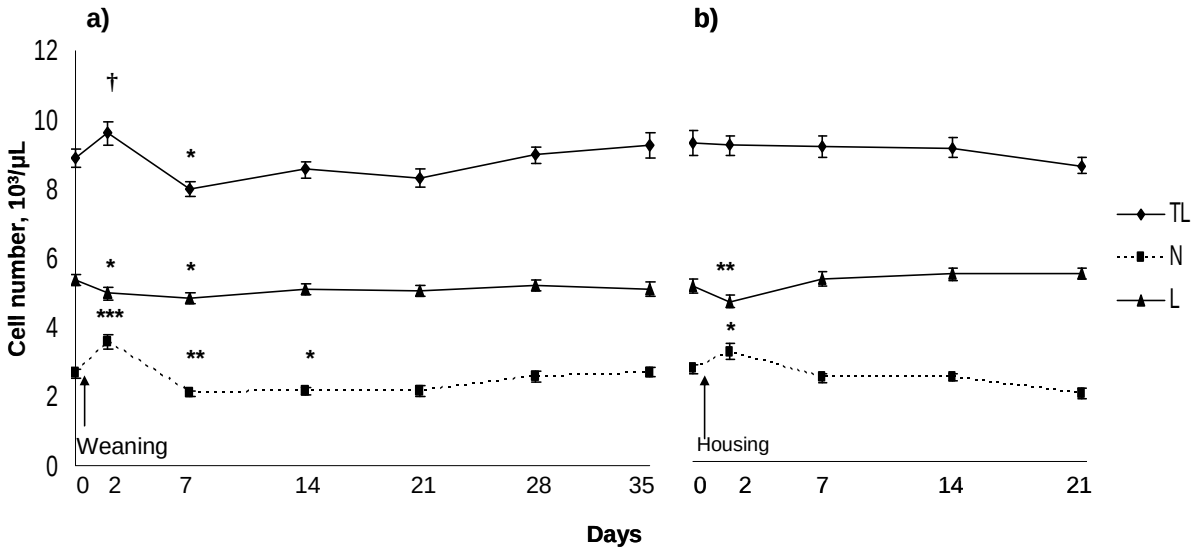

Figure 4.1. Effect of (i) abrupt weaning and (ii) subsequent housing on circulating total leukocyte number, neutrophil number and lymphocyte number in beef cows (n = 18 cows per treatment).

There was a tendency for a significant increase (P = 0.07) for total leukocyte number in beef cows at d 2 compared with d 0 and is denoted by †. Significant differences between d 0 (pre-weaning) and d 35 baselines (pre-housing) are denoted by asterisks; * = P < 0.05, ** = P < 0.01 and *** P < 0.001. TL = Total leukocyte number, L = lymphocyte number and N = neutrophil number.

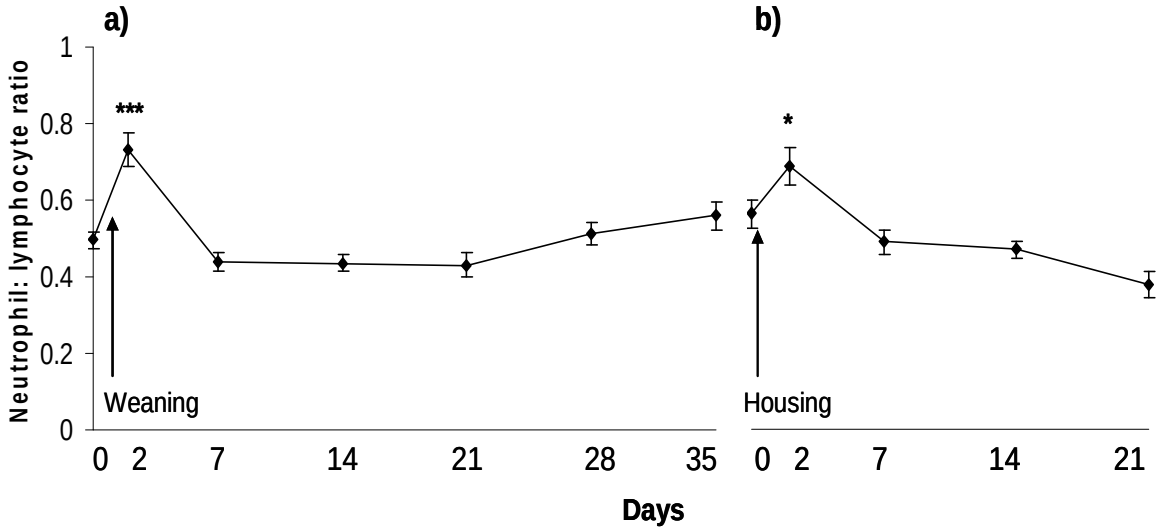

Figure 4.2. Effect of (i) abrupt weaning and (ii) subsequent housing on neutrophil: lymphocyte ratio in beef cows (n = 18 cows per treatment).

Significant differences between d 0 (pre-weaning) and d 35 baselines (pre-housing) are denoted by asterisks; * = $P < 0.05$ and *** $P < 0.001$.

Table 4.3. Least squares means (s.e.m.) for monocyte, eosinophil and basophil number in whole blood in beef cows following abrupt weaning and subsequent housing compared with pre-weaning and pre-housing baselines respectively.

	Pre-weaning	Days post-weaning						Pre-housing	Days post-housing			
Variable	**0**	**2**	**7**	**14**	**21**	**28**	**35**	**0**	**2**	**7**	**14**	**21**
Monocytes (×10^{3}cells/μL)	0.30 (0.013)	0.23[c] (0.012)	0.32 (0.017)	0.41[c] (0.021)	0.42[c] (0.023)	0.42[c] (0.022)	0.46[c] (0.021)	0.46 (0.021)	0.31[f] (0.015)	0.32[f] (0.017)	0.30[f] (0.019)	0.25[f] (0.017)
Eosinophils (×10^{3}cells/μL)	0.47 (0.033)	0.72[c] (0.051)	0.63[b] (0.040)	0.71[b] (0.063)	0.62[a] (0.049)	0.64[a] (0.049)	0.81[c] (0.060)	0.81 (0.076)	0.99[e] (0.076)	1.38 (0.424)	0.80 (0.067)	0.83 (0.056)
Basophils (×10^{3}cells/μL)	0.07 (0.003)	0.07 (0.003)	0.08[a] (0.004)	0.07 (0.003)	0.07 (0.04)	0.08 (0.004)	0.07 (0.003)	0.07 (0.003)	0.07 (0.003)	0.08 (0.004)	0.08 (0.004)	0.008 (0.003)

[a,b,c] Within a row, means differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

[d,e,f] Within a row, means differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

n = 18 cows per treatment.

Table 4.4. Least squares means (s.e.m.) for red blood cell number (RBC), and associated haematological variables in whole blood in beef cows following abrupt weaning and subsequent housing compared with pre-weaning and pre-housing baselines respectively.

	Pre-weaning	Days post-weaning						Pre-housing	Days post-housing			
Variable	**0**	**2**	**7**	**14**	**21**	**28**	**35**	**0**	**2**	**7**	**14**	**21**
RBC	7.4	7.5	7.2[a]	6.8[a]	6.9[a]	7.1	7.6	7.6	7.7	7.7	7.5	7.6
(×10^6/µL)	(0.10)	(0.09)	(0.11)	(0.07)	(0.08)[a]	(0.08)	(0.11)	(0.11)	(0.09)	(0.10)	(0.09)	(0.09)
HGB	11.9	12.1	12.0	11.6	11.9	12.1	12.0	13.3	13.4	13.6[d]	13.7[d]	13.7[d]
(g/dL)	(0.12)	(0.12)	(0.14)	(0.12)	(0.11)	(0.10)	(0.16)	(0.16)	(0.12)	(0.14)	(0.13)	(0.14)
HCT	31.6	31.9	31.0	30.2[a]	30.6[a]	31.9	34.1[a]	34.1	34.4	35.1[d]	32.1[d]	32.8[d]
(%)	(0.31)	(0.31)	(0.41)	(0.32)	(0.28)	(0.26)	(0.42)	(0.42)	(0.32)	(0.37)	(0.32)	(0.35)
MCV	42.6	42.6	43.1[c]	44.2[c]	44.3[c]	44.9[c]	44.9[c]	44.9	44.9	45.1	43.3[f]	43.5[f]
(fL)	(0.49)	(0.50)	(0.51)	(0.52)	(0.49)	(0.50)	(0.49)	(0.49)	(0.49)	(0.51)	(0.50)	(0.50)
MCH	16.0	16.1	16.7[c]	17.0[c]	17.2[c]	17.5[c]	17.5[c]	17.5	17.5	17.5	17.5	17.6
(pg)	(0.18)	(0.17)	(0.24)	(0.18)	(0.18)	(0.18)	(0.18)	(0.18)	(0.18)	(0.18)	(0.19)	(0.18)
MCHC	37.7	37.8	38.5[c]	38.8[c]	38.8[c]	38.9[c]	38.9[c]	38.9	38.9	38.8	40.4[f]	40.5[f]
(g/dL)	(0.07)	(0.09)	(0.25)	(0.09)	(0.08)	(0.08)	(0.08)	(0.08)	(0.09)	(0.11)	(0.09)	(0.12)

[a,b,c]Within a row, means differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

[d,e,f]Within a row, means differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

n = 18 cows per treatment.

4.3.3. In vitro lymphocyte production of interferon-γ (IFN-γ)

Following weaning, PHA-induced *in vitro* lymphocyte IFN-γ production decreased ($P < 0.05$) on d 2 (mean (s.e.m.) 0.17 (0.03) OD at 450nm) and d 35 (mean (s.e.m.) 0.20 (0.04)) compared with pre-weaning baseline (mean (s.e.m.) 0.34 (0.05)). The Con A-induced production of IFN-γ decreased ($P < 0.05$) on d 35 (mean (s.e) 0.30 (0.03)) compared with pre-weaning baseline (mean (s.e.m.) 0.59 (0.08)).

Post-housing, IFN-γ production was unchanged ($P > 0.05$) for both mitogens (Con A: mean (s.e.m.) 0.23 (0.04), and PHA: mean (s.e.m.) 0.24 (0.04)) on d 2 compared with pre-housing baseline (Con A: mean (s.e.m.) 0.29 (0.03), and PHA: mean (s.e.m.) 0.20 (0.4)). On d 21, Con A-induced IFN-γ production increased ($P < 0.05$, mean (s.e.m.) 0.58 (0.06)) compared with pre-housing baseline (mean (s.e.m.) 0.30 (0.03)), whereas PHA-induced IFN-γ production was unchanged ($P > 0.05$) from pre-housing baseline.

4.3.4. Acute phase proteins (fibrinogen and haptoglobin)

Concentrations of fibrinogen increased ($P < 0.05$) on d 2 to 35 (mean (s.e.m.) over this period was 476 (17.4) mg/dL) and haptoglobin increased ($P < 0.01$) on d 2 to 35 (mean (s.e.m.) over this period was 0.56 (0.034) mg/dL) compared with pre-weaning baseline (fibrinogen: mean (s.e.m.) 408 (15.2) mg/dL, and haptoglobin: mean (s.e.m.) 0.33 (0.013) mg/dL). Maximum peak concentrations of mean (s.e.m.) 493 (15.7) mg/dL and mean (s.e) 0.72 (0.071) mg/dL were recorded for fibrinogen on d 21 and for haptoglobin on d 14, respectively (Table 4.5). Post housing, concentration of fibrinogen decreased ($P < 0.05$) on d 2 to mean (s.e.m.) 411 (20.1) mg/dL and subsequently increased ($P < 0.001$) on d 14 to mean (s.e.m.) 587 (24.9) mg/dL compared with pre-housing mean (s.e.m.) baseline of 467 (20.1) mg/dL.

Concentration of haptoglobin was unchanged on d 2 and 7 ($P > 0.05$) but decreased ($P < 0.05$) on d 14 (mean (s.e.m.) 0.49 (0.017) mg/dL) and 21 (mean (s.e.m.) 0.54 (0.069) mg/dL) compared with pre-housing baseline (mean (s.e.m.) 0.68 (0.038) mg/dL) (Table 4.5).

Table 4.5. Least squares means (s.e.m.) for concentration of acute phase proteins (plasma fibrinogen and haptoglobin) in beef cows following abrupt weaning and subsequent housing compared with pre-weaning and pre-housing baselines, respectively.

	Pre-weaning	Days post-weaning						Pre-housing	Days post-housing			
Variable	**0**	**2**	**7**	**14**	**21**	**28**	**35**	**0**	**2**	**7**	**14**	**21**
Fibrinogen	408	458[a]	489[a]	489[a]	493[a]	462[a]	467[a]	467	411[d]	488	587[f]	364[f]
(mg/dL)	(15.2)	(16.9)	(18.6)	(20.7)	(15.7)	(12.8)	(20.1)	(20.1)	(20.1)	(22.2)	(24.9)	(27.2)
Haptoglobin	0.33	0.43[b]	0.53[b]	0.72[b]	0.46[b]	0.57[b]	0.67[b]	0.68	0.74	0.68	0.49[d]	0.54[d]
(mg/dL)	(0.013)	(0.031)	(0.025)	(0.071)	(0.016)	(0.025)	(0.038)	(0.038)	(0.037)	(0.032)	(0.017)	(0.069)

[a,b,c] Within a row, means differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

[d,e,f] Within a row, means differ from pre-housing baseline by $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

n = 18 cows per treatment.

4.4. Chapter Summary and Discussion

4.4.1. Summary of the main findings

Stress related variables were measured in beef cows to characterise the extended physiological and immunological responses to abrupt weaning and subsequent housing. Post-weaning, concentration of cortisol and dehydroepiandrosterone were unchanged ($P > 0.05$). Rectal body temperature, neutrophil number and neutrophil: lymphocyte ratio increased ($P < 0.01$) on d 2 compared with pre-weaning baseline. There was a tendency for total leukocyte number to increase ($P = 0.07$) on d 2 post-weaning. Lymphocyte and neutrophil number decreased ($P < 0.05$) on d 2 to 7 and d 7 to 21, respectively, compared with pre-weaning baseline. Interferon-γ production decreased ($P < 0.05$) on d 2 compared with pre-weaning baseline. An increase ($P < 0.05$) in acute phase proteins, fibrinogen and haptoglobin was evident on d 2 to 35 compared with pre-weaning baseline. Concentration of glucose increased on d 2 to 28, whereas non-esterified fatty acid decreased on d 2 to 35 compared with pre-weaning baseline. Post-housing, concentrations of cortisol, rectal body temperature, total leukocyte number, and glucose were unchanged ($P > 0.05$). On d 2 post-housing, neutrophil number and neutrophil: lymphocyte ratio increased ($P < 0.05$), whereas lymphocyte number and concentrations of dehydroepiandrosterone, fibrinogen and non-esterified fatty acid decreased ($P < 0.05$) compared with pre-housing baseline. Concentration of haptoglobin increased ($P < 0.05$) on d 14 to 21 post-housing.

4.4.2. Discussion

To our knowledge, no other study has characterised the extended physiological and immunological responses in beef cows to abrupt weaning or subsequent housing.

The effect of post-weaning management practice [previously grazed calves were abruptly weaned and either immediately housed in a slatted floor shed or returned to the grazing area for 35 d and then housed] on physiological, haematological and immunological responses in the progeny of the cows used in the present study was also measured up to d 35 post-weaning and d 21 post-housing (Lynch *et al.*, 2008b, reported in chapter 3 of this thesis). Behavioural research to date has shown that breaking the mother-offspring bond alone represents a stressful experience for both mother and young (Newberry and Swanson, 2008). Following separation from their calves, beef cows generally exhibit similar, less intense, reinstatement behaviours (increased locomotor activity and vocalisations) to their young, in an effort to reunite (Price *et al.*, 2003). Consequently, no behavioural measurements were examined as part of this study.

The milk yield of the cow breed type (beef × dairy) used in the present study remains relatively high at weaning (McGee *et al.*, 2005b) and milk would have constituted a significant proportion of the calves' total diet. There is no information in the literature on the extent to which drying off induces a systemic inflammatory response in beef cows, however, there is considerable literature on systemic and local inflammatory responses during mastitis in dairy cows (Nickerson, 1989; Sordillo *et al.*, 1997; Sordillo, 2005; Singh *et al.*, 2005; Rainard and Pollet, 2006). During the drying off period, the mammary gland continues to synthesise and secrete milk which accumulates in the gland. An increase in intra-alveolar pressure as a result of milk accumulation is thought to trigger involution. Involution-associated ultrastructural changes in bovine mammary cells are reported to commence within 48 hours after cessation of milk removal (Holst *et al.*, 1987; Hurley, 1989) and by day 28 the collapsed alveolar structures remaining are considerably smaller than during lactation.

In cows, the alveolar structure is maintained throughout involution and there is no evidence for extensive tissue degeneration that is found in other species, such as rodents (Walker *et al.*, 1989; Huang and Ip, 2001). The impact of mammary cell apoptosis in the bovine is not fully characterised. In the present study, all animals were monitored daily and no signs of ill-health were recorded. The small increase in rectal body temperature recorded post-weaning was not of clinical significance (Duff and Galyean, 2007).

In accord with the findings of Lefcourt and Elsasser (1995), where cows were separated from their calves when calves were 4 to 6 months of age, weaning was not associated with increased plasma cortisol concentration in cows. In contrast, Whisnant *et al.* (1985), using more frequent blood sampling collection time points relative to cow-calf separation than those used in our study, reported increased concentration of cortisol in cows that were separated from their calves 35 days post-partum. The large difference in age at weaning may account for the disparity in cortisol response observed between these studies. The absence of an effect of housing on cortisol concentration contrasts with the findings of Higashiyama *et al.* (2007) who reported an increase in plasma concentration in previously grazed cows that were housed. However, in the latter study, cows were tethered, whereas in the present study cows were loose-housed in pens. In the present study, concentration of DHEA was not affected by weaning. This hormone is episodically co-secreted with cortisol from the adrenal gland and is also a potential precursor for androgen and oestrogen synthesis in cattle (Marinelli *et al.*, 2007). Post-housing, concentration of DHEA decreased without a concomitant increase in cortisol. Increased cortisol: DHEA ratio was reported in lame dairy cows (Almeida *et al.*, 2007) and following transportation of young bulls (Buckham Sporer *et al.*, 2008) and was suggested as a potential biomarker

of stress. The unaltered cortisol: DHEA ratio found in the present study suggests that the practice of weaning and housing were insufficient to elicit a physiological stress response, whereby a shift occurred in the steroidogenic pathway towards cortisol at the expense of DHEA. Further research investigating the use of cortisol: DHEA ratio as a biomarker of stress is warranted.

Peripheral blood cells are sensitive indicators of patho-physiological responses and may be used to define subclinical disease states in cattle (Jones and Allison, 2007). Alterations in circulating leukocyte subsets have been documented in beef cattle under various management practices such as restricted space allowance during housing of steers (Gupta *et al.*, 2007), transportation of bulls (Buckham Sporer *et al.*, 2007a) and weaning of calves (Lynch *et al.*, 2008b Chapter 3; Blanco *et al.*, 2009). In agreement with the latter studies using calves, an increased total leukocyte number, as defined by increased neutrophil number and decreased lymphocyte number (increased N:L ratio), was evident in cows post-weaning. The magnitude of the increase in neutrophil number was higher (33 % versus 18 %) on d 2 post-weaning compared with d 2 post-housing in cows. The changes observed in RBC number, HGB concentration, HCT percentage, mean corpuscular concentration, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration in cows were negligible and within the normal physiological range for multiparous beef cows (Kramer, 2006) suggesting that there were no negative consequences of weaning or housing on these variables.

The decrease in IFN-γ production on d 2 compared with pre-weaning baseline was consistent with the findings of Lynch *et al.* (2008b; Chapter 3) who weaned calves and returned them to pasture and with Hickey *et al.* (2003a) and Lynch *et al.* (2008b; Chapter 3) using weaned calves that were immediately housed. The relative decrease in IFN-γ production on d 2 compared with pre-weaning baseline was lower (48%

versus 67 %) in cows compared with the decrease found in their calves (Lynch *et al.*, 2008b; Chapter 3). An absence of a decrease in IFN-γ production in cows on d 2 post-housing suggests that housing was insufficient to elicit a stress response, and these findings are consistent with Lynch *et al.* (2008b; Chapter 3) who reported unchanged IFN-γ production post-housing in calves that were previously grazed.

Overall, these results suggest that cows were more sensitive to the effects of weaning than to the effects of housing due to the less marked neutrophil response and unchanged IFN-γ production observed post-housing. Cows may also be less sensitive to weaning- and housing-associated stress, as indicated by the relatively lower change in neutrophil number and IFN-γ production, compared with their calves (Lynch *et al.*, 2008b; Chapter 3).

Post-weaning, cows had increased concentrations of plasma fibrinogen and haptoglobin, which is in agreement with the results obtained for calves following abrupt weaning (Lynch *et al.*, 2008b; Chapter 3). Following housing, no clear trend was evident for the acute phase protein response. The response most housing may have been influenced by the change in dietary crude protein concentration.

Post-weaning, decreased albumin concentrations were evident, and are most likely attributed to the acute phase response and associated with elevated globulin concentrations (Thomas, 2006), as reported in this chapter. The ratio of albumin: globulin (AG ratio) is relatively consistent in healthy cattle and the reference interval has been defined as 0.84-0.94 (Kaneko, 1997).

Blood metabolite concentrations indicate the extent of metabolism of energy, protein and other nutrients and thus, can provide information on the nutritional status of cattle. Typically, stressful events result in reduced feed intake and depletion of energy stores in cattle (Duff and Galyean, 2007). In the present study, weaning and

subsequent housing caused transient changes in blood metabolites. Increased concentration of glucose is often attributed to glycogenolysis, which is associated with increased catecholamines and glucocorticoid secretion at the onset of a stressor (McDowell, 1983). However, due to the unaltered cortisol response throughout the study, it is unlikely that the increased concentration of glucose is solely a consequence of weaning as a stressor, but rather an indication of a more positive nutritional status in the cow due to the cessation of nursing. Generally, higher concentrations of NEFA and βHB and lower concentrations of glucose are associated with negative energy balance in beef cows (McGee *et al.*, 2005a). Thus, the opposing trend of concentrations of plasma glucose and NEFA suggests that removal of the calf from the cow resulted in a more positive nutritional state in the cow.

Elevated creatine kinase activity can be used to identify increased levels of activity or exercise, with excessive elevations of plasma creatine kinase a signature of muscle membrane damage, as has been reported following the transportation of cattle (Tarrant, 1990). The increased creatine kinase activity observed in the present study may represent a period of increased locomotor activity immediately post-weaning as the cows search for their calves. Although, no behavioural data were collected during this experiment, literature has shown the cows are highly motivated to reunite with the calves post-weaning, and spend considerable time walking and vocalising, albeit that their responses are not as marked in terms of intensity as those documented for calves (Price *et al.*, 2003). As a result of confinement within their pens at housing, creatine kinase activity was lower than that when at pasture, following 7 d of housing.

Under the conditions of the present study, animal was the experimental unit and was specified as a repeated measures effect in the statistical analysis and most of the physiological and immunological variables which changed as a consequence of i)

weaning and ii) housing had recovered to baseline values on completion of the study. In the present study, while changes were found in physiological and immunological variables in response to weaning and subsequent housing, the values were within the normal physiological ranges for cattle (Radostisis, 2000; Earley and Crowe, 2002; Kramer, 2006; Jones and Allison, 2007).

In conclusion, abrupt weaning results in neutrophilia and lymphopenia, coupled with reduced *in vitro* lymphocyte production of IFN-γ and increased acute phase protein response suggests that weaning elicited a transitory stress response in beef cows. Moreover, increased concentration of glucose coupled with decreased concentration of NEFA indicate improved nutritional status in the cow post-weaning. Post-housing, transitory neutrophilia and lymphopenia were evident, however unchanged total leukocyte number and IFN-γ production and the lack of a clear trend in the acute phase protein response indicates that housing did not elicit a stress response as great as weaning in the beef cow.

Chapter 5

Effect of abrupt weaning at housing on leukocyte distribution, functional activity of neutrophils, and the acute phase protein response of beef calves

5.1 Introduction

Weaning is an inherent husbandry practice in cow-calf beef production systems that imposes physical, psychological, and nutritional stressors on calves. Integrated calf-to-beef production systems, such as seasonal grass-based systems, often combine weaning and housing (Drennan and McGee, 2009), whereas non-integrated systems often combine weaning with additional stressors such as transportation and marketing, prior to entry into feedlots (Duff and Galyean, 2007). Alterations in calf behaviour (Price *et al.*, 2003; Haley *et al.*, 2005), hormonal mediators of stress (Lefcourt and Elsasser, 1995; Blanco *et al.*, 2009) and consequently measures of immune function (Hickey *et al.*, 2003a; Arthington *et al.*, 2005; 2008) are evident post-weaning. Furthermore, weaning is considered to be a predisposing factor to bovine respiratory disease (BRD) (Callan and Garry, 2002; Snowder *et al.*, 2006; Snowder 2009).

Neutrophils provide the first line of cellular defense against pathogens, whereas lymphocytes are of pivotal importance in cell-mediated and humoral immunity (Paape *et al.*, 2003; Janeway *et al.*, 2005). Although studies have examined neutrophil and lymphocyte function and distribution following transport (Riondato *et al.*, 2008; Buckham Sporer *et al.*, 2007a), and during natural and experimental cases of BRD (McInnes *et al.*, 1999; Coomber *et al.*, 2001; Sandbulte and Roth, 2002), none have investigated the direct effects of weaning on these immune variables in beef calves. Additional information on the immune status of newly weaned calves at a time when pathogen exposure is heightened may be useful for identifying animals likely to succumb to infection.

Thus, the objectives of the study were to examine the effect of abrupt weaning at housing on (i) peripheral leukocyte and differential number, (ii) granulocyte positive neutrophils and lymphocyte immunophenotypes, (iii) phagocytic and oxidative burst

activity, and surface expression of CD62L of neutrophils, and (iv) the acute phase protein response in beef calves. The experimental design is described in Section 2.2.2.3.

5.2 Effect of abrupt weaning on physiological responses in beef calves

5.2.1 Rectal body temperature

There was no effect of treatment ($P = 0.4$) or treatment × sampling time interaction ($P = 0.3$), whereas sampling time was significant ($P = 0.048$) for rectal body temperature (Figure 5.1). On d 2, rectal body temperature increased ($P = 0.02$) compared with baseline (mean (s.e.m.) 38.9 (0.09) °C versus mean (s.e.m.) 38.5 (0.07) °C).

5.2.2 Cortisol and dehydroepiandrosterone (DHEA)

There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction for cortisol or DHEA (Table 5.1). Concentration of cortisol and DHEA remained lower than 2.1 and 1.0 ng/mL, respectively, in all calves for the duration of the study. The mean (s.d.) concentration of cortisol and DHEA over the entire experimental period were 1.8 (0.66) ng/mL and 0.78 (0.081) ng/mL, respectively.

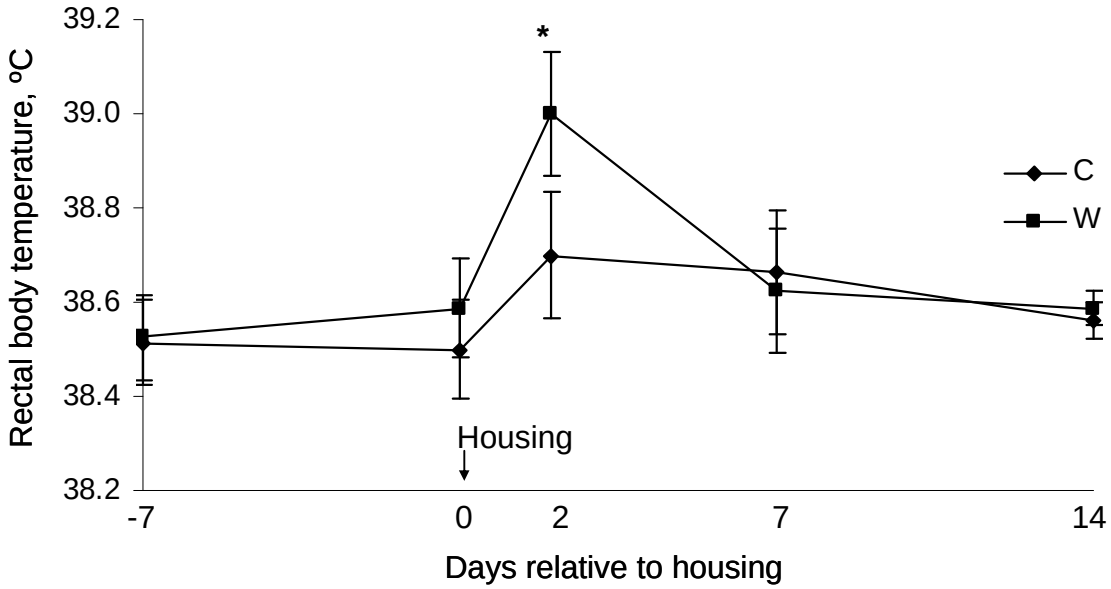

Figure 5.1. Effect of abrupt weaning at housing on rectal body temperature in beef calves.

There was no treatment × sampling time interaction for rectal body temperature (P = 0.3). Sampling time was significant (P = 0.048), it increased (P = 0.02) on d 2 and subsequently returned (P > 0.05) to baseline. W = abruptly weaned calves (n = 8), C = non-weaned (control) calves (n = 8). T × S = treatment × sampling time point interaction, S = sampling time. * = P < 0.05. Baseline is defined as d 0.

Table 5.1. Effect of abrupt weaning at housing concentration of cortisol and dehydroepiandrosterone[1] (least squares means ± s.e.m., ng/mL) in beef calves.

		Days relative to housing					***P*-values[2]**		
Variable		**-7**	**0**	**2**	**7**	**14**	**T**	**S**	**T×S**
Cortisol	C	1.9 ± 0.24	1.9 ± 0.33	1.8 ± 0.18	1.3 ± 0.07	1.9 ± 0.17	NS	***	NS
	W	2.1 ± 0.24	1.8 ± 0.34	1.9 ± 0.18	1.5 ± 0.07	1.7 ± 0.17			
DHEA[1]	C	0.72 ± 0.011	0.72 ± 0.030	0.70 ± 0.024	0.71 ± 0.030	0.71 ± 0.037	NS	NS	NS
	W	0.71 ± 0.012	0.70 ± 0.031	0.71 ± 0.025	0.71 ± 0.025	0.72 ± 0.037			

[1]DHEA = dehydroepiandrosterone.

[2]T = treatment, S = sampling time, T × S = treatment × sampling time interaction.

P-values; NS = $P > 0.05$, ** = $P < 0.01$.

C = non-weaned (control) calves (n = 8), W = abruptly weaned (n = 8).

5.3 Effect of abrupt weaning on immunological responses in beef calves

5.3.1 Total leukocyte and differential number

There was a treatment × sampling time interaction (P = 0.01) for total leukocyte number whereby on d 2, it increased (P = 0.0002) in W (mean (s.e.m.) 13.3 (0.47) × 10^3 cells/μL), whereas C (mean (s.e.m.) 10.7 (0.47) × 10^3 cells/μL) did not differ (P > 0.05) from baseline (mean (s.e.m.) 10.4 (0.61) × 10^3 cells/μL) (Table 5.2). On d 7 and 14, total leukocyte number in W and C did not differ from baseline.

There were treatment × sampling time interactions for neutrophil (P < 0.0001) and lymphocyte (P = 0.002) number (Table 5.2). On d 2, neutrophil number increased (P < 0.001, mean (s.e.m.) 6.6 (0.25) × 10^3 cells/μL) and lymphocyte number decreased (P < 0.001, mean (s.e.m.) 5.2 (0.23) × 10^3 cells/μL) in W, whereas C (neutrophil: mean (s.e.m.) 3.3 (0.25) × 10^3 cells/μL; lymphocyte: mean (s.e.m.) 6.7 (0.22) × 10^3 cells/μL) did not differ from baseline (C: neutrophil: mean (s.e.m) 2.8 (0.11) × 10^3 cells/μL, lymphocyte: mean (s.e.m.) 7.0 (0.19) × 10^3 cells/μL) (Table 5.2).

There were no effects (P > 0.05) of treatment and sampling time, or treatment × sampling time interactions for monocyte, eosinophil and basophil number (Table 5.2). Monocyte, eosinophil, and basophil number ranged (minimum - maximum) from 1.9 – 14.2 × 10^2 cells/μL, 0.5 – 1.5 × 10^2 cells/μL, and 0.4 – 3.0 × 10^2 cells/μL respectively.

5.3.2. Red blood cell number and associated variables

There were no effects of treatment or treatment × sampling time (P > 0.05) for RBC number, concentration of HGB, HCT percentage, MCV, MCH and MCHC post-housing (Table 5.3). Sampling time was significant (P = 0.005) for MCHC only. Mean corpuscular haemoglobin concentration decreased (P < 0.05) on d 2, 7 and 14

compared with pre-weaning baseline. Over the experimental period, RBC number, concentration of HGB, and percentage HCT ranged (minimum – maximum) from 8.6 - 12.2 × 10^6 cells/μL, 10.3 - 15.6 g/dL, and 27 - 39.1 % respectively. Corresponding means (s.d.) were 10.5 (0.87) × 10^6 cells/μL, 13.2 (1.41) g/dL, and 33.7 (2.70) % for RBC number, HGB concentration and HCT percentage, respectively. Mean corpuscular volume, MCH, and MCHC ranged from (minimum – maximum) 28.4 - 35.0 fL, 11.2 – 13.6 pg, and 37.2 - 41.9 %, respectively. Corresponding means (s.d.) for these variables were 32.0 (1.96) fL, 12.6 (0.72) pg, and 39.3 (0.94) %, respectively.

Table 5.2. Effect of abrupt weaning at housing on total leukocyte, neutrophil, lymphocyte, monocyte, eosinophil, and basophil number (least squares means ± s.e.m., $\times 10^3$ cells/μL) in beef calves.

		Days relative to housing					*P*-values[1]		
Cell type		**-7**	**0[2]**	**2**	**7**	**14**	**T**	**S**	**T×S**
Total leukocytes	C	10.3 ± 0.61	10.0 ± 0.31	10.7^{x} ± 0.47	10.5 ± 0.92	10.2 ± 0.32	*	***	**
	W	10.6 ± 0.62	10.2^{a} ± 0.31	$13.3^{b,c,y}$ ± 0.47	$11.2^{a,c}$ ± 0.92	10.0^{a} ± 0.32			
Neutrophils	C	2.8 ± 0.11	2.9 ± 0.17	3.2^{x} ± 0.65	2.9 ± 0.64	2.5 ± 0.23	***	***	***
	W	2.7 ± 0.11	2.5^{a} ± 0.18	$6.6^{b,y}$ ± 0.68	2.7^{a} ± 0.64	2.7^{a} ± 0.24			
Lymphocytes	C	7.0 ± 0.19	6.7 ± 0.21	6.6^{x} ± 0.22	6.8 ± 0.49	6.9 ± 0.36	NS	***	**
	W	6.9 ± 0.19	6.9^{a} ± 0.22	$5.2^{b,y}$ ± 0.22	7.3^{a} ± 0.49	6.7^{a} ± 0.37			
Monocytes	C	0.64 ± 0.10	0.46 ± 0.05	0.59 ± 0.07	0.54 ± 0.11	0.47 ± 0.05	NS	NS	NS
	W	0.60 ± 0.11	0.48 ± 0.06	0.59 ± 0.06	0.59 ± 0.10	0.50 ± 0.05			
Eosinophils	C	0.29 ± 0.05	0.33 ± 0.10	0.31 ± 0.12	0.59 ± 0.11	0.35 ± 0.07	NS	NS	NS
	W	0.21 ± 0.05	0.31 ± 0.11	0.38 ± 0.13	0.29 ± 0.10	0.24 ± 0.07			
Basophils	C	0.12 ± 0.02	0.15 ± 0.02	0.13 ± 0.01	0.11 ± 0.02	0.12 ± 0.01	NS	NS	NS
	W	0.11 ± 0.02	0.12 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.13 ± 0.01			

[1]T = treatment, S = sampling time, T × S = treatment × sampling time interactions.

[2]Baseline is defined as d 0 for each variable.

C = non-weaned (control) calves (n = 8), W = abruptly weaned (n = 8).

P-values; NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

[a,b,c]Lsmeans within rows differ from pre-weaning baseline by $P < 0.05$.

[x,y]Within day for cell type, Lsmeans differ by $P < 0.001$.

Table 5.3. Effect of abrupt weaning at housing on red blood cell (RBC) number and associated variables (least squares means ± s.e.m.) in beef calves.

Variable		Days relative to housing					*P*-values[1]		
		-7	0[2]	2	7	14	T	S	T×S
RBC, ×10^6 cells/µL	C	10.9 ± 0.30	10.3 ± 0.31	10.4 ± 0.31	10.5 ± 0.29	10.3 ± 0.27	NS	NS	NS
	W	10.4 ± 0.30	10.3 ± 0.31	10.8 ± 0.31	10.9 ± 0.28	10.5 ± 0.27			
HGB[3], g/dL	C	14.1 ± 0.37	13.5 ± 0.40	13.4 ± 0.42	13.5 ± 0.35	13.3 ± 0.30	NS	NS	NS
	W	12.5 ± 0.37	12.6 ± 0.41	13.2 ± 0.42	13.3 ± 0.35	13.05 ± 0.30			
HCT[4], %	C	35.5 ± 0.88	33.8 ± 1.06	34.0 ± 0.98	34.4 ± 0.76	33.8 ± 0.70	NS	NS	NS
	W	32.3 ± 0.88	32.0 ± 1.06	33.7 ± 0.98	34.2 ± 0.75	33.3 ± 0.69			
MCV[5], fL	C	32.5 ± 0.66	32.7 ± 0.66	32.7 ± 0.62	32.9 ± 0.60	32.8 ± 0.66	NS	NS	NS
	W	31.2 ± 0.66	31.1 ± 0.66	31.3 ± 0.62	31.4 ± 0.60	31.8 ± 0.66			
MCH[6], pg	C	12.9 ± 0.20	13.0 ± 0.21	12.9 ± 0.23	12.9 ± 0.22	12.9 ± 0.22	NS	NS	NS
	W	12.0 ± 0.20	12.2 ± 0.21	12.2 ± 0.23	12.2 ± 0.22	12.4 ± 0.22			
MCHC[7], %	C	39.8 ± 0.40	39.8 ± 0.30	39.4 ± 0.26	39.3 ± 0.24	39.3 ± 0.29	NS	**	NS
	W	38.7 ± 0.40	39.5 ± 0.30	39.1 ± 0.26	38.6 ± 0.24	39.1 ± 0.29			

[1]T = treatment, S = sampling time, T × S = treatment × sampling time interactions.

P-values, NS = $P > 0.05$, ** = $P < 0.01$.

[2]Baseline is defined as d 0 for each variable.

C = non-weaned (control) calves (n = 8), W = abruptly weaned (n = 8).

[3]HGB = haemoglobin concentration,

[4]HCT = haematocrit percentage,

[5]MCV = mean corpuscular volume,

[6]MCH = mean corpuscular haemoglobin,

[7]MCHC = mean corpuscular haemoglobin concentration.

5.3.3. Granulocyte positive neutrophils

There was no effect of treatment or treatment × sampling time interaction ($P > 0.05$) for percentage $G1^+$ neutrophils. Sampling time was significant ($P < 0.0001$), on d 2, it increased (mean (s.e.m.) 48.5 (1.71) MFI) $P < 0.001$) and subsequently returned to baseline (mean (s.e.m.) 37.3 (1.7) MFI) by d 7 (Table 5.4).

5.3.4. Neutrophil functional activity

5.3.4.1. Surface expression of L-selectin (CD62L)

There was a treatment × sampling time interaction ($P = 0.002$) for MFI of $CD62L^+$ neutrophils (Table 5.4). On d 2, MFI decreased ($P < 0.001$) in W (mean (s.e.m.) 10.0 (2.31) MFI) and subsequently returned to baseline (mean (s.e.m.) 22.6 (1.82) MFI), whereas C did not differ ($P > 0.05$) from baseline (mean (s.e.m.) 20.9 (1.82) MFI). On d 14, MFI of $CD62L^+$ neutrophils did not differ ($P > 0.05$) in W (mean (s.e.m.) 21.3 (1.50) MFI) and C (mean (s.e.m.) 20.4 (1.50) MFI) compared with baseline.

5.3.4.2. Neutrophil phagocytic activity

There was a treatment × sampling time interaction ($P = 0.02$) for percentage of neutrophils performing phagocytosis (Table 5.5). On d 2, percentage neutrophils performing phagocytosis decreased ($P < 0.001$, mean (s.e.m.) 61.3 (3.20) %) in W, whereas it did not differ ($P > 0.05$) in C compared with baseline (W: mean (s.e.m.) 87.0 (2.01) %, C: 87.2 (2.01) %). Subsequently, it returned to baseline ($P > 0.05$).

5.3.4.3. Neutrophil oxidative burst activity

There was no effect ($P > 0.05$) of treatment, sampling time, or treatment × sampling time interaction for the percentage neutrophils positive for oxidative burst activity

(Table 5.5). The mean (s.e.m.) percentage of neutrophils performing oxidative burst activity over the experimental period was 39.5 (3.38) % of total neutrophils.

Table 5.4. Effect of abrupt weaning at housing on percentage $G1^+$ neutrophils and surface expression of L-selectin (CD62L) in beef calves.

		Days relative to housing					*P*-values[1]		
Neutrophils		**-7**	**0[2]**	**2**	**7**	**14**	**T**	**S**	**T×S**
$G1^+$, %	C	27.6 ± 0.68	27.3[a] ± 0.83	35.0[b,c,x] ± 0.77	30.8[a,c] ± 0.84	27.7[a] ± 0.85	NS	***	NS
	W	28.9 ± 0.68	27.4[a] ± 0.83	41.9[b,y] ± 0.77	31.4[a] ± 0.84	28.2[a] ± 0.85			
$CD62L^+$, MFI[3]	C	20.9 ± 1.81	21.1 ± 1.91	19.8[x] ± 2.31	22.3 ± 2.02	20.4 ± 1.49	NS	***	**
	W	22.6 ± 1.81	22.2[a] ± 1.91	10.0[b,y] ± 2.31	19.3[a] ± 2.02	11.3[a] ± 1.49			

[1]T = treatment, S = sampling time, T×S = treatment × sampling time interactions.

P-values, NS = $P > 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

[2]Baseline is defined as d 0 for each variable.

[3]MFI = mean fluorescence intensity

W = abruptly weaned (n = 8), C = non-weaned (control) calves (n = 8).

[a,b,c]Within a row, least squares means without a common superscript differ ($P < 0.05$).

[x,y]Within a column (day) for each variable, least squares means without a common superscript differ ($P < 0.01$).

Table 5.5. Effect of abruptly weaning at housing on percentage neutrophils performing phagocytic and oxidative burst activity in beef calves.

		Days relative to housing					*P*-values[1]		
Neutrophils, %		**-7**	**0[2]**	**2**	**7**	**14**	**T**	**S**	**T×S**
Phagocytosis positive	C	87.2 ± 2.00	88.8 ± 1.98	79.4^{x} ± 3.20	87.4 ± 2.48	89.6 ± 2.18	**	***	*
	W	87.0 ± 2.00	89.6^{a} ± 1.98	61.3^{b,y} ± 3.20	79.7^{a} ± 2.48	89.3^{a} ± 2.18			
Oxidative burst positive	C	36.5 ± 3.93	36.6 ± 2.14	45.1 ± 4.83	46.1 ± 5.24	40.3 ± 5.82	NS	NS	NS
	W	34.7 ± 3.93	37.0 ± 2.14	41.3 ± 4.83	37.2 ± 5.24	40.1 ± 5.82			

[1]T = treatment, S = sampling time, T×S = treatment × sampling time interactions.

P-values, NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

[2]Baseline is defined as d 0 for each variable.

W = abruptly weaned (n = 8), C = non-weaned (control) calves (n = 8).

[a,b,c]Within a row, least squares means without a common superscript differ ($P < 0.01$).

[x,y]Within a column (day) for each variable, least squares means without a common superscript differ ($P < 0.05$).

5.3.5. Lymphocyte immunophenotypes

There was a treatment × sampling time interaction for percentage $CD4^+$ ($P = 0.0002$) and $CD8^+$ ($P < 0.0001$) lymphocytes. On d 2, percentage $CD4^+$ and $CD8^+$ lymphocytes decreased ($P < 0.001$) in W ($CD4^+$: mean (s.e.m.) 15.1 (0.87) %; $CD8^+$: mean (s.e.m.) 8.0 (0.34) %), and subsequently returned to baseline ($P > 0.05$; $CD4^+$: mean (s.e.m.) 21.7 (0.76) %; $CD8^+$: mean (s.e.m.) 11.8 (0.36) %), whereas C did not differ ($P > 0.05$) from baseline ($CD4^+$: mean (s.e.m.) 19.4 (0.76) %; $CD8^+$: mean (s.e.m.) 10.7 (0.36) %) (Figure 5.2a and b). There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction for CD4:CD8 ratio (Figure 5.2c). Sampling time was significant ($P = 0.005$), whereby CD4:CD8 ratio increased ($P < 0.05$) on d 7 (mean (s.e.m.) 1.9 (0.055)) compared with baseline (mean (s.e.m.) 1.8 (0.04)) (Figure 5.2c).

There was a treatment × sampling time interaction for $WC1^+$ ($P < 0.001$) and MHC Class II^+ ($P = 0.002$) lymphocytes. On d 2, the percentage $WC1^+$ lymphocytes decreased ($P < 0.001$) in W and C to mean (s.e.m.) 12.3 and 19.9 (0.64) %, respectively, however the decrease was greater ($P < 0.001$) in W (Figure 5.3a). Subsequently, the percentage $WC1^+$ lymphocytes returned ($P > 0.05$) to baseline (W: mean (s.e.m.) 24.3 (0.49) %; C: mean (s.e.m.) 23.1 (0.49) %) in both treatments. Conversely, on d 2, percentage MHC Class II^+ lymphocytes increased ($P < 0.001$) in W and C to mean (s.e.m.) 30.3 and 21.9 (1.07) % respectively, but the increase was greater ($P < 0.001$) in W than C, before returning to baseline ($P > 0.05$; W: mean (s.e.m.) 14.0 (1.01) %, C: mean (s.e.m.) 13.4 (1.01) %) (Figure 5.3b).

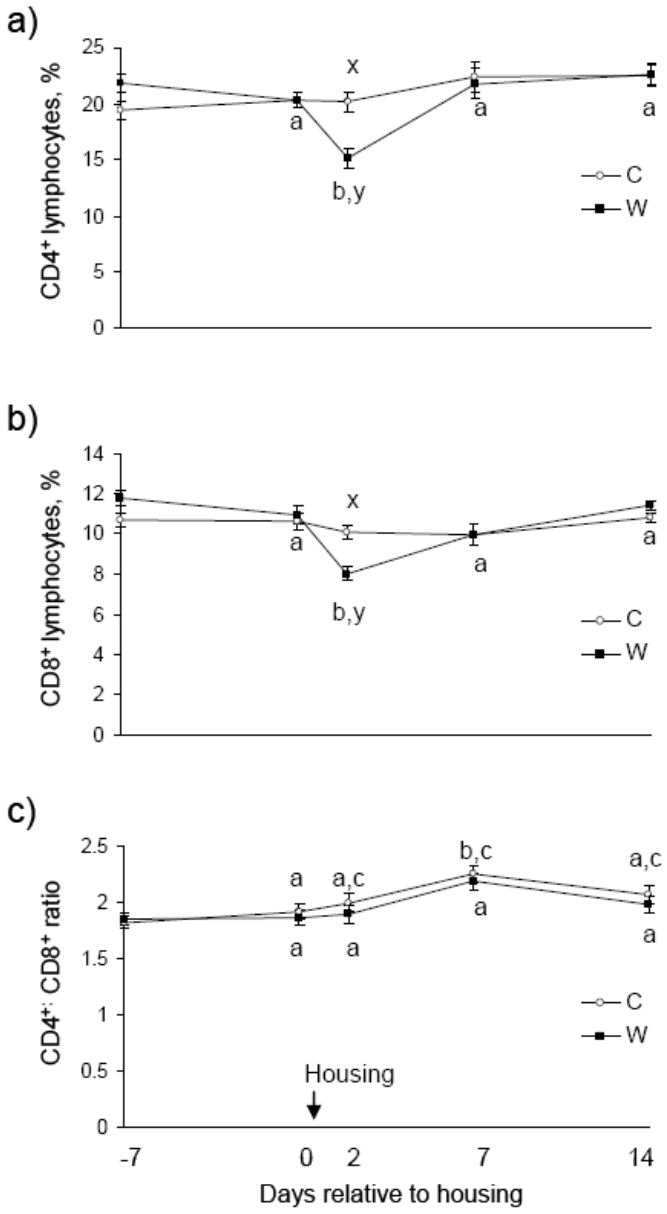

Figure 5.2. Effect of abrupt weaning at housing on percentage (a) CD4$^+$ and (b) CD8$^+$ lymphocytes (least squares mean ± s.e.m., %) and (c) CD4: CD8 ratio (least squares means ± s.e.m.) in beef calves.

W = abruptly weaned calves (n = 8), C = non-weaned (control) calves (n = 8). ^{a,b,c}Between days, least squares means without a common superscript differ ($P < 0.05$). ^{x,y}Within a day, least squares means without a common superscript differ ($P < 0.05$). T × S = treatment × sampling time point interaction. Baseline is defined as d 0 for each variable.

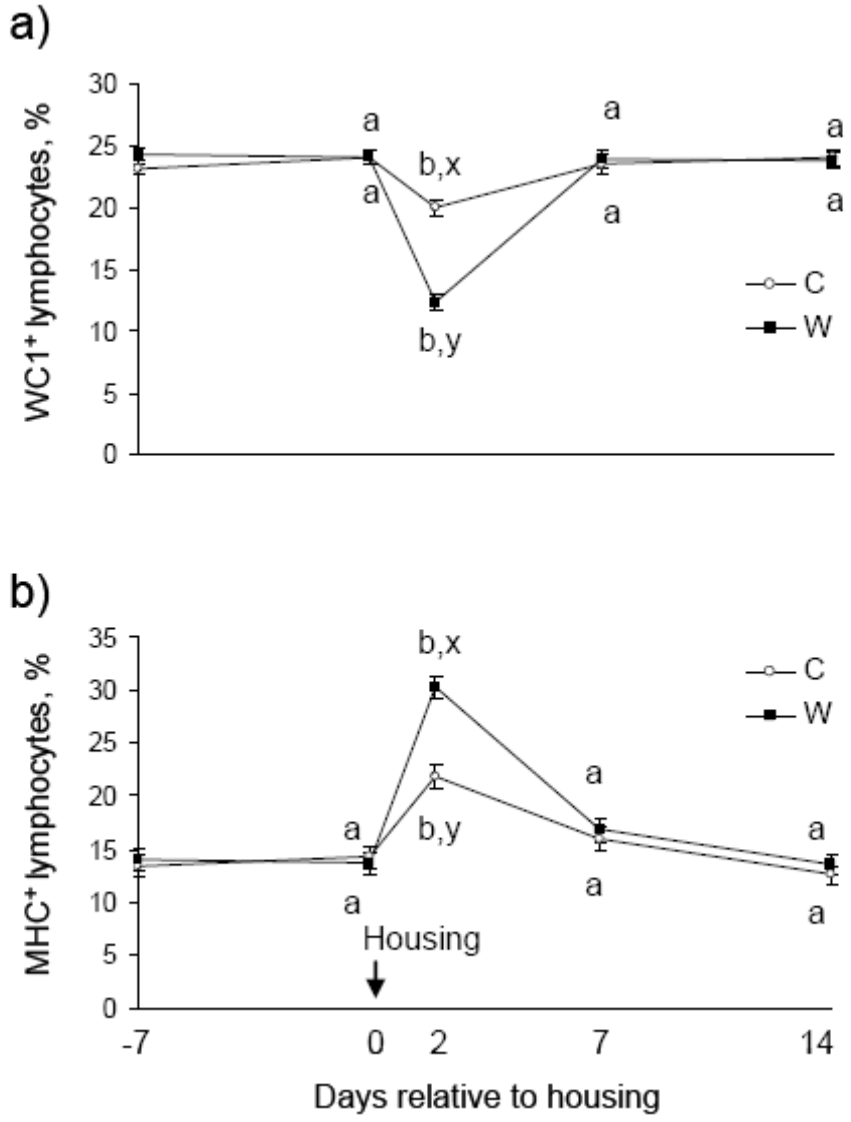

Figure 5.3. Effect of abrupt weaning at housing on (a) WC1^{+} and (b) MHC Class II^{+} lymphocytes (least squares mean ± s.e.m., %) in beef calves.

W = abruptly weaned calves (n = 8), C = non-weaned (control) calves (n = 8). ^{a,b,c}Between days, least squares means without a common superscript differ (P < 0.001). ^{x,y}Within a day, least squares means without a common superscript differ (P < 0.01). T × S = treatment × sampling time point interaction. Baseline is defined as d 0 for each variable.

5.3.6. Acute phase proteins

There were no effects ($P > 0.05$) of treatment or treatment × sampling time interactions for concentrations of fibrinogen and haptoglobin (Figure 5.4.). Sampling time was significant ($P = 0.0002$) for haptoglobin, whereby on d 2, concentration increased ($P < 0.002$, mean (s.e.m.) 0.48 (0.03) mg/mL) compared with baseline (means (s.e.m.) 0.32 (0.023) mg/mL). Sampling time was not significant ($P > 0.05$) for concentration of fibrinogen.

5.3.7. In vitro lymphocyte production of interferon-γ (IFN-γ)

There were no effects of sampling time, treatment or treatment × sampling time interactions ($P > 0.05$) for PHA- and Con A-induced IFN-γ production (Table 5.6).

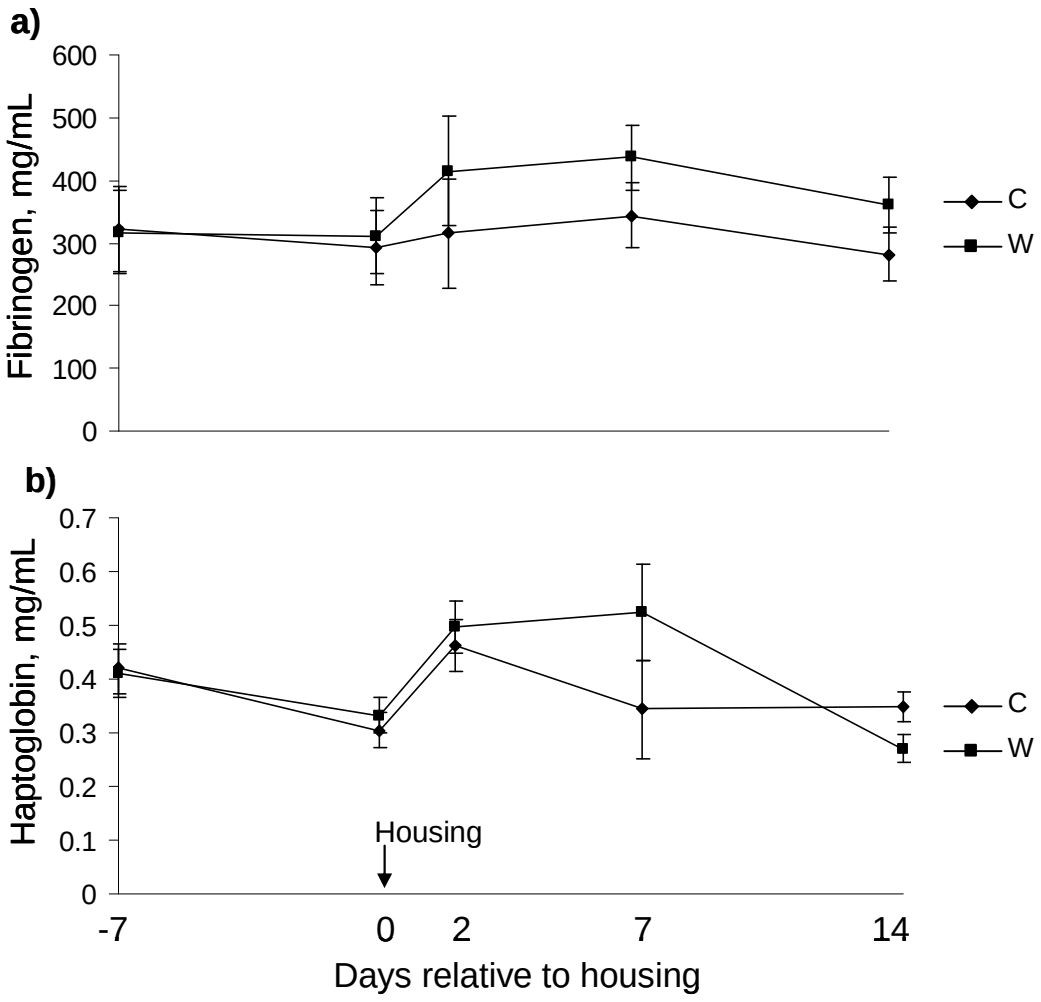

Figure 5.4. Effect of abrupt weaning at housing on concentration of a) fibrinogen and b) haptoglobin (least squares mean ± s.e.m., mg/mL) in beef calves.

W = abruptly weaned calves (n = 8), C = non-weaned (control) calves (n = 8). T × S = treatment × sampling time point interaction. There was an effect of sampling time (*P* = 0.002) for concentration of haptoglobin. Baseline is defined as d 0 for each variable.

Table 5.6. Effect of abruptly weaning at housing on phytohaemagglutinin- and concanavalin A-induced INF-γ production (least squares means ± s.e.m., OD at 450nm) in beef calves.

		Days relative to housing					*P*-values[1]		
Variable		**-7**	**0**	**2**	**7**	**14**	**T**	**S**	**T×S**
PHA[2]	C	0.06 ± 0.057	0.05 ± 0.052	0.04 ± 0.040	0.05 ± 0.049	0.04 ± 0.033	NS	NS	NS
	W	0.10 ± 0.057	0.06 ± 0.052	0.08 ± 0.040	0.03 ± 0.049	0.08 ± 0.033			
ConA[3]	C	0.27 ± 0.061	0.16 ± 0.049	0.12 ± 0.039	0.16 ± 0.042	0.06 ± 0.029	NS	NS	NS
	W	0.18 ± 0.061	0.20 ± 0.049	0.17 ± 0.039	0.14 ± 0.042	0.17 ± 0.029			

[1]T = treatment, S = sampling time, T×S = treatment × sampling time interactions.

P-values, NS = $P > 0.05$.

[2] PHA = Phytohaemagglutinin.

[3]ConA = Concanavalin A.

W = abruptly weaned calves (n = 8), C = non-weaned (control) calves (n = 8).

5. 4. Chapter Summary and Discussion

5.4.1. Summary of main findings

Sixteen, spring-born, single suckled, castrated male calves of Limousin × Holstein-Friesian and Simmental × Holstein-Friesian dams respectively, were used to investigate the effect of weaning on total leukocyte and differential number, neutrophil functional activity, lymphocyte immunophenotypes, and acute phase protein response. Calves grazed with their dams until the end of the grazing season when they were housed in a slatted floor shed. On the day of housing, calves were assigned to a treatment, (i) abruptly weaned (W: n = 8) or (ii) non-weaned (controls) (C: n = 8). Weaned calves were housed in pens without their dams, whereas non-weaned (control) calves were housed with their dams. Blood was collected on day -7, 0 (housing), 2, 7, and 14 to determine total leukocyte and differential number and concentration of fibrinogen and haptoglobin. Lymphocyte immunophenotypes were characterised using selected surface antigens ($CD4^+$, $CD8^+$, $WC1^+$ (γδ T cells), MHC Class II^+ lymphocytes), and the functional activities of neutrophils (surface expression of L-selectin (CD62L), phagocytic and oxidative burst activity) were investigated using flow cytometry. Treatment × sampling time interactions ($P < 0.05$) were detected for total leukocyte and neutrophil number, all lymphocyte subsets, mean fluorescence intensity of $CD62L^+$ neutrophils, and percentage neutrophils performing phagocytosis. On d 2, total leukocyte and neutrophil number increased ($P < 0.001$), and percentage $CD4^+$ and $CD8^+$ lymphocytes, percentage phagocytic neutrophils, mean fluorescence intensity of $CD62L^+$ neutrophils decreased ($P < 0.05$) in W compared with baseline (d 0), whereas they were unchanged ($P > 0.05$) in C. On d 2, percentage $WC1^+$ lymphocytes decreased ($P < 0.05$), whereas percentage MHC class II^+ lymphocytes increased ($P < 0.05$) in W and C, however the magnitude of change was greater in W

than C. There were no treatment × sampling time interactions ($P > 0.05$) for monocyte, eosinophil, and basophil number, percentage $G1^+$ neutrophils, or percentage oxidative burst positive neutrophils. Abrupt weaning resulted in increased neutrophil number and impaired trafficking and phagocytic function. Together with the changes in lymphocyte subsets, the results suggest that there was a greater transitory reduction in immune function at housing in abruptly weaned than non-weaned beef calves.

5.4.2. Discussion

The immune variables measured in the present study showed that weaning together with movement of beef calves from a pasture environment to a housing environment elicited transient neutrophilia, impaired neutrophil phagocytic function, decreased peripheral lymphocyte number and altered immunophenotypes. To the authors' knowledge, this is the first study to collectively examine these immune responses to weaning and housing in the beef calf.

Stress-induced changes in immune function have been documented in cattle, with alterations to cell-mediated and humoral immunity having a significant impact on immunocompetence which may render an animal more susceptible to infection (Kelley, 1980; Griffin, 1989; Chirase *et al.*, 2004; Carroll and Forsberg, 2007). In the present study, neutrophil surveillance and phagocytic activity were affected by weaning, however bactericidal activity was unaffected. Post-weaning, peripheral neutrophil number increased which was most likely resultant of the decreased surface expression of L-selectin as defined by the reduced MFI CD62L on blood neutrophils. Reduction of this adhesion molecule from the surface of neutrophils, where it is constitutively expressed on resting cells prior to activation, suggests that weaning may have had a negative impact on the ability of these cells to roll along and adhere to the

endothelium lining of vessels and thus, more cells remained in circulation as evidenced by neutrophilia. This phenomenon has been documented in physiologically stressed cattle, most notably coinciding with parturition (Lee and Kehrli, 1998; Monfardini *et al.*, 2002; Burton *et al.*, 2005) and following transportation (Yagi *et al.*, 2004).

Additionally, weaned calves had reduced percentage of neutrophils performing phagocytosis compared with control calves. This transient depression in phagocytosis was short lived with cells recovering this function by 7 d post-housing. This finding concurs with the other findings that report a transient decrease in neutrophil phagocytic activity in weaned foals (Sarwar *et al.*, 2008). Decreased phagocytosis has also been reported in horses following transportation (Raidal *et al.*, 1997), in cows that were milked only once-a-day (Llamas Moya *et al.*, 2008), and in dairy cattle following administration of ACTH and dexamethasone (Paape *et al.*, 1981; Roth and Kaeberle, 1981; Roth *et al.*, 1982). Although phagocytic activity of neutrophils was impaired immediately post-weaning, the ability of these cells to perform oxidative burst activity was unaffected, implying that any internalised particles or bacteria could be efficiently degraded despite the depression in particle uptake by neutrophils. Unaltered respiratory burst activity was reported in 14 mo old Holstein-Friesian bulls that were subjected to either Burdizzo castration or hydrocortisone infusion (Pang *et al.*, 2009). Moreover, *in vitro* studies examining pharmacologically high doses of infused hydrocortisone and therapeutic doses of glucocorticoids did not affect the oxidative burst activity of neutrophils isolated from high milk yielding Holstein 2 – 5 weeks post-partum (Hoeben *et al.*, 1998).

Appropriate lymphocyte responses provide immunoprotection against bacterial and viral antigens but can also critically influence immunopathogenesis of disease (Glaser and Kiecolt-Glaser, 2005; Gershwin, 2007; Salak-Johnson and McGlone,

2007). In agreement with other studies (Hickey *et al.*, 2003a; Blanco *et al.*, 2009; Lynch *et al.*, 2008b; Chapter 3), weaning decreased peripheral lymphocyte count. Further investigation of the lymphocyte immunophenotypes showed that there was a greater reduction in the percentage of γδ T cells than αβ T cells post-weaning. Transient decreases in proportion of $WC1^+$ lymphocytes were observed in both treatment groups, with the greatest degree of change observed when weaning and housing were combined. Calves held in conventional housing had significantly lower percentage $WC1^+$ T cells compared with calves that were allowed free-range grazing and access to their dams (Baldwin *et al.*, 2000). Moreover, a decrease in $WC1^+$ T cells in the free-ranging calves coincided with a change from an outdoor to indoor environment following a period of transportation. Contrary to the present findings, percentage $WC1^+$ T cells did not return to baseline in that study, rather they remained decreased and after 3 weeks post-housing were not different from calves that were permanently housed (Baldwin *et al.*, 2000). The additional stressor of transportation may have contributed to the prolonged response observed in the latter study. Our findings are in agreement with other studies that have shown that γδ T cells are most sensitive to physiological and pharmacological stressors with reductions in percentage $WC1^+$ lymphocytes observed following transportation of beef steers (Riondato *et al.*, 2008), parturition in dairy cows (Meglia *et al.*, 2005) and dexamethasone treatment in dairy and beef bulls (Burton and Kehrli, 1996; Menge and Dean-Nystrom, 2008).

In the present study, the decrease in percentage αβ T cells post-weaning was evidenced by transient decreases in percentage $CD4^+$ and $CD8^+$ lymphocytes. Decreases in both peripheral $CD4^+$ and $CD8^+$ lymphocytes have been reported in calves at unloading after 14 h truck transportation, following which they returned to pre-transport values by 24 h (Riondato *et al.*, 2008), and in beef steers following

induction into a feedlot environment (Fell *et al.*, 1999). Some studies examining the effect of stress in domestic animals have utilised the $CD4^+$: $CD8^+$ ratio as an indicator of stress-induced immunosuppression, however conflicting evidence exists in cattle (van Kampen and Mallard, 1997; Anderson *et al.*, 1999; Ishizaki *et al.*, 2005; Degabriele and Fell, 2001). Our findings suggest that movement of beef calves from a pasture environment to a housing environment was able to elicit a transient increase in $CD4^+$:$CD8^+$ ratio. This increase may reflect recovery of immune competence in an attempt to restore homeostasis following the initial reaction to the onset of stress. An overall rise in $CD4^+$: $CD8^+$ ratio was reported in sheep that were housed either in isolation or in groups (Degabriele and Fell, 2001). These authors attributed the rise in $CD4^+$:$CD8^+$ ratio to recovery of immune compensation following perturbation to lymphocyte subsets due to a stressful change in environment. Thus, $CD4^+$:$CD8^+$ ratio may provide more information on the recovery of homeostasis rather than occurrence of immunosuppression. Further research into the utility of this ratio to describe a stress scenario in domestic animals is warranted.

In the present study, percentage MHC class II^+ lymphocytes in peripheral blood increased significantly in weaned calves and to a lesser extent in control calves indicating that a change in environment in combination and without weaning resulted in the activation of lymphocytes. This increase in percentage MHC class II^+ lymphocytes may be due to increased B cells or activated T cells in circulation (Quade *et al.*, 1999). Increase in MHC class II^+ lymphocytes may be due to expansion of NK cells bearing MHC class II molecules (Storset *et al.*, 2004), however, this supposition cannot be confirmed by the findings of the present study as NK cells were not investigated. Our findings concur with Riondato *et al.* (2008) who reported increased percentage MHC class II^+ cells in transported beef steers, but in that study the response

was more prolonged (> 7 d). Holstein bulls challenged with pharmacological doses of dexamethasone were reported to have increased percentage MHC class II^+ cells compared with non-treated controls (Saama *et al.*, 2004). Furthermore, increased percentage MHC class II^+ lymphocytes have been reported following repeated restraint and isolation stress in lambs (Minton *et al.*, 1992).

The reduction in lymphocyte subsets is most likely attributable to a redistribution of these cells from the peripheral circulation to immune compartments or tissues of greater importance during a stressful event. Trafficking of cells is an important and dynamic factor for effective cell-mediated immunity and stress has been shown to influence this process (Kehrli *et al.*, 1999; Bauer *et al.*, 2001; Viswanathan and Dhabhar, 2005; Dhabhar, 2009).

It is important to note that despite the numerous changes in leukocyte number, neutrophil function and lymphocyte immunophenotypes observed, generally these perturbations were transient and homeostasis was restored by 7 d post-weaning. Additionally, changes in some of the immune-related biomarkers, namely the proportion of γδ T cells and MHC class II^+ cells in peripheral circulation and the phagocytic function of blood neutrophils, were evident following change in environment alone. Further research is warranted to investigate the potential use of these measures as prognostic biomarkers of stress sensitive and consequently, infection susceptible animals in response to other stressors.

In line with previous study, total leukocyte number increased following weaning (Blanco *et al.*, 2009). Monocyte, basophil and eosinophil number were not sensitive to weaning or change in environment as evidenced by the unaltered profiles pre- and post-weaning.

Previous studies have shown that weaning induces an acute phase protein response in beef calves (Arthington *et al.*, 2003; 2008; Carroll *et al.*, 2009), whereas in the present study, although there was a numerical increase in fibrinogen and haptoglobin concentration, it was not statistically significant. Increased and unchanged concentration of fibrinogen and haptoglobin, respectively in weaned bull calves has been reported (Hickey *et al.*, 2003a), whereas elevated responses in weaned steers have been reported for fibrinogen (Arthington *et al.*, 2003; Blanco *et al.*, 2009) and haptoglobin (Arthington *et al.*, 2003; 2005; 2008).

In conclusion, abrupt weaning at housing affected total leukocyte, neutrophil and lymphocyte number, lymphocyte immunophenotypes and functional activity of neutrophils in beef calves. Neutrophilia was evident post-weaning, however, crucially, the functional capacity of these cells to effectively traffic from the periphery and phagocytose bacteria was temporarily decreased. Additionally, weaning resulted in a temporary reduction in lymphocyte subsets, most notably of γδ lymphocytes. Considered together, abrupt weaning transiently impaired immune function in beef calves and thus, in terms of immunocompetence, new and additional husbandry practices with the potential to induce stress in beef calves, such as housing, should be avoided immediately post-weaning.

Chapter 6

Effect of pre-weaning concentrate supplementation on the peripheral distribution of leukocytes, the functional activity of neutrophils and the acute phase protein responses of abruptly weaned beef calves

6.1. Introduction

Within seasonal, grassland-based suckler beef production systems in Ireland, calves are generally spring-born and reared with their dam at pasture for approximately 8 months until the end of the grazing season in autumn when they are weaned. At or shortly after weaning, calves are housed indoors over the winter period and offered grass silage, which is generally supplemented with concentrates (Drennan and McGee, 2009). Concentrate supplementation of nursing, grazing beef calves prior to weaning is commonly referred to as 'creep feeding', and serves to compensate for decreasing milk yield and forage, and to improve calf weaning weights (Martin *et al.*, 1981; Prichard *et al.*, 1989; Wright, 1992; McGee *et al.*, 1996; Myers *et al.*, 1999). Additionally, this practice is often advocated as a means of reducing weaning stress in calves through the familiarisation to a palatable feed, such as concentrates (Lardy, 2007) and has been reported to decrease morbidity in feedlots (Myers *et al.*, 1999).

Weaning has been well established as a stressful event in the calf's lifetime with alterations in behaviour (Veissier and Le Neindre, 1989a; Price *et al.*, 2003; Enriquez *et al.*, 2010), hormonal mediators of stress (Hickey *et al.*, 2003a; Blanco *et al.*, 2009) and immune function (Arthington *et al.*, 2005; 2008; Lynch *et al.*, 2009a) evident post-weaning. Although, some studies have examined the effects of pre-weaning calf management, such as two-stage weaning with nose-clips (Haley *et al.*, 2005; Boland *et al.*, 2008), on the stress response of beef calves post-weaning, none have examined the effects of offering concentrate supplementation for a period prior to weaning on the physiological and immunological responses of weaned beef calves.

Previously, we have shown that abrupt weaning causes transitory neutrophilia, mediated by reduced surface expression of L-selectin, and transient lymphopenia, characterised by decreased percentage $CD4^+$, $CD8^+$ and $WC1^+$ lymphocytes and

increased MHC class II^+ cells. Additionally, neutrophil phagocytic function was reduced, whereas oxidative burst activity was not affected in abruptly weaned beef calves.

Thus, the objectives of the present study were to examine the effects of offering concentrate supplementation to beef calves (CS) for 26 days prior to abrupt weaning on (i) the peripheral distribution of leukocytes, (ii) function activities of neutrophils, and (iii) the acute phase protein response compared with calves that were not offered concentrate supplementation (NCS) prior to weaning. The metabolic and behavioural responses and performance of calves were also characterised to provide supplementary information on the effects of concentrate supplementation prior to weaning on abruptly weaned beef calves. The experimental design is described in Section 2.2.2.4.

6.2. Effect of concentrate supplementation pre-weaning on physiological responses in abruptly weaned beef calves

6.2.1 Rectal body temperature

There was no treatment × sampling time interaction ($P > 0.05$) for rectal body temperature. Rectal body temperature was greater ($P < 0.01$) in NCS calves than CS calves (Table 6.1.). On d 1 and 2, rectal temperature increased ($P < 0.01$; mean (s.e.m.) 39.0 (0.11) °C) and subsequently did not differ ($P > 0.05$) compared with pre-weaning baseline (d 0) (mean (s.e.m.) 38.4 (0.07) °C).

Table 6.1. Effect of concentrate supplementation for 26 days pre-weaning on rectal body temperature in abruptly weaned beef calves.

		Days post-weaning							*P*-values[1]		
		0	**1**	**2**	**3**	**7**	**14**	**Pooled s.e.m.**	**T**	**S**	**T×S**
Rectal body temperature, °C	CS	38.3[a]	39.0[b]	38.7[b]	38.5[a]	38.6[a]	38.4[a]	0.11	**	**	NS
	NCS	38.5[a]	39.1[b]	38.9[b]	38.6[a]	38.6[a]	38.6[a]				

[1]Levels of significance: NS = $P > 0.05$, ** = $P < 0.01$.

T= treatment, S = sampling time, T × S = treatment × sampling time interaction.

CS = concentrate supplement prior to abrupt weaning (n = 10).

NCS = no concentrate supplement prior to abrupt weaning (n = 10).

[a,b]Within a row, least squares means without a common superscript differ ($P < 0.05$).

6.3 Effect of concentrate supplementation pre-weaning on immunological responses in abruptly weaned beef calves

6.3.1. Total leukocyte and differential number

There was no effect ($P > 0.05$) of treatment, sampling time or treatment × sampling time interaction for total leukocyte number (Table 6.2). The mean total leukocyte number (s.e.m.) for CS and NCS over the experimental period was 10.3 (0.37) $\times 10^3$ cells/μL and 10.0 (0.37) $\times 10^3$ cells/μL, respectively. There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction for neutrophil, lymphocyte, monocyte, eosinophil or basophil number (Table 6.2). However, neutrophil number increased ($P < 0.001$) on d 1(mean (s.e.m.) 4.5 (0.26) $\times 10^3$ cells/μL) and 2 (mean (s.e.m.) 3.6 (0.17) $\times 10^3$ cells/μL), whereas lymphocyte number decreased ($P < 0.001$) on d 1 (mean (s.e.m.) 5.1 (0.23) $\times 10^3$ cells/μL) and 2 (mean (s.e.m) 5.8 (0.20) $\times 10^3$ cells/μL), and subsequently did not differ ($P < 0.05$) compared with pre-weaning baseline (neutrophil: mean (s.e.m.) 2.5 (0.14) $\times 10^3$ cells/μL and lymphocytes: mean (s.e.m.) 7.1 (0.23) $\times 10^3$ cells/μL). Monocyte number decreased on d 14 compared with pre-weaning baseline. The mean eosinophil and basophil number (s.d.) over the experimental period were 0.20 (0.010) $\times 10^3$ cells/μL and 0.10 (0.033) $\times 10^3$ cells/μL, respectively.

6.3.2. Red blood cell number and associated variables

There was no effect ($P > 0.05$) of treatment and treatment × sampling time interaction for RBC number, percentage HCT, MCV concentration and platelet number post-weaning (Table 6.3). The mean (s.d.) RBC number, percentage HCT, and concentration of MCV during the experimental period was 10.2 (1.54) $\times 10^6$ cells/μL,

33.5 (1.53) %, and 33.2 (2.76) fL, respectively. There was a treatment × sampling time interaction ($P < 0.05$) for concentration of HGB whereby, on d 7, concentration of HGB increased ($P = 0.07$) in CS (mean (s.e.m.) 13.1 (0.34) g/dL), whereas it did not change ($P > 0.05$) in NCS compared with pre-weaning baseline (mean (s.e.m.) 12.5 (0.29) g/dL). The mean (s.d.) MCH and MCHC was 12.3 (1.55) pg and 36.8 (1.19) %, respectively.

Table 6.2. Effect of concentrate supplementation for 26 days pre-weaning on total leukocyte and differential populations in abruptly weaned beef calves.

		Days post-weaning							*P* values[1]		
Cell type, $\times 10^3$cells/ µL		**0**	**1**	**2**	**3**	**7**	**14**	**Pooled s.e.m.**	**T**	**S**	**T×S**
Total leukocytes	CS	10.5	11.3	10.4	10.1	10.0	9.3	0.52	NS	NS	NS
	NCS	10.3	10.7	9.6	9.9	9.8	10.0				
Neutrophils	CS	2.5^{a}	4.2^{b}	3.7^{b}	2.5^{a}	2.8^{a}	2.3^{a}	0.31	NS	***	NS
	NCS	2.6^{a}	4.8 ^{b}	3.9^{c}	2.7^{a}	2.7^{a}	2.7^{a}				
Lymphocytes	CS	7.3^{a}	5.2^{b}	5.3^{b}	6.6^{a}	6.7^{a}	6.6^{a}	0.33	NS	**	NS
	NCS	6.7^{a}	4.9^{b}	5.0^{b}	6.0^{a}	7.1^{a}	6.4^{a}				
Monocytes	CS	0.68^{a}	0.61^{a}	0.58^{a}	0.61^{a}	0.63^{a}	0.37^{b}	0.063	NS	**	NS
	NCS	0.73	0.69	0.70	0.73	0.82	0.51				
Eosinophils	CS	0.21	0.26	0.21	0.17	0.10	0.16	0.048	NS	NS	NS
	NCS	0.22	0.20	0.24	0.26	0.15	0.19				
Basophils	CS	0.09	0.11	0.12	0.11	0.08	0.13	0.029	NS	NS	NS
	NCS	0.11	0.10	0.09	0.09	0.08	0.10				

[1]Levels of significance: NS = $P > 0.05$, ** = $P < 0.01$, *** $P < 0.001$.

T = treatment, S = sampling time, T × S = treatment × sampling time interaction.

CS = concentrate supplement prior to abrupt weaning (n = 10).

NCS = no concentrate supplement prior to abrupt weaning (n = 10).

[a,b,c]Within a row, least squares means without a common superscript differ ($P < 0.05$).

Table 6.3. Effect of concentrate supplementation for 26 d pre-weaning on red blood cell number and associated haematological variables in abruptly weaned beef calves.

Variable		Days post-weaning							*P*-values[1]		
		0	1	2	3	7	14	Pooled s.e.m.	T	S	T×S
RBC[2], ×10[6]cells/µL	CS	10.3	10.5	10.3	9.2	10.1	10.0	0.41	NS	NS	NS
	NCS	10.2	10.3	10.4	9.8	10.9	10.2				
HGB[3], g/dL	CS	12.5	12.8	12.8	12.9	12.9	12.5	0.32	NS	**	*
	NCS	12.1[a]	12.1[a]	12.3[a]	12.3[a]	13.1[b]	12.3[a]				
HCT[4], %	CS	34.3	34.8	34.4	38.9	34.8	33.5	0.91	NS	***	NS
	NCS	32.8	33.3	33.6	32.3	35.6	33.0				
MCV[5], fL	CS	33.5	33.3	33.5	33.5	33.9	33.5	0.83	NS	NS	NS
	NCS	32.6	32.5	32.8	33.6	32.9	32.5				
MCH[6], pg	CS	12.1	12.3	12.2	13.4	12.6	12.5	0.40	NS	***	***
	NCS	12.2	11.8	11.9	13.1	12.1	12.1				
MCHC[7], %	CS	36.4	36.8	36.2	37.3	37.2	37.4	0.32	NS	**	***
	NCS	37.2	36.3	36.5	36.3	36.7	37.4				
PLT[8], ×10[3]cells/µL	CS	567	695	685	644	572	786	56.4	NS	***	NS
	NCS	602	727	710	624	655	750				

[1]Levels of significance: NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. T = treatment, S = sampling time, T × S = treatment × sampling time interaction.

[2]RBC = red blood cell, [3]HGB = haemoglobin concentration, [4]HCT = haematocrit percentage, [5]MCV = mean corpuscular volume, [6]MCH = mean corpuscular haemoglobin, [7]MCHC = mean corpuscular haemoglobin concentration, [8]PLT = platelet number. CS = concentrate supplement prior to abrupt weaning (n = 10). NCS = no concentrate supplement prior to abrupt weaning (n = 10). [a,b,c]Within a row, least squares means without a common superscript differ ($P < 0.05$).

6.3.3. Neutrophil functional activity

6.3.3.1. Surface expression of L-selectin (CD62L) and percentage CD62L$^+$ neutrophils

There were no effects of treatment or treatment × sampling time interactions for neutrophil CD62L MFI or percentage CD62L$^+$ neutrophils (Table 6.4). On d 2, neutrophil CD62L MFI decreased ($P < 0.001$, mean (s.e.m.) 24.2 (1.93) MFI), and subsequently did not differ ($P > 0.05$) compared with pre-weaning baseline (mean (s.e.m.) 35.7 (1.43) MFI). The mean (s.e.m.) percentage of CD62L$^+$ neutrophils pre- and post-weaning was 91.0 (0.98) % (Table 6.4).

6.3.3.2 Neutrophil phagocytic activity

There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction, and sampling time was significant ($P < 0.05$) for percentage of neutrophils performing phagocytosis (Table 6.4). On d 2, percentage neutrophils performing phagocytosis decreased ($P < 0.03$) in CS (mean (s.e.m.) 71.1 (1.72) %) and NCS (mean (s.e.m.) 66.2 (1.72) %) compared with pre-weaning baseline (mean (s.e.m.) 79.3 (1.34) %), however there was a tendency for the decrease to be greater ($P = 0.06$) in NCS than CS. Subsequently, it did not differ ($P > 0.05$) compared with pre-weaning baseline.

There was no effect ($P > 0.05$) of treatment, sampling time or treatment × sampling time interaction for the MFI of neutrophils performing phagocytosis (mean (s.e.m.) 13.7 (0.96) MFI) (Table 6.4).

6.3.3.3 Neutrophil oxidative burst activity

There was no effect ($P > 0.05$) of treatment, sampling time or treatment × sampling time interactions for the percentage and MFI of neutrophils performing oxidative burst

activity (Table 6.4). The mean (s.d.) percentage and MFI of neutrophils performing oxidative burst activity was 29.5 (8.56) % and 13.8 (1.95) MFI, respectively.

Table 6.4. Effect of concentrate supplementation for 26 d pre-weaning neutrophil surface expression of CD62L, and neutrophil phagocytic and oxidative burst activity in abruptly weaned beef calves.

Variable		Days post-weaning					*P*-values[1]		
		0	2	7	14	Pooled s.e.m.	T	S	T×S
CD62L^{+}[2], MFI[3]	CS	35.2^{a}	20.19^{b}	35.6^{a}	35.2^{a}	2.24	NS	*	NS
	NCS	36.2^{a}	17.3^{b}	36.0^{a}	34.0^{a}				
Phagocytosis, %	CS	81.1^{a}	71.2^{b}	78.0^{a}	83.2^{a}	1.99	NS	*	NS
	NCS	80.5^{a}	73.2^{b}	74.8^{a}	80.0^{a}				
Phagocytosis, MFI[3]	CS	12.9	10.7	16.8	14.3	0.97	NS	NS	NS
	NCS	14.7	12.9	14.8	13.01				
Oxidative burst, %	CS	26.0	29.4	33.1	27.3	2.34	NS	NS	NS
	NCS	24.2	23.9	26.4	25.0				
Oxidative burst, MFI[3]	CS	13.0	10.7	16.8	14.3	0.71	NS	NS	NS
	NCS	14.8	12.9	14.8	13.0				

[1]Levels of significance: NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

T = treatment, S = sampling time, T × S = treatment × sampling time interaction.

[2]CD62L = L-selectin marker, [3]MFI = mean fluorescence intensity.

CS = concentrate supplement prior to abrupt weaning (n = 10).

NCS = no concentrate supplement prior to abrupt weaning (n = 10).

[a,b,c]Within a row, least squares means without a common superscript differ ($P < 0.05$).

6.3.4. Lymphocyte immunophenotypes

There was a treatment × sampling time interaction ($P < 0.05$) for percentage $CD4^+$ lymphocytes (Figure 6.1). On d 2, percentage of $CD4^+$ lymphocytes decreased ($P < 0.001$) in CS (mean (s.e.m.) 20.3 (1.21) %) and NCS (mean (s.e.m.) 20.9 (1.21) %) but, subsequently, increased ($P < 0.01$) in CS (mean (s.e.m.) 29.4 (1.06) %) on d 7, whereas it remained unchanged ($P > 0.05$) in NCS compared with pre-weaning baseline (mean (s.e.m.) 25.8 (0.42) %). There was no effect ($P > 0.05$) of treatment, sampling time or treatment × sampling time interaction for percentage $CD8^+$ lymphocytes and the CD4: CD8 ratio (Table 6.5). The mean (s.d.) percentage $CD8^+$ lymphocytes and $CD4^+$:$CD8^+$ ratio for the experimental period was 9.9 (2.86) % and 2.7 (0.16).

There was a treatment × sampling time interaction ($P < 0.02$) for percentage $WC1^+$ lymphocytes. On d 2 and 7, $WC1^+$ lymphocytes decreased ($P < 0.05$) in CS (mean (s.e.m.) 14.1 (1.02) %) and NCS (mean (s.e.m.) 10.4 (1.02) %) however the decreased was greater ($P < 0.05$) in NCS than CS, and subsequently did not differ ($P > 0.05$) from pre-weaning baseline (mean (s.e.m. 19.1 (1.14) %) (Figure 6.2a).

There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction for percentage MHC class II^+ cells. However, on d 2, percentage MHC class II^+ lymphocytes increased ($P < 0.01$), and subsequently returned ($P > 0.05$) to pre-weaning baseline (Figure 6.2a).

Table 6.5. Effect of concentrate supplementation for 26 d pre-weaning percentage $CD8^+$ lymphocytes, $CD4^+$:$CD8^+$ ratio in abruptly weaned beef calves.

Variable		Days post-weaning					*P*-values[1]		
		0	2	7	14	Pooled s.e.m.	T	S	T×S
$CD8^+$	CS	10.1	8.4	9.9	10.7	0.85	NS	NS	NS
	NCS	10.8	7.9	10.6	10.8				
$CD4^+$:$CD8^+$ ratio	CS	2.6	2.6	3.1	2.8	0.24	NS	NS	NS
	NCS	2.7	3.0	2.7	2.4				

[1]Levels of significance: NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

T = treatment, S = sampling time, T × S = treatment × sampling time interaction.

CS = concentrate supplement prior to abrupt weaning (n = 10).

NCS = no concentrate supplement prior to abrupt weaning (n = 10).

[a,b,c]Within a row, least squares means without a common superscript differ ($P < 0.05$).

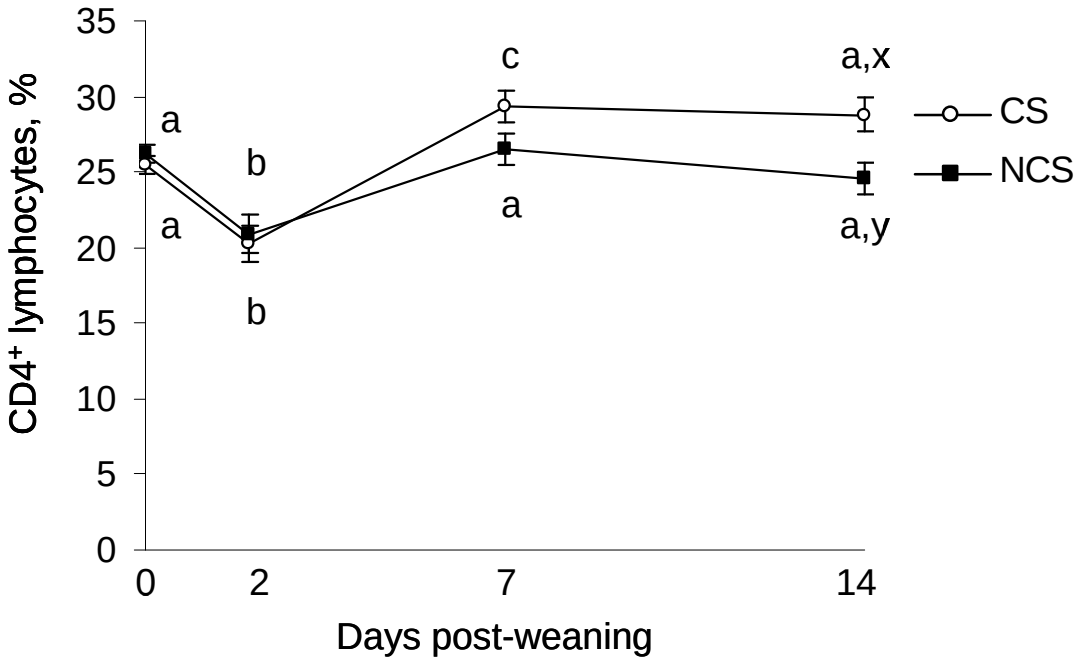

Figure 6.1. Effect of concentrate supplementation for 26 d pre-weaning on percentage CD4$^+$ in abruptly weaned beef calves.

CS = concentrate supplement prior to abrupt weaning (n = 10), NCS = no concentrate supplement prior to abrupt weaning (n = 10). ^{a,b,c}Between days, least squares means without a common superscript differ ($P < 0.05$). ^{x,y}Within a day, least squares means without a common superscript differ ($P < 0.01$).

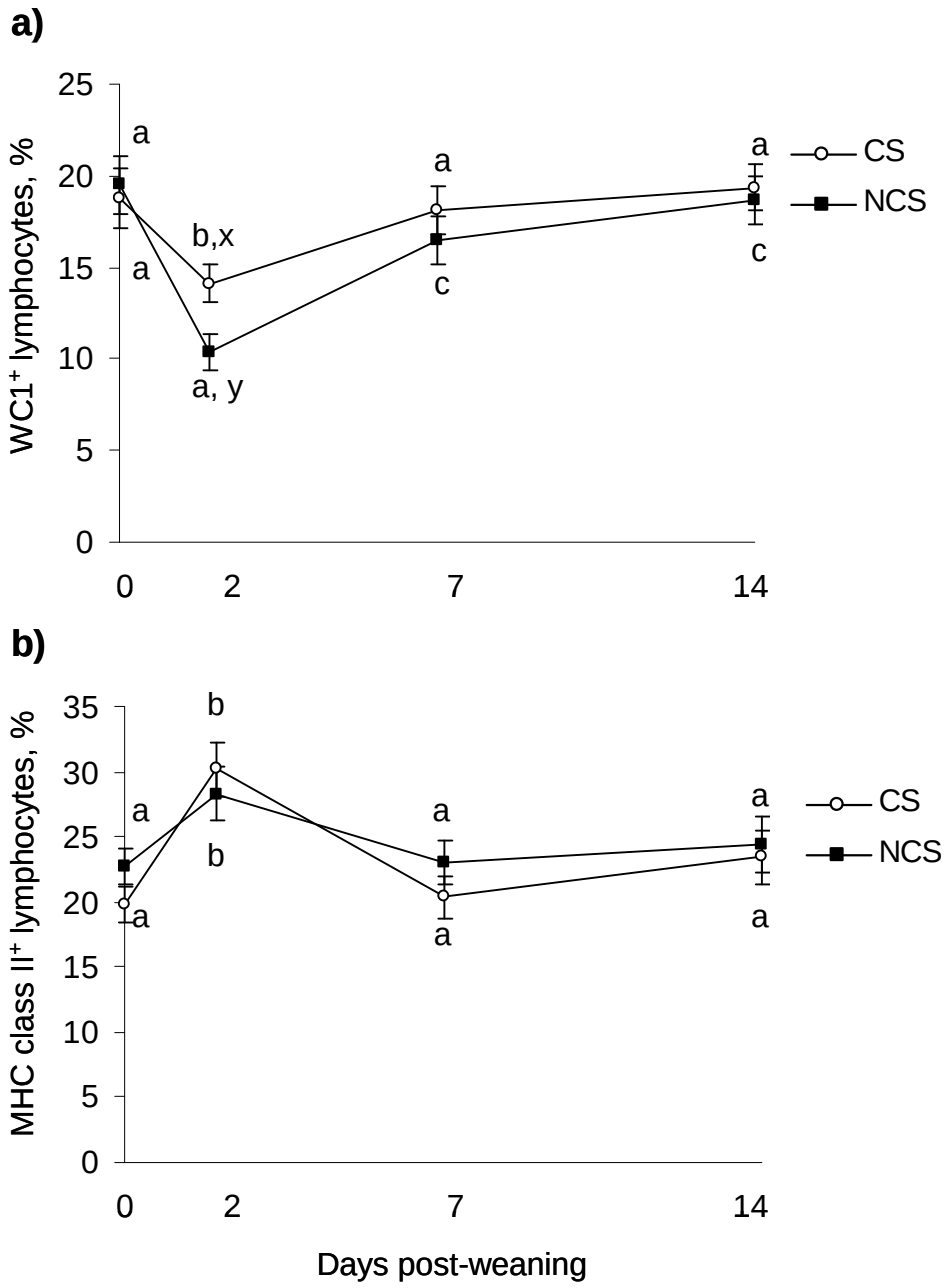

Figure 6.2. Effect of concentrate supplementation for 26 d pre-weaning on percentage (a) WC1$^+$ and (b) MHC Class II$^+$ lymphocytes (least squares mean ± s.e.m., %) in abruptly weaned beef calves.

CS = concentrate supplement prior to abrupt weaning (n = 10). NCS = no concentrate supplement prior to abrupt weaning (n = 10). ^{a,b,c}Between days, least squares means without a common superscript differ (P<0.001). ^{x,y}Within a day, least squares means without a common superscript differ (P < 0.001). There was a significant treatment × sampling time interaction for percentage WC1$^+$ lymphocytes.

6.3.5. Acute phase proteins

There was no effect ($P > 0.05$) of treatment or treatment × sampling time interactions for concentration of fibrinogen and haptoglobin or sampling time ($P > 0.05$) for concentration of fibrinogen (Table 6.5). However, concentration of haptoglobin increased ($P < 0.05$) on d 2 (mean (s.e.m.) 0.71 (0.072) mg/mL) compared with pre-weaning baseline (mean (s.e.m.) 0.37 (0.009) mg/mL). The mean (s.d.) concentration of fibrinogen over the experimental period was 464 (12.7) mg/dL.

6.3.6. In vitro lymphocyte production of interferon-γ (IFN-γ)

There was no effect of treatment, sampling time, and treatment × sampling time interaction ($P > 0.05$) for PHA- and Con A-induced INF-γ production (Table 6.5).

Table 6.6. Effect of concentrate supplementation pre-weaning on concentration of fibrinogen and haptoglobin and phytohaemagglutinin (PHA) - and concanavalin A (ConA)-induced INF-γ production in abruptly weaned beef calves.

Mitogen		Days post-weaning					*P*-values[1]		
		0	2	7	14	Pooled s.e.m.	T	S	T×S
Haptoglobin, mg/mL	**CS**	0.38	0.62	0.48	0.47	0.068	NS	*	NS
	NCS	0.35[a]	0.80[b]	0.67[a]	0.42[a]				
Fibrinogen, mg/dL	**CS**	379	456	495	432	24.9	NS	NS	NS
	NCS	439	524	545	486				
PHA[2], OD (450nm)	**CS**	0.11	0.10	0.16	0.15	0.030	NS	NS	NS
	NCS	0.09	0.08	0.19	0.08				
ConA[3], OD (450nm)	**CS**	0.24	0.31	0.43	0.38	0.052	NS	NS	NS
	NCS	0.27	0.25	0.32	0.35				

[1]Levels of significance: NS = $P > 0.05$. * = $P < 0.05$.

T = treatment, S = sampling time, T × S = treatment × sampling time interaction.

[2]PHA = Phytohaemagglutinin.

[3]ConA = Concanavalin A.

CS = creep-fed prior to abrupt weaning (n = 10),

NCS = not creep-fed prior to abrupt weaning (n = 10).

[a,b]Within a row, least squares means without a common superscript differ ($P < 0.05$).

6.4. Effect of concentrate supplementation pre-weaning on metabolic responses in abruptly weaned beef calves

6.4.1. Total protein, albumin and globulin

There was a treatment × sampling time interaction for concentration of globulin whereby on d 2, the increase in concentration of globulin was greater ($P < 0.05$) in CS (mean (s.e.m.) 38.9 (0.52) g/dL) compared with NCS (mean (s.e.m.) 37.6 (0.52) mg/dL), and subsequently there was no difference between treatments (Table 6.6). There were no ($P > 0.05$) treatment × sampling time interactions for concentrations of total protein and albumin. Concentration of total protein was greater ($P < 0.05$) in CS (mean (s.e.m.) 72.0 (0.74) mg/dL) compared NCS (mean (s.e.m.) 69.7 (0.74) mg/dL). On d 2 and 7, concentration of albumin increased ($P < 0.05$), and subsequently did not differ ($P > 0.05$) compared with pre-weaning baseline (Table 6.).

6.4.2. Urea

There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction for concentration of urea (Table 6.6). Concentration of urea decreased ($P < 0.05$) compared with pre-weaning baseline (mean (s.e.m) 4.3 (0.09) mmol/L versus mean (s.em.) 2.9 (0.13) mmol/L).

6.4.3. Creatine kinase

There was no effect ($P > 0.05$) of treatment, sampling time or treatment × sampling time interactions for creatine kinase activity (Table 6.6). The mean (s.d.) creatine kinase activity over the experimental period was 319 (144.4) U/L.

6.4.4. Glucose, NEFA and βHB

There was no ($P > 0.05$) treatment × sampling time interactions for concentration of glucose, βHB, and NEFA (Table 6.6). On d 2, concentration of NEFA increased ($P < 0.05$; mean (s.e.m.) 0.28 (0.036) mmol/L), and subsequently decreased on d 14 ($P < 0.05$; mean 0.11 (0.005) mmol/L) compared with pre-weaning (mean (s.e.m.) 0.15 (0.018) mmol/L). Concentration of βHB increased ($P < 0.05$) post-weaning (mean (s.e.m.) 0.26 (0.011) mmol/L) compared with pre-weaning baseline (mean (s.e.m.) 0.18 (0.006) mmol/L). The mean (s.d.) concentration of glucose over the experimental period was 4.4 (0.32) mmol/L.

Table 6.7. Effect of concentrate supplementation for 26 d pre-weaning on plasma metabolites in abruptly weaned beef calves.

Metabolite		Days post-weaning					*P*-values[1]		
		0	2	7	14	Pooled s.e.m.	T	S	T×S
Total protein, g/dL	CS	70.3^{a}	73.9^{b,x}	72.8^{a}	70.6^{a}	0.98	*	†	NS
	NCS	68.3^{a}	70.9^{b,y}	72.0^{b}	67.6^{a}				
Albumin, g/dL	CS	34.0^{a}	33.9^{a}	35.4^{a}	31.6^{b}	0.45	†	*	NS
	NCS	33.2^{a}	33.4^{a}	33.4^{a}	31.0^{b}				
Globulin, g/dL	CS	36.6^{a}	38.9^{b,x}	37.7^{a}	38.9^{b}	0.80	NS	**	*
	NCS	35.1^{a}	37.6^{b,y}	38.4^{b}	36.6^{a}				
Creatine kinase, U/L	CS	295	304	359	317	42.7	NS	NS	NS
	NCS	307	297	324	350				
Urea, mmol/L	CS	4.2^{a}	3.1^{b}	3.1^{b}	2.5^{c}	0.17	NS	***	NS
	NCS	4.3^{a}	3.3^{b}	3.0^{b}	2.5^{c}				
Glucose, mmol/L	CS	4.5	4.6	4.3	4.3	0.08	NS	NS	NS
	NCS	4.5	4.8	4.3	4.3				
βHB[2], mmol/L	CS	0.19^{a}	0.27^{b}	0.25^{a}	0.30^{b}	0.014	NS	*	NS
	NCS	0.17^{a}	0.24^{b}	0.23^{a}	0.28^{c}				
NEFA[3], mmol/L	CS	0.17	0.24	0.17	0.13	0.023	NS	*	NS
	NCS	0.13^{a}	0.33^{b}	0.20^{a}	0.09^{a}				

[1]Levels of significance: NS = $P > 0.05$, † = $P = 0.06$, * = $P = 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. T = treatment, S = sampling time, T × S = treatment × sampling time interaction. [2]βHB = β-hydroxybutyrate, [3]NEFA = non-esterified fatty acids. CS = concentrate supplement prior to abrupt weaning (n = 10), NCS = no concentrate supplement prior to abrupt weaning (n = 10). [a,b,c]Within a row, least squares means without a common superscript differ ($P < 0.05$). [x,y]Within a column (day) for each variable, least squares means without a common superscript differ ($P < 0.05$).

6.5. Effect of concentrate supplementation pre-weaning on behaviour and performance of abruptly weaned beef calves

6.5.1. Active, lying and standing behaviour

There was no difference for percentage of time spent lying and active and number of steps taken from d 8 to 14, hence only data from d 0 to 7 are shown. There was a treatment × day interaction ($P < 0.001$) for lying behaviour. On d 0, 2, and 5, CS spent 8 % ($P < 0.01$, 1 h 55 min), 9 % ($P = 0.002$, 2 h 10 min) and 3 % ($P < 0.05$, 43 min) more time lying, respectively (and consequently, less time standing), compared with NCS. Time spent lying increased ($P < 0.001$) for all calves on d 1 to 7 compared with d 0 (Figure 6.3a). There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction for percentage of time spent active post-weaning. However, time spent active decreased ($P < 0.001$) by 13 % and 28 % on d 1 and 2, respectively, and remained decreased ($P < 0.05$) up to d 7 compared with d 0 (Figure 6.3b).

6.5.2 Walking behaviour

There was no treatment × sampling time interaction ($P > 0.05$) for the number of steps taken per day. On d 1 and 2, the number of steps taken decreased ($P < 0.001$) by 37 % and 58 %, respectively, compared with d 0 (Figure 6.3c).

6.5.3. Feeding behaviour

There was no effect ($P > 0.05$) of treatment or treatment × sampling time interaction for the percentage time spent at the silage feed face or concentrate trough post-weaning (Figure 6.4). Sampling time was significant ($P < 0.05$) for percentage time spent at the silage feed face and concentrate trough with calves spending more time at these feeding points on d 7 and 14 compared with d 0.

6.5.4. Calf performance

Pre-weaning ADG for the period between d -26 and 0 was 1.1 (s.e.m.) (0.26) kg/d and 1.0 (s.e.m.) (0.26) kg/d for CS and NCS respectively. Post-weaning, ADG for the period between d 0 and 14 was 0.1 (s.e.m.) (0.14) kg/d and -0.1 (s.e.m.) (0.14) for CS and NCS respectively.

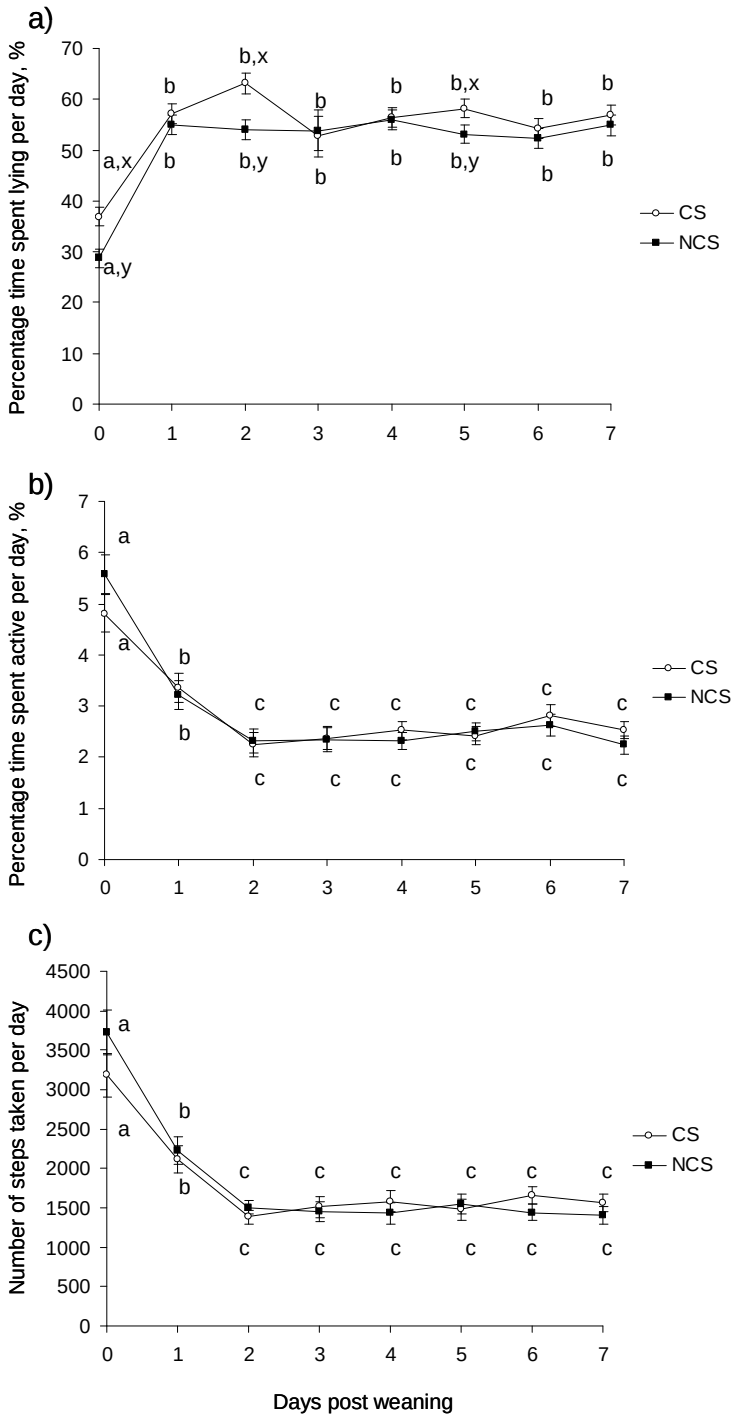

Figure 6.3. Effects of concentrate supplementation for 26 d pre-weaning on percentage time spent a) lying and b) active, and c) the number of steps taken per day by abruptly weaned beef calves. CS = concentrate supplement prior to abrupt weaning (n = 10), NCS = no concentrate supplement prior to abrupt weaning (n = 10). [a,b]Between days, least squares means without a common superscript differ ($P < 0.05$). [x,y]Within a day, least squares means without a common superscript differ ($P < 0.05$).

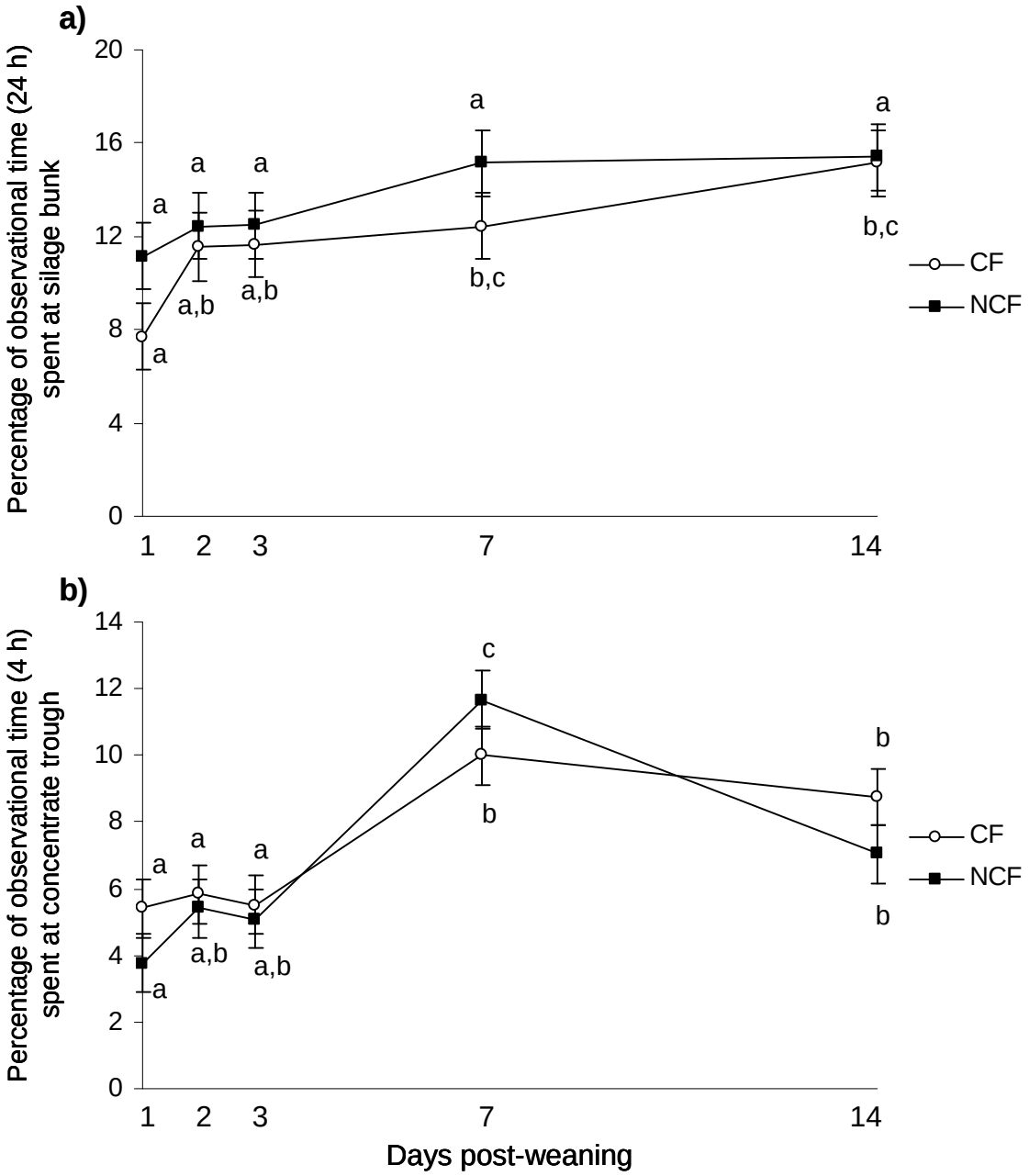

Figure 6.4. Effect of concentrate supplementation for 26 d pre-weaning on percentage of observational time spent at the silage feed face or concentrate trough by abruptly weaned beef calves. CS = concentrate supplement prior to abrupt weaning (n = 10), NCS = no concentrate supplement prior to abrupt weaning (n = 10). [a,b,c]Between days, least squares means without a common superscript differ ($P < 0.05$). The observational period was 24 h and 4 h for attendance at the silage feed face and concentrate trough, respectively.

6.6. Chapter Summary and Discussion

6.6.1 Summary of main findings

The effect of pre-weaning concentrate supplementation on peripheral distribution of leukocytes, functional activity of neutrophils, the acute phase protein response, metabolic and behavioural response, and performance of abruptly weaned beef calves was investigated. Calves were grazed with their dams until the end of the grazing season when they were housed in a concrete slatted floor shed. Twenty-six days prior to weaning, 20 spring-born (mean date of birth (s.d.) 23 March (12.7) d), singled suckled, pure-bred Simmental male (non-castrated), (n = 10, m) and female (n = 10, f) calves were randomly assigned one of two treatments (i) concentrate supplement (CS: n = 10 (5 m and 5 f), mean age (s.d.) 201 (12.8) d, mean weight (s.d.) 258 (20.2) kg) or (ii) no concentrate supplement (controls) (NCS: n = 10, (5 m and 5 f), mean age (s.d.) 201 (13.4) d, mean weight (s.d.) 257 (19.6) kg) pre-weaning. On d 0 (weaning), all calves were housed in a slatted floor shed and offered grass silage *ad libitum* plus concentrates. There was a treatment × sampling time interaction ($P < 0.05$) for $CD4^+$ lymphocytes, whereby following a decrease ($P < 0.01$) on d 2, it increased ($P < 0.05$) in CS on d 7 and 14, whereas it did not differ ($P > 0.05$) in NCS compared with d 0. There was treatment × sampling time interaction ($P < 0.05$) for percentage $WC1^+$ lymphocytes, whereby on d 2 it decreased ($P < 0.05$) in CS and NCS compared with d 0, however the decrease was greater ($P < 0.05$) in NCS than CS. Post-weaning, rectal body temperature, neutrophil and platelet number, percentage MHC class II^+ lymphocytes, concentrations of haptoglobin, total protein, NEFA and βHB increased ($P < 0.05$), whereas lymphocyte number, MFI of $CD62L^+$ neutrophils, percentage phagocytic neutrophils, and concentrations of urea decreased ($P < 0.05$) compared

with d 0. There was a treatment × sampling time interaction ($P = 0.048$) for globulin, whereby on d 2 it increased ($P < 0.001$) in CS and NCS, however, the increase was greater ($P < 0.01$) in CS than NCS, and subsequently it remained increased ($P < 0.05$) in NCS and did not differ ($P > 0.05$) in CS compared with d 0. There was no effect ($P > 0.05$) of treatment, sampling time or treatment × sampling time interactions for monocyte, eosinophil, basophil, and red blood cell number, percentage $CD8^+$ lymphocytes, CD4: CD8 ratio, oxidative burst activity of neutrophils, interferon-γ production, concentration of glucose and creatine kinase activity. Calves that were supplemented with concentrates pre-weaning spent more time lying post-weaning compared with non-supplemented calves, and the number of steps taken post-weaning did not differ between treatments. In conclusion, calves supplemented with concentrate prior to weaning had a lesser reduction in $WC1^+$ lymphocytes, and had increased percentage $CD4^+$ lymphocytes and concentrations of total protein, and spent increased percentage time lying post-weaning, however, other immunological, metabolic and behavioural measures ($CD8^+$and MHC class II^+ lymphocytes, IFN-γ production, creatine kinase activity, urea, glucose, NEFA, βHB, percentage of time spent active, feeding behaviour), were not influenced by the provision of concentrate pre-weaning.

6.6.2. Discussion

To our knowledge, this is the first study to investigate the effects of pre-weaning concentrate supplementation on the peripheral distribution of leukocytes, the functional activity of neutrophils amd the acute phase response in abruptly weaned beef calves. The ability of calves to cope with weaning stress may affect their subsequent health and performance thus, reducing the negative impact of weaning

stress through the use of management strategies designed to optimize the health and welfare are important considerations.

In the present study, weaning resulted in neutrophilia and concurrent lymphopenia which is in accord with other studies (Hickey *et al.*, 2003a; Blanco *et al.*, 2009; Lynch *et al.*, 2009; Chapter 5). Neutrophil number increased by approximately 50 % in both treatments in the present study, which is less than the magnitude of neutrophilia observed in calves that were not offered concentrates pre- and post-weaning (164 % increase) in other studies (Lynch *et al.*, 2009; Chapter 5). The provision of concentrates for 26 d pre-weaning did not result in discernible differences in neutrophil number between the treatments in the present study immediately post-weaning. Findings by Lynch *et al.* (2009) reported that increased total leukocyte number was defined by profound neutrophilia. In the present study, the total leukocyte number was unchanged, and this was related to a less marked neutrophilia response. In line with previous research (Lynch *et al.*, 2009; Chapter 5), abrupt weaning decreased percentage of neutrophils performing phagocytosis and did not affect neutrophil oxidative burst activity in the present study, and moreover, the provision of concentrates did not affect these functions.

In the present study, similar decreases in lymphocyte number were observed in abruptly weaned calves that were supplemented with concentrate pre- and post-weaning and post-weaning only. Further examination of lymphocyte subsets revealed that calves offered concentrates pre-weaning not only displayed a lesser reduction in percentage $WC1^+$ lymphocytes compared with calves not offered concentrates pre-weaning in the present study, but also had a lesser reduction in percentage $WC1^+$ lymphocytes compared with calves that not offered concentrates pre- or post-weaning (Lynch *et al.*, 2009; Chapter 5). Additionally, calves that were offered concentrates

pre-weaning had unaltered percentage of $CD8^+$ lymphocytes and increased percentage $CD4^+$ lymphocytes post-weaning compared with those that were not offered concentrates post-weaning, and those that were never supplemented with concentrates (Lynch *et al.*, 2009; Chapter 5). Collectively, these findings suggests that offering concentrates may provide some level of protection, manifested as a lesser reduction in γδ T cells and more stable αβ T cell population, and which may prove beneficial for calf health immediately post-weaning when pathogen exposure is heightened after the social mixing and regrouping of unfamiliar calves during transportation and marketing.

Monocyte and eosinophil number were not altered by offering pre-weaning concentrates or by weaning in the present study, which is in accordance with the stiudy described in Chapter 5 (Lynch *et al.*, 2009). Alterations in haematological variables reported in this study were found to be within the normal physiological range for calves of this age, and thus no negative effects on animal welfare were determined using these measures. Furthermore, the difference in rectal body temperature between supplemented and non-supplemented calves was not of clinical significance (Duff and Galyean, 2007).

Acute phase protein response was not affected by offering pre-weaning concentrates in the present study. Although, significant differences were not realised between supplemented and non-supplemented calves, concentration of haptoglobin increased post-weaning, in line with other studies (Arthington *et al.*, 2003; Lynch *et al.*, 2009; Chapter 5). Hickey *et al.* (2003a) reported increased concentration of fibrinogen in bull calves that were not supplemented and abruptly weaned. Differences in the gender of calves as well as the provision of concentrates pre-weaning used in the present study may account for the differences in fibrinogen response observed compared with the aforementioned study.

Metabolic responses can provide valuable information on the nutritional status of an animal and can inform on global deficiencies and malnutrition. The diet of all calves, supplemented or not, were not limited in any means. As stated, milk yield and consumption at weaning, and grass quality and quantity were not limited. Additionally, there is a period of adaptation as the calf ceases one diet where milk intake was a sizable proportion of total intake, and the calf adapts to a new, completely solid diet. Thus, examination of the metabolic profile may be confounded by this period of adaptation as the calf acclimates to their new diet, hence it can be difficult to conclude definitively on the effects of stress on the metabolic response. It was not surprising that concentration of urea decreased in both treatments post-weaning as the crude protein content of the diet decreased. Calves that were supplemented with concentrates had greater concentrations of total protein compared with non-supplemented calves prior to- and immediately post-weaning which suggests that these calves may have had greater protein intakes pre-weaning and were habituated to concentrates, consuming more protein for the first 7 d post-weaning than those unfamiliar with concentrates as a feedstuff. However, differences in feeding behaviour, as time spent at the silage feed face or concentrate trough, did not differ between treatments post-weaning in the present study. As feed intake was not measured, it is difficult to directly attribute the increased concentration of total protein to increased intake. Increased concentration of total protein has been reported in abruptly weaned beef calves (Phillips *et al.*, 1987), however these animals were fasted for 24 h post-weaning, and dehydration likely contributed to the elevated concentrations of total protein. Activation of the stress response is an energy-dependent process (Nieuwenhuizen and Rutters, 2008). However due to issues discussed earlier it is difficult to say whether the increase in energy-related metabolites

(glucose, NEFA and βHB) are attributable to weaning stress or adaptation to new diet. It is most likely that both these factors contribute the response observed. Further research is required in disentangle these elements and to provide additional information on the dietary adaptation of weaned beef calves. Consistent with the profile described by Boland *et al.* (2008), NEFA increased initially and subsequently decreased in abruptly weaned beef calves in the present study. Elevated NEFA and βHB represent a shift in energy balance in the cattle, and may suggest a greater mobilisation of energy reserves in weaning-stressed calves. Elevated concentrations of NEFA and βHB have been associated with reduced neutrophil function *ex vivo* (Moyes *et al.*, 2009) in dairy cattle under negative energy balance, and this may contribute the reduced phagocytic capacity of neutrophils post-weaning in the present study.

In the present study, calves that were offered pre-weaning concentrate and those not offered pre-weaning concentrate were equally active in terms of number of steps taken per day post-weaning, however concentrate supplemented calves spent more time resting post-weaning than non-supplemented calves suggesting that this group was less restless than their peers. In agreement with other studies (Haley *et al.*, 2005; Boland *et al.*, 2008), on the day of abrupt weaning, calves in the present study spent approximately 32 % of their time resting. Following the day of abrupt weaning, calves spent increased percentage of their time lying which is consistent with Enríquez *et al.* (2010) but contrary to Boland *et al.* (2008). Where excessive space is allocated, weaned calves spend more time active and less time resting post-weaning (Boland *et al.*, 2008), and it is likely that this factor contributed to the discrepancies between the present and aforementioned studies, as calves in the present study and those used by Enriquez *et al.* (2010) were restricted to a space allowance of 3.7 m^2 per animal in slatted floor pens and 2.1 m^2 per animal in a corral, respectively, whereas weaned

calves were allocated to open space paddocks in which they had a greater area to move as described by Boland *et al.* (2008). Within the confines of the present study, feeding behaviour did not differ between treatments with both groups spending similar percentage time at the silage feed face and concentrate trough post-weaning. Although, calves that were not offered pre-weaning concentrates were initially slower to spend time at the concentrate trough on d 1, this difference was short lived and was not evident by d 2, with both groups fully adapted to the provision of concentrates in a feeding trough by d 7 post-weaning. Walker *et al.* (2007) reported that paddock weaned calves that were introduced to a feedlot were slower to find the feed bunk than their yard weaned counterparts, however similar to the present study this difference in feeding behaviour was short-lived, persisting for only a few days and had no overall effect on performance.

Calves that were offered concentrates had a numerically higher rate of average daily gain for the 26 d pre-weaning period compared with those that were not offered concentrates. The relative poor response to concentrates in comparison with other studies (Wright, 1992; McGee *et al.*, 1996), may have been affected by the inclement weather that was experienced by the calves at pasture. Post-weaning, there was a difference of 0.24 kg/d ADG between calves that had been supplemented with concentrates pre-weaning compared with non-supplemented calves which may indicate a greater intake post-weaning.

In conclusion, compared with calves that were not offered concentrates pre-weaning, calves that were offered concentrates for 26 d prior to weaning had a lesser reduction in percentage $WC1^+$ lymphocytes and increased percentage $CD4^+$ lymphocytes post-weaning. Calves that were offered concentrate supplementation pre- and post-weaning weaning had a less marked neutrophilia response than calves that did

not receive concentrate supplementation pre- or post-weaning. In terms of immunocompetence, this lymphocyte subset profile may confer enhanced resistance to weaning stress. Additionally, concentrate supplemented calves spent more time resting post-weaning compared with non-supplemented calves.

Chapter 7

General Discussion

7.1. Introduction

In Ireland, seasonal grassland-based beef production systems typically comprise of a grazing season followed by a period of indoor housing over winter. The majority of calves are spring born and are reared with their dam at pasture and are allowed unlimited nursing, until the end of the grazing season in autumn when they are weaned and generally housed. In non-integrated systems, housing can be preceded by transportation and social regrouping of unfamiliar animals at livestock markets. As such, weaning is an inherent husbandry practice in beef production systems, serving to maximise the reproductive potential of the dam by hastening the return to reproductive cycling and allowing for the marketing and specialised feeding of the calves.

Stress has been linked to many detrimental effects in cattle including immune suppression, increased disease susceptibility and decreased reproductive performance resulting in huge economic losses to the beef industry every year. Weaning has been identified as one of the main stressors implicated in these negative effects due to its multifaceted nature whereby, depending upon the management practices employed, physical, psychological, and nutritional stressors can be imposed on the calf simultaneously. Heightened distress responses and activation of the neuroendocrine-immune axis are hallmarks of stress, and weaning has been demonstrated to induce alterations in behavioural responses (Price *et al.*, 2003; Haley *et al.*, 2005; Enriquez *et al.*, 2010), hormonal mediators of stress (Lefcourt and Elsasser, 1995; Hickey *et al.*, 2003a) and consequently, alterations in immune function in beef calves (Hickey *et al.*, 2003a; Arthington *et al.*, 2005; 2008; Blanco *et al.*, 2009) as reviewed in Chapter 1. It is these alterations in immune function that have been associated with increased susceptibility to infection in weaned beef calves, with bovine respiratory disease being the most commonly reported disease (Duff and Galyean, 2007).

Occurrence of this disease complex has major ramifications in terms of animal health and welfare, and has major economic implications, incurring costs attributable to vaccinations, loss in live weight gains, antibiotic treatments, and mortality (Gunn and Scott, 1998; Thomson and White, 2006).

It is important to study weaning as a stressor in order to determine its effects and the mechanisms through which it exerts these effects. The knowledge gained may identify effective weaning strategies to prevent or treat disease. With the ultimate aim of improving the health and welfare of beef calves calves around the time of weaning, this thesis set out to gain a better understanding of the effects of weaning stress in beef calves and to discover novel biomarkers and characterise their profile of change. Moreover, it was hoped that from these profiles, potential biomarkers of stress-susceptible animals, likely to succumb to infection post-weaning could be identified. These biomarkers may also provide potential targets for therapeutics and may provide information to improve vaccination protocol efficacy. Additionally, on a practical implications level, this research was designed and conducted to incorporate typical 'on farm' practices, in order to provide practical information and solutions which could be readily disseminated to farmers to improve pre- and post-weaning management practices. Therefore, taking these goals into consideration the work of this thesis set out to address the following 4 objectives:

1. Examine the effect of post-weaning management practices (i.e. abruptly weaned and housed and offered grass silage *ab libitum* plus concentrates versus abruptly weaned and returned to pasture with no concentrates offered) and subsequent housing on the physiological and immunological responses of beef calves (Chapter 3).

2. Characterise the physiological and immunological response in beef cows to abrupt weaning and subsequent housing (Chapter 4).
3. Examine the effects of abrupt weaning at housing on peripheral leukocyte distribution, functional activity of neutrophils, and the acute phase response of beef calves (Chapter 5).
4. Examine the effects of offering pre-weaning concentration supplementation on the peripheral leukocyte distribution, functional activity of neutrophils and the acute phase protein response of abruptly weaned beef calves (Chapter 6).

7.2. Discussion

There has been limited research on the effects of weaning alone on beef calves. The majority of the literature has focused on the combined effects of weaning and transportation together since often both of these stressors occur at around the same time in a farm setting. However, abrupt weaning is reported to induce stress in beef calves and is therefore an important area to investigate further (Hickey *et al.*, 2003a). Through the examination of these key areas, this thesis sought to address some of the current gaps in the knowledge pertaining to weaning stress in beef cattle. Presently, there is somewhat limited information on the physiological, adrenocortical and immunological responses of beef cattle following abrupt weaning. Examination of the effects of weaning on beef cows have been largely neglected by the literature. Research todate has focused on the effects of early weaning, when the calf is approximately 80 – 90 days of age, and on the nutritional and reproductive status of the cow. Early weaning is implemented in production systems where forage may be limiting due to unfavourable climatic conditions to improve body condition score in

beef cows, increasing pregnancy rate and decreasing postpartum anoestrous period (Arthington and Minton, 2004; Rasby, 2007).

In Chapter 3, a practical consideration regarding post-weaning management practices of beef calves was addressed to ascertain whether a weaning strategy, whereby less stressors are imposed on the calf at the time of weaning could have a positive effect on the physiological and immunological responses post-weaning. The findings of this chapter indicated that beef calves that were abruptly weaned and returned to familiar pasture had a less marked stress response than calves that were abruptly weaned and introduced to a new environment of a slatted floor shed and were offered a new diet of grass silage *ab libitum* plus supplementary concentrates simultaneously. The more marked stress response was attributed to decreased number of lymphocytes, the attenuated production of IFN-γ and greater concentrations of the acute phase protein, fibrinogen, post-weaning in abruptly weaned and housed calves compared with those that were abruptly weaned and returned to pasture. Thus, reducing the cumulative effects of multiple stressors by deferring housing of beef calves for a period of time post-weaning may have beneficial effects on the stressed calf.

In line with the findings of Chapter 3 and other studies examining the effects of weaning stress in beef calves (Hickey *et al.*, 2003a; Blanco *et al.*, 2009), weaning resulted in neutrophilia and concurrent lymphopenia which was coupled with decreased in vitro production of IFN-γ, and increased acute phase response in beef cows. Thus, it is apparent that the beef cow is stressed by the abrupt separation from her calf. However, when the magnitude and duration of these perturbations in the cow are compared with those in the calf, it appears that stress response is activated to a lesser degree and for a shorter period. The practice of early weaning has not been

adopted in Ireland due to favourable climatic conditions for grass growth. Traditionally, cow-calf separation occurs when calves are approximately 7 – 9 months of age. Observations have confirmed that distinct distress behaviours, such as increased vocalisations and locomotor activity, are expressed by beef cows following abruptly separated from their calves (Price *et al.*, 2003), therefore it is reasonable to assume that physiological and immunological processes similar to those altered in abruptly weaned beef calves would also be altered in beef cows following abrupt separation from their offspring.

Additionally, the findings of Chapter 3 and 4 have demonstrated that movement from a pasture environment to a housing environment in a slatted floor shed is capable of inducing a transient stress response in beef calves and cows however, homeostatic mechanisms are capable of regulating this response with no ramifications on animal health or welfare. Collectively, these chapters identified a profile in which neutrophilia and concurrent lymphopenia are evident for a brief period following relocation.

Due to the pronounced neutrophilia and lymphopenia observed in Chapters 3 and 4, a more in depth examination of the effects of abrupt weaning on neutrophil function and lymphocyte immunophenotypes was undertaken in Chapter 5. To the author's knowledge, no other studies have examined these biomarkers in abruptly weaned beef calves. The main findings of Chapter 5 showed that transient neutrophilia was evident post-weaning, and that this population of circulating neutrophils are less active in terms of phagocytosis than the neutrophil population in circulation prior to weaning (Table 7.1). Previous studies examining the effects of neutrophils in response to glucocorticoids have reported that neutrophilia is attributed to an influx of immature neutrophils released from the bone marrow into circulation, decreased in migration

along endothelial cells (Burton *et al.*, 1995; Weber *et al.*, 2001; and as evidenced in the work of this thesis), and by decreased neutrophil apoptosis (Change *et al.*, 2004; Burton *et al.*, 2005; Madsen-Boutrese *et al.*, 2006). The decrease in percentage neutrophils performing phagocytosis post-weaning may be related to the age and stage of maturity of circulating neutrophil at this time. Future work should examine the stage of maturity of neutrophils present at time of neutrophilia in order to determine if these or other factors are responsible for the decrease in bacterial uptake. It is interesting to note that the respiratory burst activity of neutrophils was not affected by weaning, suggesting that these cells are still capable of generating and releasing toxic reactive oxygen species and proteolytic enzymes. Due to decreased apoptosis of neutrophils in response to glucocorticoids, neutrophils have prolonged contact with their localised microenvironment and may secrete noxious agents as the cells proceed through necrosis resulting in localised tissue damage. This paradox, whereby the protective actions of neutrophils may cause tissue damage and exacerbate disease when improperly regulated, has been documented in cattle (Burton *et al.*, 2005) and appears to play a role in overall dysregulated response in stressed cattle (Buckham *et al.*, 2007a; 2007b). The findings of Chapter 5 also showed that abrupt weaning induced a redistribution of peripheral lymphocyte immunophenotypes in beef calves. Transient decreases in the proportion of $CD4^+$, $CD8^+$, and γδ T cells reported immediately post-weaning. Trafficking of lymphocytes is an important and dynamic factor for effective cell-mediated immunity and stress has been shown to influence this process (Kehrli *et al.*, 1999; Viswanathan and Dhabhar, 2005; Dhabhar, 2009) and has important implications for vaccination protocols (Esser *et al.*, 2003). As stated, these alterations were transient in the beef calf which is in agreement with other studies that

reported rapid reversion to pre-stress levels following the cessation of stressor in bovine and rodent studies (Dhabhar *et al.*, 1995; Riondato *et al.*, 2008).

Utilising the profile of changes observed in neutrophil number and function and lymphocyte subsets identified in Chapter 5 as sensitive biomarkers of weaning stress in beef calves, the provision of concentrate supplementation pre-weaning was examined to determine if this practice could ameliorate the stress response post-weaning.

The main findings of Chapter 6 showed that calves that were offered concentrate supplementation pre-weaning had a lesser degree of neutrophilia compared with calves that were not supplemented pre- and post-weaning (Chapter 5). Additionally, a lesser reduction in percentage $WC1^+$ lymphocytes and increased percentage $CD4^+$ lymphocytes were observed post-weaning in supplemented calves compared with calves that were not supplemented. The common alterations in neutrophil number and function, and lymphocytes number and subsets observed in Chapter 5 and 6 are summarised in Table 7.1.

As the predominant lymphocyte phenotype in bovine peripheral blood, $CD4^+$ T cells play an important role directing the immune response towards a cell-mediated, pro-inflammatory or a humoral (antibody) response via the secretion of specific cytokine subsets by T helper 1 (Th1) or T helper 2 (Th2) cells (Kampen *et al.*, 2006; Tanaka *et al.*, 2007). Progression to either response is important for immunoprotection against viral and bacterial antigens and also immunopathogenesis of disease (Glaser and Kiecolt-Glaser, 2005). The percentage $CD4^+$ lymphocytes was decreased in calves post-weaning and subsequently, returned to, and was increased, compared with pre-weaning baseline in Chapter 5 and 6, respectively. Stress has been shown to disrupt the balance between Th1 and Th2 cells in an attempt to restore homeostasis in rodent

and human studies (Elenkov and Chrousos, 2002) by influencing the production of cytokines for each subset (Elenkov *et al.*, 1999). Th1 cells are involved in cell-mediated immunity whereas Th2 cells are involved in antibody production (Yang *et al.* 2000). A shift away from a Th1 cell mediated response can result in increased susceptibility to viral infections (Elenkov *et al.* 1996).

The bias for a Th2 response following stress may enhance protection against bacterial pathogens while dampening the response to viral antigens (Salak-Johnson and McGlone, 2007). This has important implications for weaned beef calves as BRD is typically characterised by an initial viral infection followed by a secondary bacterial infection. Future work should address the complexity of the relationship between stress and Th1/Th2 balance in weaned beef calves and those likely to succumb to BRD following transportation and housing.

The term biomarker denotes a substance used as an indicator of a biological state and this can be very useful when identifying and defining weaning stress. In terms of potential biomarkers that may be useful to identify stress sensitive beef calves following abrupt weaning, it is evident from the work of this thesis that no biomarker can be used in isolation, but rather a panel of biomarkers defining a specific profile may be used to identify stressed animals. The author suggests that a collective examination of neutrophil and lymphocyte number, functional activity (neutrophil phagocytosis), and proportion of $CD4^+$ and $WC1^+$ lymphocytes may be useful over the post-weaning period. Failure of these biomarkers to return to pre-weaning levels as time progresses post-weaning may signal disturbed homeostasis that may infer reduced immunocompetence in calves at a time when pathogen exposure is heightened.

Table 7.1. Summary of the profile of changes in peripheral neutrophil number and function, and lymphocyte number and subsets in beef calves following abrupt weaning

Biomarker	Chapter	Treatment	T × S[1]	Response to weaning	Range	Biomarker	Chapter	Treatment	T × S[1]	Response to weaning	Range
Neutrophils, $\times 10^3$cell/μL	5	W	***	↑↑	2.5 – 6-6	Lymphocytes, $\times 10^3$cell/μL	5	W	***	↓	5.2 – 7.3
		C		↔				C		↔	
	6	CS	NS	↑	2.3 - 4.8		6	CS	NS	↓	5.0 – 7.3
		NCS		↑				NCS		↓	
L-selectin, MFI[2]	5	W	***	↓	25 – 40	$CD4^+$, %	5	W	***	↓	15 – 22
		C		↔				C		↔	
	6	CS	NS	↓	25 - 35		6	CS	*	↓ followed by ↑	21 – 29
		NCS		↓				NCS		↔	
Phagocytosis, %	5	W	***	↓	61 – 87	$CD8^+$, %	5	W	***	↓	8 – 12
		C		↔				C		↔	
	6	CS	NS	↓	66 – 79		6	CS	NS	↔	8 – 11
		NCS		↓				NCS		↔	
Oxidative burst, %	5	W	NS	↔	20 - 40	$WC1^+$, %	5	W	***	↓↓	12 – 24
		C	NS					C		↓	
	6	CS	NS	↔	23 - 33		6	CS	*	↓	10 – 19
		NCS	NS					NCS		↓↓	
						MHC class II^+, %	5	W	***	↑↑	13 – 30
								C		↑	
							6	CS	NS	↑	21 – 25
								NCS		↑	

[1]T × S = Treatment × sampling time interaction. [2]MFI = mean fluorescence intensity. W = abruptly weaned and housed, C = not weaned (controls) and housed, CS = concentrate supplemented pre-weaning, NCS = not concentrate supplemented pre-weaning.

The acute stress response produced by abrupt social, nutritional, and environmental changes associated with weaning may disrupt homeostasis and thus compromise well-being. Some researchers have argued that the magnitude of the acute phase response may be a key indicator of subsequent productivity, especially during the initial period of specialised feeding (Arthingon *et al.*, 2005; Qiu *et al.*, 2007). Moreover, the magnitude of the acute phase reaction is greater in these cases of clinical morbidity compared with calves that did not become morbid (Carter *et al.*, 2002). Variable results on the effect of abrupt weaning on the acute phase protein response in beef cattle were documented in this thesis. In Chapters 3 and 4, the acute phase response was elevated for an extended period post-weaning, however in Chapter 5 and 6 concentration of haptoglobin increased whereas fibrinogen was not affected by weaning. Hickey *et al.* (2003a) reported that weaning had no effect on concentration of haptoglobin in calves, whereas Arthington *et al.* (2003) evaluated the effect of weaning and weaning combined with transport in calves and found an increase in the concentration of haptoglobin in calves weaned but not in those weaned and transported, concluding that it is not necessary to have an inflammatory process to increase the concentration of this protein. These authors and the author of this thesis acknowledge that husbandry stressors may differentially elicit varied acute phase responses based on the age, breed, and gender of the animal and nature of the stimulus, and thus, further research on the effect of husbandry practices on the acute phase response in cattle is warranted.

Examination of the metabolic profiles, in terms of protein and energy metabolism, in beef cattle post-weaning showed that health and welfare of these animals was not compromised by abrupt weaning. Altered metabolic profiles signal a shift in protein and energy metabolism as the animal adapts to a new diet.

7.3. Main conclusions

In conclusion, the main findings of this thesis are:

1. Abrupt weaning is a stressful event for the beef calf and cow as evidenced by the altered physiological and immunological responses, namely increased neutrophil number and decreased lymphocyte number (Chapter 3 and 4).
2. Abrupt weaning stress altered the plasma protein profile of calves and many significant differences were found between the physiological haematological and metabolic profiles of calves undergoing abrupt weaning versus calves which had not been weaned.
3. In addition to increased neutrophil number and decreased lymphocyte number, neutrophil phagocytic activity and percentage $CD4^+$, $CD8^+$ and $WC1^+$ lymphocytes are transiently decreased in abruptly weaned beef calves (Chapter 5 and 6).
4. Examination of the biomarker panel consisting of neutrophil and lymphocyte number, percentage neutrophils performing phagocytosis, and percentage $CD4^+$ and $WC1^+$ lymphocytes may be indicative of calves sensitive to stress and consequently more susceptible to infection post-weaning (Chapter 5 and 6). This finding has made an important contribution to the understanding of effects of weaning stress on immune homeostasis as well as identifying novel biomarkers of weaning stress.
5. Variable acute phase protein responses were reported in beef calves post-weaning. Despite, the lack of significant differences, concentration of haptoglobin and fibrinogen increased numerically post-weaning in beef calves indicating that an inflammatory response is induced in weaned calves (Chapter 3, 5, 6). The results of these studies may help to study weaning stress in relation to disease susceptibility.

6. An extended acute phase protein response was observed in beef cows post-weaning which may be attributable to the weaning stress and the systemic responses in mammary gland involution (Chapter 4).
7. Metabolic profiles are altered for a period post-weaning in weaned beef calves, these alteration are reflective of adaptation to new, entirely solid diet and may be confounded by levels of intake post-weaning (Chapter 3 and 6).
8. A metabolic profile indicating positive nutritional status in beef cows was evidenced post-weaning by increased concentrations of glucose and decreased concentrations of NEFA thus, suggesting that energy was redirected from milk production to other functions following the removal of the calf and cessation of nursing (Chapter 4).
9. Housing resulted in a transient stress response as evidenced by increased neutrophil and lymphocyte number (Chapter 3 and 4) in beef calves and cows, and by reduced percentage $WC1^{+}$ lymphocytes (Chapter 5) in beef calves. These alterations were resolved rapidly post-housing thus housing had no ramifications on animal health or welfare.
10. Reducing the cumulative effects of weaning stress by deferring housing for a 35 d period post-weaning and by offering concentrates for a period pre-weaning resulted in less marked stress responses, in calves post-weaning compared with those that are weaned and housed simultaneously (Chapter 3) and those that were not offered concentrates pre-weaning (Chapter 5 and Chapter 6).

7.4. Implications and future work

The data presented in this thesis has identified many practical implications pertaining to weaning in beef cattle. Reducing the cumulative stressors at the time of weaning may allow for smoother transition from social and nutrition dependence on the dam to complete social and nutritional independence of the calf post-weaning. Manipulation of pre- and post-weaning management practices can reduce weaning stress as evidenced by the deferral of housing of a period post-weaning and by the provision of concentrate supplementation pre-weaning. The practicality of these strategies will be dictated by environmental and economic factors, namely grass availability at pasture and the costs associated with feeding supplementary concentrates pre-weaning.

It is evident from the findings of Chapter 5 and 6 that homeostasis is altered at numerous levels which may affect immunocompetence in beef calves. Extended and more profound perturbations to neutrophil number and function, greater than those observed in this thesis, may induce a state, whereby the protective actions of these phagocytosing cells begin to damage tissue, thus, allowing opportunistic bacteria to become resident locally. Moreover, adaptive immune responses may be attenuated by the redistribution of lymphocyte subsets reducing the capacity for surveillance of foreign antigens. Both these factors could influence the efficacy of vaccination protocols in weaned beef calves.

The following section outlines the possible future directions of study arising from the findings of these chapters.

- Due to changes observed in peripheral immune responses post-weaning in calves, it would be worthwhile to investigate whether these changes reduce immunocompetence when calves are challenged with pathogens. These studies

could incorporate *in vivo* challenge models where calves are challenged with viral strains associated with BRD, such as BRSV or PI-3 or IBR with and without subsequent challenge with bacterial agents, such as *M. haemolytica*. In order to determine immunocompetence, viral titres and specific antibodies against the viral strain employed in challenge model could be measured.

- A combined approach using genomic, cellular and proteomic analyses could investigate the effects of weaning on candidate genes that have been reported to be altered by other husbandry practices in cattle (e.g. transportation). These studies could focus on genes relating to innate immune response, apoptosis, signal transduction, and wound healing.
- Future work investigating *in vitro* leukocyte function and cytokine and chemokine profile characterisation in weaned beef calves could provide vital information on the key mediators in the stress-immune axis and how this response is regulated in cattle.
- To gain a greater understanding of the complex interactions between stress and the immune system in cattle, *in vitro* and *ex vivo* studies could be carried out to examine the pro-inflammatory cytokine signalling directing the response. Cytokine responses could be measured using a candidate gene approach and quantitative real-time PCR and by intracellular staining techniques utilising flow cytometry.
- In light of the synergistic actions of viral and bacterial pathogens in which cattle become infected with BRD, it would be of interest to address the complexity of the relationship between weaning stress and the Th1/Th2 balance. In the future, it may be possible to identify a greater number of significant biomarkers and to use both *in vivo* and *ex vivo* models to gain a

greater understanding of stress related disease susceptibility in weaned beef calves.

Chapter 8

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Chapter 9

Appendix

Appendix 1. Criteria underpinning the Welfare Quality® assessment systems

1. Animals should not suffer form prolonged hunger, i.e. they should have a sufficient and appropriate diet.
2. Animals should not suffer form prolonged thirst, i.e. they should have a sufficient and accessible water supply.
3. Animals should have comfort around resting.
4. Animals should have thermal comfort, i.e. they should neither be too hot nor too cold.
5. Animals should have enough space to be able to move around freely.
6. Animals should be free of physical injuries.
7. Animals should be free of disease, i.e. farmers should maintain high standards of hygiene and care.
8. Animals should not suffer pain induced by inappropriate management, handling, slaughter, or surgical procedures (e.g. castration, dehorning).
9. Animals should be able to express normal, non-harmful, social behaviours, e.g. grooming.
10. Animals should be able to express other normal behaviours, i.e. it should be possible to express species-specific natural behaviours such as foraging.
11. Animals should be handled well in all situations, i.e. handlers should promote good human-animal relationships.
12. Negative emotions such as fear, distress, frustration or apathy should be avoided whereas positive emotions such as security or contentment should be promoted.

Appendix Table A.1. Effect of vitamin and mineral status and supplementation on immunological biomarkers in cattle

Animal	Treatment / Source	Biomarkers examined	Results	References
Vitamin (Vit) A				
Crossbred Angus steers, 7 months, (n = 20)	Low: no supplemented vit A fed, High: 2,200 IU /kg DM Vit A fed	Antibody response to ovalbumin vaccine (1 mg/mL) administered s.c.	Ovalbumin antibody titre not affected by vit A restriction	Gorocica-Buenfil *et al.*, 2008
Japanese Black steers, 18 – 21 months, (n = 70)	Low vit A status steers: 30 IU/d vit A vs. Control: normal vit A status	Leukocyte population and cytokine mRNA expression	Low status steers had lower $WC1^+$ γδ T cells, MHC Class II^+CD14^- B cells and Th1 to Th2 cytokine shift (IFN-γ: IL-4)	Yano *et al.*, 2009
Vitamin E				
Feedlot steers	Feed supplement	Acute phase protein response and animal health/treatment costs	Supplementation attenuated serum amyloid A and α-1-acid glycoprotein response	Carter *et al.*, 2002
Continental × British steers, mean (s.e.) BW = 173 (1.3) kg, (n = 18)	Feed supplement of 285, 570, or 1,140 IU vit E/animal daily	Antibody response to ovalbumin vaccine (4 mg/mL) administered s.c.	Linear increase in antibody titre s was associated with increasing vit E supplementation., maximum response noted on d 21	Rivera *et al.*, 2002
Angus, Angus × Hereford, and Angus × Shorthorn heifer calves, mean (s.e.) BW = 193 (30.0) kg, (n = 52)	Feed supplement of 15 or 185 IU vit E/kg DM	BHV-1 challenge (virus titre 6.9_{10}/mL)	Lower ceruloplasmin concentration associated with BHV-1 challenged calves supplemented with vit E	Cusack *et al.*, 2005
Selenium (Se)				
Crossbred Angus steer calves, mean (s.e.) BW = 200 (5.2) kg, (n = 18)	Feed supplement of 0 Se, 26 ppm of Se from sodium Se, and 26 ppm from Se yeast.	PHA-induced skin swelling response, macrophage phagocytosis, lymphocyte proliferation, IFN-γ production	Macrophage phagocytosis increased in steers supplemented with Se yeast. There was no effect on lymphocyte proliferation or IFN-γ production	Beck *et al.*, 2005

Appendix Table A.1. (cont.)

Animal	Treatment / Source	Biomarkers examined	Results	References
Selenium (Se)				
Crossbred Angus steers, (n = 30)	Feed supplement of 0, 1.7 mg/steer organic or 1.7 mg/steer inorganic Se daily	Macrophage phagocytosis and lymphocyte proliferation	Level and source of Se had no effect on macrophage phagocytosis or lymphocyte proliferation	Fry *et al.*, 2005
Copper (Cu)				
Steer calves deficient in Cu	Feed supplement	Neutrophil phagocytosis (*Candida albicans*)	Ability of neutrophils to phagocytose *C. albicans* restored with Cu supplementation	Boyne and Arthur, 1986
Heifer Aberdeen Angus calves, 5 months, (n = 10)	Experimental induced Cu deficiency (molybdenum (30ppm) and sulphate (225 ppm) vs. normal status (controls)	Concentration and activity of ceruloplasmin, total leukocyte counts, NBT reduction assay, neutrophil phagocytosis, and whole blood constituents	Cu deficient steers had lower concentration of ceruloplasmin and activity, lower B cell number, increased monocyte number, and reduced neutrophil function, whereas total leukocyte, neutrophil and lymphocyte number, and whole blood constituents did not differ from controls	Cerone *et al.*, 1998
Weaned Angus (n = 24) and Simmental (n = 24) steer	Normal Cu status and low Cu status	IBRV challenge, lymphocyte blastogensis	Steers with low Cu status had lower mitogenic stimulated blastogensis than controls	Wright *et al.*, 2000
Angus steers, 7 months, (n = 48)	Feed supplement of of 0, 10 mg Cu/kg (organic or non-organic), and 20 mg Cu/kg (organic or non-organic)	Antibody response to ovalbumin vaccine (4,000 μg ovalbumin) administered s.c. and i.d.	Higher Cu supplementation resulted in higher ovalbumin antibody titres, as did source (non-organic vs. organic)	Dorton *et al.*, 2003

Appendix 2. Haematological variables associated with red blood cells

Appendix 2.1. Haematocrit (%)

Proportion of blood volume occupied by erythrocytes. Also called packed cell volume.

Calculation: HCT = [Red blood cell volume / Blood volume] ×100

Appendix 2.2. Mean Corpuscular Volume (fL)

Measure of the average red blood cell volume.

Calculation: MCV = [HCT (%) / RBC ($\times 10^6$ /μL)] × 10

Appendix 2.3. Mean corpuscular haemoglobin (pg)

An estimate of the average amount of haemoglobin in the average red blood cell in a sample of blood.

Calculation: MCH = [HGB (g/dL) / RBC ($\times 10^6$ /μL)] × 10

Appendix 2.4. Mean corpuscular haemoglobin concentration (g/dL)

An estimation of the concentration of haemoglobin in grams per 100 mL of packed red blood cells, derived from the ratio of the haemoglobin to the haematocrit.

Calculation: MCHC = HGB (g/dL) / HCT (%)] × 100

Appendix Table A.2. Normal blood values of erythrocytes and leukocytes in cattle.

Erythrocyte Series			Leukocyte Series		
	Range	**Average**		**Range**	**Average**
Erythrocytes, $\times 10^6/\mu l$	5.0 - 10.0	7.0	Leukocytes, $\times 10^3/\mu l$	4.0 – 12.0	8.0
Haemoglobin, g/dl	8.0 - 15.0	11.0	Neutrophil,	0.6 – 4.0	2.0
Haematocrit, %	24.0 - 46.0	35.0	Lymphocytes	2.5 – 7.5	4.5
Mean corpuscular volume (MCV), fL	40.0 – 60.0	52.0	Monocytes	0.25 – 0.84	0.4
Mean corpuscular haemoglobin concentration (MCHC), pg	11.0 – 17.0	14.0	Eosinophils	0 – 2.4	0.7
MCHC, %	30.0 – 36.0	32.7	Basophils	0 – 0.2	0.05
			Percentage distribution		
			Neutrophils	15 - 45	28.0
			Lymphocytes	45 - 75	58.0
			Monocytes	2 - 7	4.0
			Eosinophils	0 - 20	9.0
			Basophils	0 - 2	0.5

(Adapted from Radostitis, 1994)